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# Characterization of gut microbiota in hepatic patients: Hepatitis B virus infection and alcoholic hepatitis

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# THÈSE DE DOCTORAT

Soutenue à Aix-Marseille Université  
Le 24 novembre 2023 par :

**Reham MAGDY WASFY**

## Caractérisation du microbiote intestinal chez les patients hépatiques : Infection par le virus de l'hépatite B et hépatite alcoolique

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# AFFIDAVIT

I, undersigned, **Reham MAGDY WASFY**, hereby declare that the work presented in this manuscript is my own work, carried out under the scientific supervision of Professor **Mathieu MILLION**, in accordance with the principles of honesty, integrity and responsibility inherent to the research mission. The research work and the writing of this manuscript have been carried out in compliance with both the French national charter for Research Integrity and the Aix-Marseille University charter on the fight against plagiarism.

This work has not been submitted previously either in this country or in another country in the same or in a similar version to any other examination body.

Place Marseille, date 15th September 2023

*Reham MAGDY*

# LIST OF PUBLICATIONS AND CONFERENCE PARTICIPATION

## ➤ List of publications related to the thesis project:

### Published Articles:

1. **Magdy Wasfy R**, Zoaiter M, Bilen M, Alou MT, Lo CI, Bellali S, Caputo A, Alibar S, Andrieu C, Raoult D, Fournier PE and Million M. 2023. Description of *Agathobaculum massiliense* sp. nov., a new bacterial species prevalent in the human gut and predicted to produce indole and tryptophan based on genomic analysis. *Antonie van Leeuwenhoek*, 116(6), 541–555. <https://doi.org/10.1007/s10482-023-01824-1>
2. **Magdy Wasfy R**, Mbaye B, Borentain P, Alou MT, Murillo Ruiz ML, Caputo A, Andrieu C, Armstrong N, Million M, and Gérolami R. Ethanol-Producing *Enterocloster bolteae* Is Enriched in Chronic Hepatitis B-Associated Gut Dysbiosis: A Case-Control Culturomics Study. *Microorganisms*. 2023 Sep 28;11(10):2437. doi: 10.3390/microorganisms11102437.
3. Zoaiter M, **Magdy Wasfy R**, Caputo A, Fenollar F, Zeaiter Z, Fournier PE and Houhamdi L. 2023 . *Streptococcus bouchesdurhonensis* sp. nov. isolated from a bronchoalveolar lavage of a patient with pneumonia. *Arch Microbiol* 205, 3. <https://doi.org/10.1007/s00203-022-03348-0>.
4. Iwaza R, **Magdy Wasfy R**, Dubourg G, Raoult D and Lagier J-C. 2022. *Akkermansia muciniphila*: The state of the art, 18 years after its first discovery. *Front. Gastroenterol*. 1:1024393. doi: 10.3389/fgstr.2022.1024393.
5. Hamame A., **Magdy Wasfy R**, Lo CI, Fenollar F, Raoult D, Fournier PE and Houhamdi L. 2023. Taxonogenomics of *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov. new bacteria isolated from human stool microbiota. *Sci Rep* 13, 8465. <https://doi.org/10.1038/s41598-023-35443-7>.
6. Mbaye B, **Magdy Wasfy R**, Alou MT, Borentain P, Andrieu C, Caputo A, Raoult D, Gérolami R and Million M. 2023. *Limosilactobacillus fermentum*, *Lactococcus lactis* and *Thomasclavelia ramosa* are enriched and *Methanobrevibacter smithii* is depleted in patients with non-alcoholic steatohepatitis. *Microbial pathogenesis*, 180, 106160. <https://doi.org/10.1016/j.micpath.2023.106160>.
7. Mbaye B, Borentain P, **Magdy Wasfy R**, Alou MT, Armstrong N, Mottola G, Meddeb L, Ranque S, Gérolami R, Million M and Didier R. 2022. Endogenous Ethanol and Triglyceride Production by Gut *Pichia kudriavzevii*, *Candida albicans* and *Candida glabrata* Yeasts in Non-Alcoholic Steatohepatitis. *Cells*; 11(21):3390. <https://doi.org/10.3390/cells11213390>.
8. Mbaye B, **Magdy Wasfy R**, Borentain P, Tidjani Alou M, Mottola G, Bossi V, Caputo A, Gerolami R and Million M (2023) Increased fecal ethanol and enriched ethanol-producing gut bacteria *Limosilactobacillus fermentum*, *Enterocloster bolteae*, *Mediterraneibacter gnavus* and *Streptococcus mutans* in

nonalcoholic steatohepatitis. *Front. Cell. Infect. Microbiol.* 13:1279354. [doi: 10.3389/fcimb.2023.1279354](https://doi.org/10.3389/fcimb.2023.1279354).

#### **Submitted Articles:**

9. **Magdy Wasfy R**, Alou MT, Borentain P, Ranque S, Raoult D, Gérolami R, and Million M. Gut Dysbiosis Signatures Associated with Hepatitis B Virus-Related Diseases: An Insightful Review Using AI-Powered Research Tools. ([Submitted to Microbial Pathogenesis; Manuscript Number: YMPAT-D-23-01822](#)).
10. Mbaye B, **Magdy Wasfy R**, Alou MT, Borentain P, Gérolami R, Dufour JC and Million M. A Catalog of Ethanol-Producing Microbes in Humans ([Submitted to Future Microbiology, Manuscript Number: FMB-2023-0250](#)).

#### **Articles in preparation:**

11. **Magdy Wasfy R**, Borentain P, Mbaye B, Alou MT, Caputo A, Andrieu C, Raoult D, Million M, and Gérolami R. *Thomasclavelia ramosa* is a signature biomarker in Patients with Alcoholic Liver Disease and Associated Hepatocellular Carcinoma: Culturomics and Metagenomics Case-Control Study.
12. **Magdy Wasfy R**, Benseddik F, Zgheib R, Borentain P, Alou MT, Caputo A, Andrieu C, Gérolami R, Raoult D and Million M. First Evidence of Candidate Phyla Radiation in Hepatitis B Virus Infected Patients: A Case-Control Metagenomic Study.

#### ➤ **Participation in conferences during the thesis period:**

1. Journée Infectiopôle Sud, Marseille : 7th July 2023 (Poster).
2. The 30th edition of the Ecole Doctorale 62 colloquium on the theme Past-Present-Future, AMU, 31st May 2023. (Oral presentation).
3. Journée Infectiopôle Sud, Marseille: 2nd July 2021 (Poster).
4. Journée Infectiopôle Sud, Marseille: 1st July 2021 (Poster).
5. Meetings of work in progress (WIP), organized every Tuesday by IHU Méditerranée Infection, Marseille (2020-2023). (Oral presentations).
6. Scientific seminar organized by IHU Méditerranée Infection, Marseille, 6th October, 2023. (Oral presentation).
7. The 5th European Congress on Infectious Diseases, London, UK, 9-10<sup>th</sup> October 2023. (Oral presentation).

# RÉSUMÉ

Les maladies du foie sont responsables d'un fardeau sanitaire mondial de plus en plus lourd. Ces dernières années, des recherches approfondies ont démontré que la dysbiose intestinale est un facteur déterminant qui contribue à la physiopathologie des maladies du foie. Des profils différents du microbiote intestinal ont été observés dans les maladies hépatiques liées à l'alcool (ALD), l'infection par le virus de l'hépatite B (VHB), ou même ces complications associées telles que la cirrhose et le carcinome hépatocellulaire (CHC). En effet, la métagénomique a révélé des altérations significatives de la composition du microbiote intestinal chez les patients infectés par le VHB ou en ALD, avec une diminution des *Clostridiales* produisant du butyrate et une augmentation des *Enterobacteriaceae* induisant l'inflammation. Cependant, peu de recherches ont étudié la dysbiose intestinale au niveau des espèces microbiennes. En outre, aucune étude n'a examiné le microbiote intestinal de ces patients à l'aide de l'approche de culturomique.

Par conséquent, dans le présent travail, nous avons d'abord réalisé des études cas-témoins pour étudier le microbiote intestinal chez les patients atteints du VHB et de l'ALD, en utilisant la culturomique et la métagénomique. En outre, nous avons d'abord effectué une analyse systématique de la littérature à l'aide d'outils de recherche assistés par ordinateur afin de décrypter les signatures du microbiote intestinal associées au VHB qui pourraient être utilisées comme biomarqueurs diagnostiques non invasifs à différents stades de la maladie.

Notre analyse qualitative dans la revue nous a permis d'identifier des résultats reproductibles pour un profil de microbiote signature dans les maladies associées au VHB, caractérisé par une augmentation constante de la plupart des membres de - *Pseudomonadota*, *Bacilli*, *Prevotella*, *Streptococcus*, *Veillonella* et une diminution constante de *Clostridia*, *Lachnospiraceae*, et *Roseburria*. En outre, les espèces de levure *Saccharomyces cerevisiae* et *Candida tropicalis* ont été très abondantes. *S. cerevisiae* et *C. tropicalis* produisent toutes les deux de grandes quantités d'éthanol. Ces résultats sont cohérents avec le rôle émergent de la production endogène d'éthanol dans les maladies du foie.

Les caractéristiques de la dysbiose associée au VHB dans notre étude ont révélé un enrichissement dans le genre *Enterocloster* (anciennement *Clostridium*), récemment apparu, dont l'importance dans l'infection par le VHB n'a pas été identifiée. Cependant, la métagénomique n'a pas pu détecter cet enrichissement en raison d'une faible résolution taxonomique. Au niveau des espèces bactériennes, les espèces *Enterocloster bolteae* étaient fortement enrichies avec deux souches produisant une quantité considérable d'éthanol (27 et 200 mM), qui est connue pour être nocive pour le foie. Il est intéressant de noter que les membres du phylum Candidate Phyla Radiation (CPR) non cultivés, tels que les *Candidatus Saccharibacteria* et le phylum *Atribacter*, ont été découverts pour la première fois en relation négative avec l'activité de la prothrombine dans la dysbiose associée au VHB.

En outre, on a constaté pour la première fois que *Thomasclavelia ramosa* (anciennement *Clostridium ramosum*) était très répandu chez les patients atteints d'ALD qui ont développé un CHC. Les approches culturomiques et métagénomiques ont validé ce résultat. Il est intéressant de noter que *T. ramosa* a déjà été trouvé chez des patients atteints de cancer colorectal et nasopharyngé, ce qui indique son rôle oncogène possible.

Dans ce contexte, la culturomique microbienne nous a permis d'isoler des espèces bactériennes viables qui ont pu être utilisées pour d'autres manipulations. Nos résultats

suggèrent que la production endogène d'éthanol par *E. bolteae* pourrait participer à la maladie hépatique liée au VHB. En outre, le rôle de la CPR dans les complications associées au VHB mérite d'être approfondi. Les études futures devraient également se concentrer sur l'élucidation des mécanismes par lesquels *T. ramosa* pourrait contribuer au carcinome hépatique.

Enfin, nos résultats ouvrent de nouvelles perspectives sur le rôle potentiel du microbiote intestinal dans la physiopathologie de l'ALD et des maladies liées au VHB. Ils ouvrent la voie au développement d'options thérapeutiques ciblées sur le microbiome et visant à restaurer un microbiote intestinal sain chez les patients hépatiques, telles que les probiotiques et la transplantation du microbiote fécal.

**Mots-clés:** Dysbiose, microbiote intestinal, virus de l'hépatite B, maladie hépatique liée à l'alcool, cirrhose, carcinome hépatocellulaire, *Saccharomyces cerevisiae*, *Candida tropicalis*, *Enterocloster bolteae*, *Thomasclavelia ramosa*, *Candidatus Saccharibacteria*, *Candidate Phyla Radiation*, Culturomics, Metagenomics.

# ABSTRACT

Liver diseases are responsible for increasing global health burden. In recent years, extensive research has demonstrated that gut dysbiosis is an instrumental factor contributing to the pathophysiology of liver diseases. Different patterns of gut microbiota have been observed in alcohol-related liver disease (ALD), hepatitis B virus (HBV) infection, or even their associated complications such as cirrhosis, and hepatocellular carcinoma (HCC). Indeed, metagenomics revealed significant alterations in gut microbiota composition in HBV-infected patients or ALD, with a decrease in butyrate-producing *Clostridiales* and an increase in inflammation-inducing *Enterobacteriaceae*. Few research, however, have studied gut dysbiosis at the species level. Furthermore, no study has investigated the gut microbiota in such patients using culturomics approach.

Therefore, in the present work, we first performed case-control studies to investigate gut microbiota in HBV and ALD patients, using culturomics and metagenomics. Moreover, we first performed a systematic literature review using artificial intelligence (AI)-powered research tools to decipher gut microbiota signatures associated with HBV that could be used as non-invasive diagnostic biomarkers in different disease stages.

Our qualitative analysis in the review allowed us to identify reproducible results for a signature microbiota profile in HBV-associated diseases characterized by a consistent increase in most members of *p\_Pseudomonadota*, *c\_Bacilli*, *g\_Prevotella*, *g\_Streptococcus*, *g\_Veillonella* and a consistent decrease in *c\_Clostridia*, *f\_Lachnospiraceae*, and *g\_Roseburria*. In addition, *Saccharomyces cerevisiae* and *Candida tropicalis* yeast species were highly abundant. Both *S. cerevisiae* and *C. tropicalis* produce high amounts of ethanol. These results are consistent with the emerging role of endogenous ethanol production in liver diseases.

The characteristics of the HBV-associated dysbiosis in our investigation revealed an enrichment in the recently emerging genus *Enterocloster* (formerly *Clostridium*), whose significance in HBV infection has not been identified. However, metagenomics could not detect this enrichment due to low taxonomic resolution. At the species level, *Enterocloster bolteae* species were highly enriched with *two* strains producing a considerable amount of ethanol (27 and 200 mM), which is known to be harmful to the liver. Interestingly, members of the uncultivated Candidate Phyla Radiation (CPR) phylum, such as *Candidatus Saccharibacteria* and *Atribacter* phylum, were discovered to be adversely connected with prothrombin activity in HBV-associated dysbiosis for the first time.

Furthermore, *Thomasclavelia ramosa* (formerly *Clostridium ramosum*) was found for the first time to be significantly prevalent in patients with ALD who developed HCC. Both culturomics and metagenomic approaches validated this result. Interestingly, *T. ramosa* has already been found in colorectal and nasopharyngeal cancer patients, indicating its possible oncogenic role.

In this context, microbial culturomics helped us isolate viable bacterial species that could be used for further manipulations. Our results suggest that endogenous ethanol production by *E. bolteae* might participate in HBV-related liver disease. Additionally, the role of CPR in HBV-associated complications deserves further investigation. Future



studies should also focus on elucidating the mechanisms by which *T. ramosa* might contribute to liver carcinoma.

Finally, our results provide insight into the potential role of those species in the pathophysiology of ALD and HBV-related disease. This helps pave the way for further development of microbiome-targeted therapeutic options restoring healthy gut microbiota in hepatic patients, such as probiotics and fecal microbiota transplantation.

**Keywords:** Dysbiosis, Gut microbiota, Hepatitis B virus, Alcohol-related liver disease, Cirrhosis, Hepatocellular carcinoma, *Saccharomyces cerevisiae*, *Candida tropicalis*, *Enterocloster bolteae*, *Thomasclavelia ramosa*, *Candidatus* Saccharibacteria, Candidate Phyla Radiation, Culturomics, Metagenomics.

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*" Science is not only a disciple of reason but also one of romance and passion."*

Stephen Hawking

# DEDICATIONS

*To the beloved Soul of my grandmother, Awatef, your unwavering love and support remained a constant presence, guiding me through the challenges and triumphs. Though you are no longer with us in the physical realm, your spirit lives on in the pages of this dissertation. I dedicate this Ph.D. thesis with profound gratitude and deep affection to your memory. Though you may not be here to witness this moment, I carry your memory in my heart and every word I write. Your spirit continues illuminating my path as I strive to honor your legacy through my academic pursuits.*

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*With boundless love and eternal gratitude,*

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# ABBREVIATIONS INDEX

AI: Artificial intelligence

ALD: Alcohol-related liver disease

BAs: Bile acids

CPR: Candidate phyla radiation

FMT: Fecal microbiota transplantation

HBeAg: Hepatitis B e antigen

HCC: Hepatocellular carcinoma

HE: Hepatic encephalopathy

HBV: Hepatitis B virus

LPS: Lipopolysaccharide

NASH: Nonalcoholic steatohepatitis

PRRs: Pattern recognition receptors

SCFAs: Short-chain fatty acids

sIgA: Secretory IgA



# INTRODUCTION

Gut microbiota is the collection of microorganisms inhabiting the gastrointestinal tract, with an estimated number of over  $10^{14}$  [1]. Many studies have reported the complicated and mutually beneficial relationship between gut microbiota and the host via playing an essential role in the host's metabolism, nutrition, pathological processes, and immune function [2]. Human gut microbiota composition is affected by multiple factors such as age, nutrition, ethnicity, disease, and medication intake [3–5]. The link between gut microbiota and its derivatives and the pathophysiology of the liver has attracted considerable interest [6,7].

The liver receives various gut-derived substances (bacterial products, environmental toxins, and food antigens) via the biliary tract, portal vein, and systemic circulation due to the structural link to the intestine known as the gut-liver axis [8]. The liver produces and transports primary bile acids (BAs) and active immunologic materials through the biliary tract to aid intestinal digestion and immunity. In contrast, the portal vein transports secondary BAs, nutrients, and gastrointestinal metabolites from the gut to the liver, where they are detoxified and biotransformed [9,10]. Usually, physical and chemical barriers in the intestine effectively block pathogens or toxins and reduce bacterial colonization [11]. Mucin proteins secreted by goblet cells, secretory IgA (sIgA) secreted by plasma cells in the lamina propria, and tight junctions between intestinal epithelial cells are the main barriers [11].

Several pathways have been proposed to investigate the mechanisms that link gut dysbiosis and liver injury in the form of fibrosis or cirrhosis, which are caused by persistent parenchymal injury and the activation of the inflammatory response, followed by liver fibrogenesis [12]. Liver damage is also caused by gut-derived microbe/antigen translocation and abnormal metabolites via the gut-liver axis [13]. Experimental mouse models of liver cirrhosis showed reduced mucus layer thickness, goblet cell loss, and pathological bacterial translocation [14]. Moreover, patients with decompensated cirrhosis had higher sIgA levels in their blood and stool, consistent with increased bacterial migration, and reduced tight junction protein expression [15]. Another mechanism is the disruption of short-chain fatty acids (SCFAs) production. Intestinal microbes produce SCFAs by degrading incomplete host digestion products to improve the energy metabolism of the colon cells [16]. SCFAs products such as acetate, propionate, and butyrate have anti-inflammatory effects [17]. Moreover, butyrate has been identified as the primary energy source of enterocytes, and a lack of butyrate has been linked to decreased intestinal barrier integrity [18]. In addition, studies have reported immune-mediated liver injury via pattern recognition receptors (PRRs) that are expressed by a wide range of immune cells, particularly macrophages [19]. Macrophages may play a role in innate immune regulation, linking microbe translocation to liver inflammation or fibrosis [20]. Recognition of PRRs sends the first signal to produce transcription factors such as nuclear factor- $\kappa$ B and cytokines [21,22]. The produced cytokines attract inflammatory cells, and enhance myofibroblast formation [23], which produce collagen, and normally are not found in healthy livers [24]. However, in response to liver injury, and several cytokines specially transforming growth factor- $\beta$  [25], myofibroblasts are transformed to activated hepatic stellate cells [26], which secrete extracellular matrix to form a fibrous scar [27].

Additionally, abnormal gut microbiota composition could affect the metabolism of BAs [28]. BAs are produced in the liver and enter the small intestine to aid in the digestion of fats and fat-soluble vitamins. While 95% of BAs are reabsorbed, the remainder may be modified by microbes and utilized in metabolic regulatory processes [18]. Recent research has investigated the link between gut microbiota and BA metabolism in chronic hepatitis [29,30]. The abundance of the bacteria genera responsible for BAs metabolism appears to decrease in patients with advanced fibrosis [29,31].

The severity of liver diseases and complications are strongly linked to endotoxemia caused by lipopolysaccharide (LPS) exposure [32]. LPS is an endotoxin and one of the component of the outer membrane of Gram-negative bacteria, primarily from the *Enterobacteriaceae* family [33]. Because many *Bacteroidetes* are gram-negative bacteria that produce LPS, whereas *Firmicutes* are gram-positive bacteria that do not produce LPS, an increased *Bacteroidetes/Firmicutes* ratio may increase a patient's LPS exposure [34]. LPS increases blood-brain barrier permeability causing hepatic encephalopathy (HE) [35]. Moreover, significantly abnormal LPS distributions were observed in patients with liver failure and were related to disease severity [36].

Altogether, it has been concluded that disruptions in these barriers can result in increased intestinal permeability and translocation of microbial metabolites (LPS, microbial DNA), allowing microbes and antigens to translocate into the portal vein to invade the liver [11], leading to the induction of systemic inflammation and severe chronic liver diseases such as alcohol-related liver disease (ALD) [37] and hepatitis B virus (HBV)-related diseases [38].

The chronicity of HBV infection is a global health burden, as there are still around 257 million people who are HBV positive [39]. HBV infection constitutes the primary driver of various complications, such as cirrhosis, hepatocellular carcinoma (HCC), and even liver transplantation [40]. Antiviral treatment such as nucleoside analogs and pegylated interferon- $\alpha$  have slowed HBV progression [41,42]. Nevertheless, the disease progression still occurs in some people even when a viral load is undetectable [43]. As a result, the pathophysiologic mechanisms driving the progression of chronic HBV infections remain unknown.

Patients with chronic HBV infection showed significant changes in gut microbiota composition using the metagenomic approach, characterized by high abundance in the *Proteobacteria* phylum [28] and *Streptococcus* [29], *Prevotella* [44], *Ruminococcus* and *Veillonella* [45,46] genera. However, detailed species profile alterations with disease progression and prognosis have not been systematically established or thoroughly investigated. Among those species is the newly emerging *Enterocloster bolteae* (formerly *Clostridium bolteae*) [47]. *E. bolteae* is an obligately anaerobic, gram-positive, rod-shaped, spore-forming gut pathogen [48]. It is known to be highly abundant in autistic children [49]. *E. bolteae* also showed high abundance in patients with fatty liver disease [50]; however, its role or abundance profile hasn't been reported in HBV infection. Interestingly, *E. bolteae* was reported to excrete metabolites that are thought to act as neurotoxins [51]. Moreover, it showed an ability to produce ethanol [52], documented to be associated with more severe liver disease [53].

Interestingly, intestinal microecological modulators, such as probiotics, are frequently used in clinical practice to treat HBV-related liver failure, particularly in patients with HE [54,55]. In addition, probiotics were reported to function by encouraging the development of beneficial bacteria and preventing liver inflammation from impeding the

advancement of HCC via antioxidant, and anti-metastatic activities [56]. However, the therapeutic efficacy varies considerably due to the differences in gut microbiota composition. Therefore, restoring the gut microbiota could improve the outcome and prevent complications of HBV infection. Fecal microbiota transplant (FMT) has proved to be a promising technique for restoring healthy microbiota in HBV-associated cirrhosis and improving hepatic encephalopathy [57,58]. Several studies have also evaluated FMT in chronic HBV infection and reported that hepatitis B e antigen (HBeAg) seroclearance, one of the critical steps toward limiting HBV replication and attaining clinical remission, can be induced with high efficacy by FMT [59,60].

Utilizing the missing repertoire of probiotics or detecting symbionts absent from patients but present in healthy control to accurately replicate a known and efficient mixed fecal microbiota requires viable strains [61]. Hence, the ability of the high throughput culturomics approach for such gut microbiota has been reported [62]. Culturomics provides viable pure cultures, unlike molecular techniques that only give information on the species level without any information regarding their viability status. In addition, microbial culture using selective media has made it possible to detect and cultivate minority bacterial populations that may pass undetected by genomic technologies [63]. By achieving this, culturomics could eliminate different limitations of metagenomics, such as extraction and bioinformatic bias, yielding difficult discrimination between species owing to amplicon length [63]. Moreover, to date, culturomics has enabled the isolation of more than 300 new bacterial species in the human gut that were previously believed to be uncultured by traditional culture methods [62,63].

Additionally, numerous bacterial taxa from the human microbiome have yet to be cultured. One of those taxa is the Candidate Phyla Radiation (CPR) superphylum, which represents more than 25% of bacterial diversity [64]. These CPR microorganisms have ultra-small cells, limited metabolic capacity, and unique ribosome compositions [65]. *Candidatus* Saccharibacteria is a known CPR phylum that has also been reported in smaller amounts in the gut microbiome [66]. CPR accounts for a large portion of bacterial diversity in general, but much of what we know about CPR is at the genomic level [67]. The role of *Candidatus* Saccharibacteria in gut microbiota has been described as predominantly anti-inflammatory [66]. However, detection of CPR in HBV infection has not been reported till now.

According to research, ALD is responsible for around 0.9% of all deaths and 47.9% of all cirrhosis-related deaths [68], moreover, the largest attributable fraction of HCC is associated with ALD [69]. The gut microbiota also plays a role in the pathogenesis of ALD, including alcoholic steatosis, steatohepatitis, fibrosis, and cirrhosis [70]. Patients with ALD showed a reduction in *Lachnospiraceae*, *Lactobacillus*, *Bifidobacterium*, *Ruminococcaceae*, *Clostridiales*, and an increase in *Enterobacteriaceae* [71]. Gut microbiota have also been reported to differ between stages of ALD and are associated with its progression where *Veillonella* showed an increase mainly in severe cases. At the same time, *Eubacterium*, *Oscillibacter* and *Prevotella* were reduced [72]. The species level was poorly investigated with no specific pattern reported in ALD-associated dysbiosis. Interestingly, *Thomasclavelia ramosa* (formerly *Clostridium ramosum*) is one of the reported species to be highly abundant in patients with colorectal [73] and nasopharyngeal cancer [74], however it has not been documented so far to be associated with HCC.

Based on the background mentioned above, my thesis targeted several objectives. The first objective was to find an agreement on a signature microbiota profile in HBV-

infected patients at different disease stages compared to healthy controls through an exhaustive literature review. The systematic review was performed by using traditional databases and Artificial intelligence (AI)-powered research tools for the first time to retrieve all the possible studies on the topic and compare the research methodology used between the two approaches. Our analysis of the studies was performed considering consistent dysregulated taxa in at least two studies as reproducible differential results. My effort in this project allowed me to infer reproducible results for a standard signature microbiota profile in HBV-associated diseases despite the challenging patient subgroups and contradicting results in many studies (Chapter I- Article. 1; **graphic abstract. 1**).

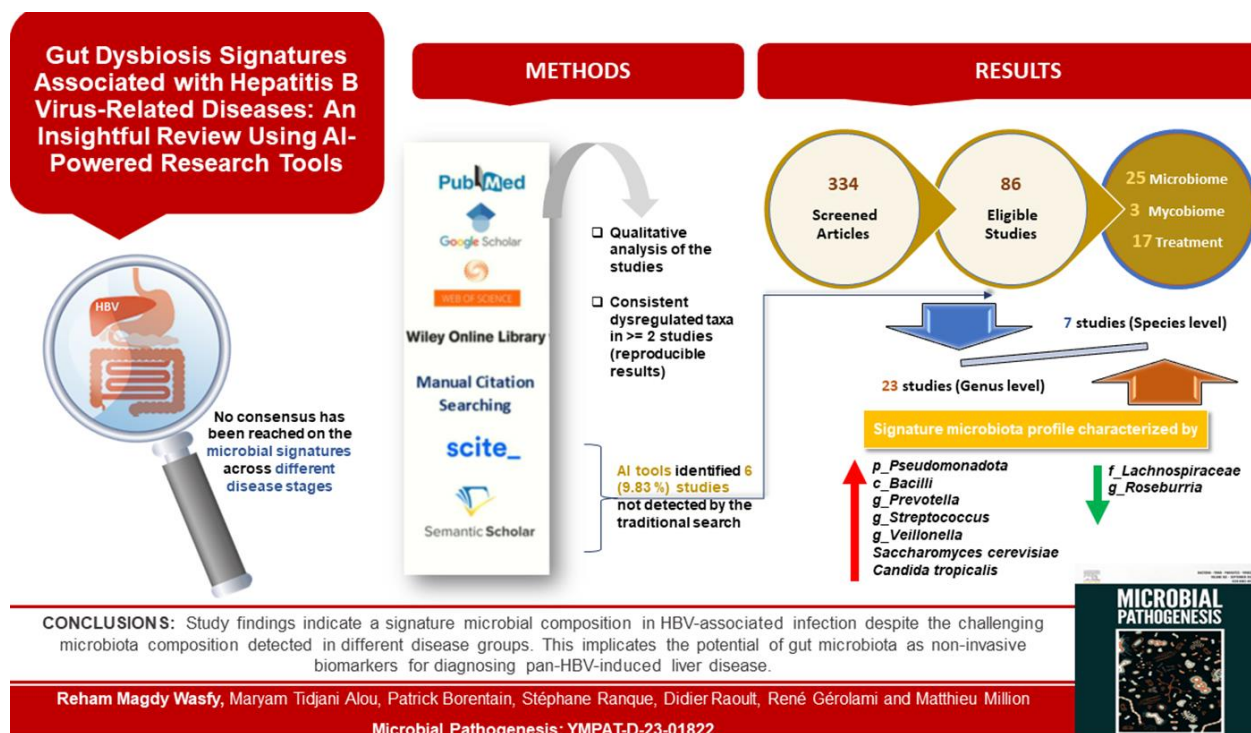
The second objective was to define gut microbiota composition in the HBV-infected patients through a designed case-control study. Notably, we are the first to describe a culturomics-based microbiome profile in HBV-infected patients in addition to metagenomics. Serendipity, we reported significantly enriched bacterial abundance in HBV patients for ethanol producing *Enterocloster bolteae* species by culturomics; however, metagenomics could not identify this enrichment. Interestingly, *E. bolteae* has not been reported before in HBV-associated gut dysbiosis (Chapter II- Article. 2; **graphic abstract. 2**).

From the perspective of culturomics done for HBV stool samples, this allowed me to identify a plethora of species already known in humans but had not been previously found in the gut or known species not previously described in humans, in addition to new species. Among those new species, one was previously isolated in our center but hasn't been described. Therefore, I learned all the possible techniques used for new species description to identify both the phenotypic and genotypic characteristics of this particular species (*Agathobaculum massiliense*) (Chapter II-Article. 3; **graphic abstract. 3**). Additionally, Members of uncultivated CPR are reported for the first time by both molecular and metagenomic approaches in HBV-associated dysbiosis such as *Candidatus* Saccharibacteria, and *Atribacter* phylum. Interestingly, their abundance was negatively correlated with prothrombin time (A blood test routinely used in clinical practice to evaluate the coagulation status of patients) (Article in preparation; **graphic abstract. 4**).

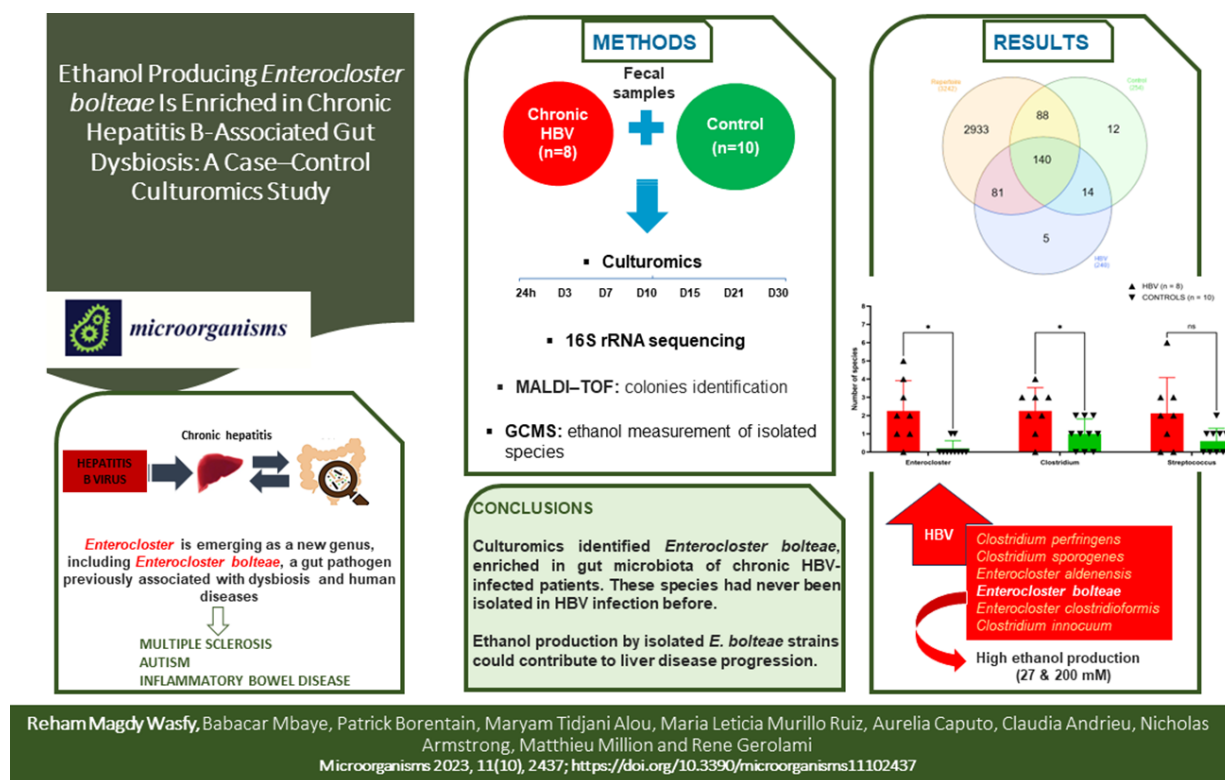
The third objective was to characterize gut microbiota in patients with ALD through a case-control study using culturomics and metagenomic approaches. Our analysis of the results showed the first significant enrichment of *Thomasclavelia ramosa* in the group of patients with ALD-associated HCC compared to the control. Interestingly, this species was highly abundant in patients with colorectal and nasopharyngeal cancer; however, it has not been reported to be associated with HCC (Draft Article. 4).

Finally, I attempted to present all the work I had the opportunity to actualize and participate in as a co-author (Chapter III). I divided it into different contexts but within the scope of the topic or the learning abilities and skills I acquired during my thesis. The contexts include conducting a systematic review on *Akkermansia muciniphila*. This human intestinal symbiont was detected in our HBV and control samples; however, no significant abundance was noted between the two groups. Interestingly, *A. muciniphila* was lost in our ALD and HCC groups (Draft Article. 4). Its administration was documented to prevent hepatic injury, steatosis, and neutrophil infiltration in a mouse model with ALD (Chapter III- Context 1). I also collaborated on another review article collecting a repertoire of all known and published ethanol-producing human microbes (Chapter III- Context 2). Moreover, I participated in performing culturomics for some non-alcoholic steatohepatitis (NASH) patients' fecal samples before establishing my

thesis projects. This collaboration yielded *three* publications (Chapter III-Context 3). I also collaborated on describing two new bacterial species, one isolated from a patient with pneumonia and the other from a healthy individual by taxonogenomics (Chapter III- Context 4).



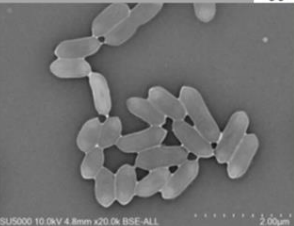
Graphic abstract. 1



Graphic abstract. 2

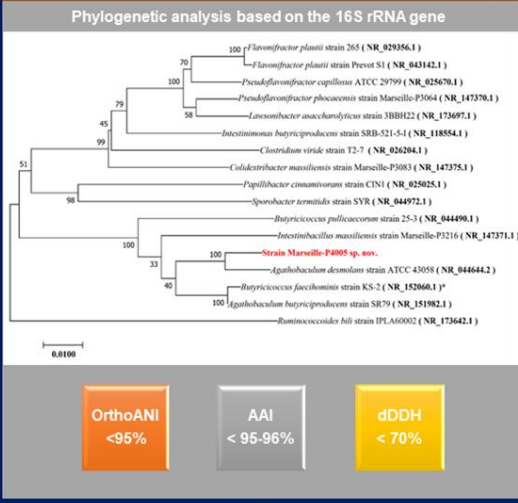
## Description Of *Agathobaculum massiliense* sp. nov., A New Bacterial Species Prevalent In The Human Gut And Predicted To Produce Indole And Tryptophan Based On Genomic Analysis

The novel bacterial strain Marseille-P4005T was isolated from a stool sample of a healthy donor



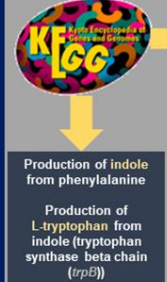
**Gram-stain negative, non-motile, non-spore-forming rod. It grew optimally at pH 7.0 on 5% sheep blood-enriched Columbia agar after preincubation in**

YCFA medium + Sheep blood + Rumen (37°C) (Ana)



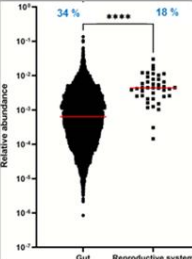
### Antibiotic resistance genes: *APH(3')-IIIa, arm(B), tet(W)*

resistance to amikacin, erythromycin, and tetracycline



Production of indole from phenylalanine

Production of L-tryptophan from indole (tryptophan synthase beta chain (*trpB*))



The novel strain could use glucose to produce L-lactate and pyruvate but not ethanol



**CONCLUSIONS:** Based on phylogenetic, phenotypic, and genomic analyses, we classify strain Marseille-P4005T (= CSUR P4005 = CECT 9669), a novel species within the genus *Agathobaculum*, for which the name of *Agathobaculum massiliense* sp. nov. is proposed

Reham Magdy Wasfy, Malak Zoaiter, Melhem Bilen, Maryam Tidjani Alou, Cheikh Ibrahim Lo, Sara Bellali, Aurelia Caputo, Stéphane Aïbar, Claudia Andrieu, Didier Raoult, Pierre Edouard Fournier and Matthieu Million  
Antonie van Leeuwenhoek  
<https://doi.org/10.1007/s10482-023-01824-1>

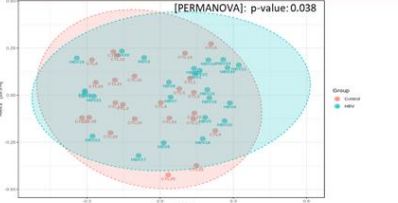
### Graphic abstract. 3

### First Evidence of Candidate Phyla Radiation in Hepatitis B Virus-Infected Patients: A Case-Control Metagenomic Study

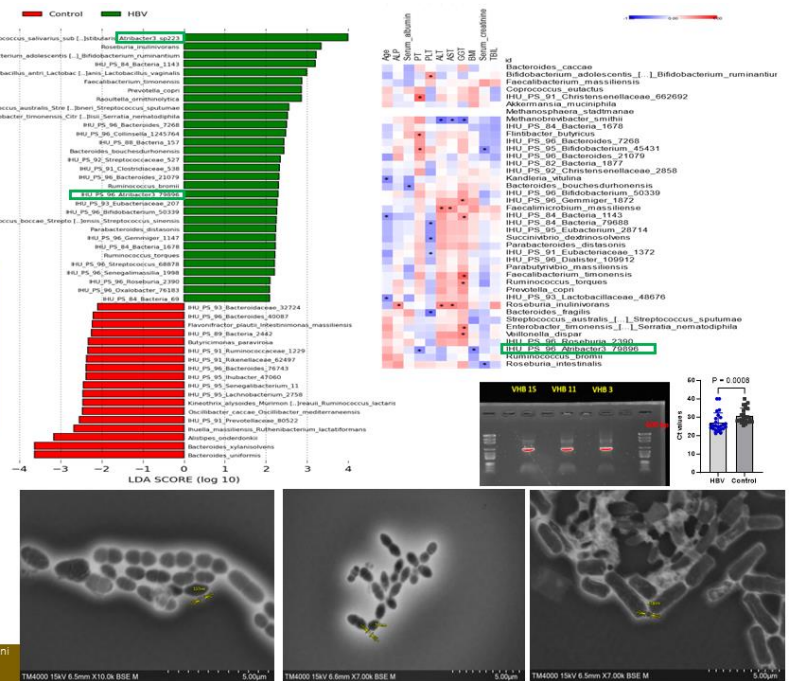
- Numerous bacterial taxa in the human microbiome have yet to be cultured, such as the CPR superphylum (25% of bacterial diversity).
- What we know about CPR is at the genomic level.
- The role of *Candidatus* Saccharibacteria in gut microbiota has been described as predominantly anti-inflammatory. However, detection of CPR in HBV infection has not been reported till now.

**RESULTS & CONCLUSIONS**

- Our study proved for the first time a significant abundance of CPR phyla, such as *Atribacter* and *Candidatus* Saccharibacteria, in HBV-infected patients, by different methods using standard PCR, qPCR, 16S rRNA sequencing, whole genome sequencing, and electron microscopy.
- Correlation analysis showed a significant negative correlation with the prothrombin index, indicating their role should be further investigated in hepatic diseases through developing specific culture methods.



Reham Magdy Wasfy, Fatma Benseddik, Rita Zheib, Patrick Borentain, Maryam Tidjani Alou, Aurelia Caputo, Claudia Andrieu, Rene Gerolami, Didier Raoult and Matthieu Million



### Graphic abstract. 4

## **CHAPTER I**

### **Literature Review: Exploring Gut Microbiota in HBV-Infected Patients**

# Preface

Increasing infection rates with HBV have been documented worldwide, with annual global mortality expected to rise by 39% in 2030 [75]. Although HBV-related diseases have been widely described in the literature, the pathophysiology of the disease progression is not entirely understood [76]. A growing body of literature suggests a crucial link between gut microbiota dysbiosis and HBV-related diseases [77–79], in addition to significant differences between HBV-related cirrhosis and healthy individuals, has been widely reported [80,81]. However, no consensus has been found on distinctive microbial signatures across different disease stages. Therefore, the objectives of the current review were to mainly summarize and discuss gut microbiota alteration in various stages of HBV infection, aiming to reach a distinctive microbiome signature in HBV-infected patients used as non-invasive biomarkers. These biomarkers might make finding novel HBV diagnostic and treatment options possible.

Moreover, available antiviral treatment can only suppress HBV DNA replication or reduce cirrhosis and HCC complications but cannot cure HBV [82,83]. In addition, emerging research documented the therapeutic efficacy of treatment options directed at HBV-associated dysbiosis [84,85]. Therefore, we discussed up-to-date microbiota-targeted therapies for HBV, addressing highlights on future research directions of clinical trials.



## **Article 1**

# **Gut Dysbiosis Signatures Associated with Hepatitis B Virus Related Diseases: An Insightful Review Using AI-Powered Research Tools**

**Reham MAGDY WASFY, Maryam TIDJANI ALOU, Patrick Borentain, Stéphane Ranque, Didier RAOULT, René Gérolami and Matthieu MILLION**

**« [Review article submitted to Microbial Pathogenesis.](#) »**

# Microbial Pathogenesis

## Gut Dysbiosis Signatures Associated with Hepatitis B Virus-Related Diseases: An Insightful Review Using AI-Powered Research Tools

--Manuscript Draft--

<b>Manuscript Number:</b>	
<b>Article Type:</b>	Review Article
<b>Keywords:</b>	Hepatitis B virus infection; Gut Dysbiosis; Fecal Microbiota; Mycobiota; Systematic Review; Artificial Intelligence
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<b>First Author:</b>	Reham MAGDY WASFY
<b>Order of Authors:</b>	Reham MAGDY WASFY Maryam TIDJANI ALOU Patrick Borentain Stéphane Ranque Didier RAOULT René Gérolami Matthieu MILLION
<b>Abstract:</b>	<p><b>Background</b></p> <p>Hepatitis B virus (HBV) has threatened public health, mainly resulting in liver fibrosis, cirrhosis, and even damage due to hepatic carcinoma. Growing evidence has supported the role of gut microbiota in different stages of liver disease. However, the mechanisms of hepatic injury from gut microbiota dysbiosis are not fully understood. Moreover, no consensus has been reached on the microbial signatures across different disease stages. This review aims to find an agreement on a signature microbiota profile in HBV-infected patients at different disease stages compared to healthy controls. These profiles may enable the identification of new diagnostic and therapeutic tools for HBV based on microbiome-targeting approaches.</p> <p><b>Methods</b></p> <p>Different literature databases and artificial intelligence (AI)-powered research tools have been investigated. Qualitative analysis of the studies was performed considering consistent dysregulated taxa in at least two studies as reproducible differential results.</p> <p><b>Results</b></p> <p>Among 334 screened articles, 86 eligible articles were identified. Interestingly, AI tools helped us identify six studies not detected by the traditional search. 25 eligible human case-control microbiome studies, three mycobiome studies, and 17 microbiota-targeted therapeutic studies were finally included and summarized. Most studies reported HBV-associated dysbiosis in different subgroups at the phyla and genus levels, while few studies referred to the species level. The results of studies were contradictory in some cases. However, we could infer reproducible results of a signature microbiota profile in HBV-associated infections characterized by an increase in p_Pseudomonadota, c_Bacilli, g_Prevotella, g_Streptococcus, g_Veillonella and a decrease in c_Clostridia, f_Lachnospiraceae, and g_Roseburria. A high abundance of Saccharomyces cerevisiae and Candida tropicalis was a constant finding. Conclusions: Study findings indicate a signature microbial composition in HBV-associated infection despite the challenging microbiota composition detected in different disease subgroups. These taxa showed diagnostic power between health and most disease stages, implicating the potential of the gut microbial as non-invasive biomarkers for diagnosing pan-HBV-</p>

	induced liver disease.
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<b>Opposed Reviewers:</b>	

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Marseille, September 13<sup>th</sup>, 2023

Dear Editor,

Kindly find enclosed our review manuscript, “Gut Dysbiosis Signatures Associated with Hepatitis B Virus-Related Diseases: An Insightful Review Using AI-Powered Research Tools,” submitted for publication.

We believe this manuscript deserves publication in your journal (**Microbial Pathogenesis**). We performed this systematic review to investigate gut microbiota alteration in patients with different HBV-related diseases. Our main goal is to reach an agreement on a signature microbiota profile in HBV patients at various stages of the disease, which has proven challenging. The exhaustive literature search, using most of the available databases in addition to artificial intelligence (AI)-powered tools, yielded 61 eligible studies, including 28 human case-control studies (microbiome and mycobiome studies). Although the results of the studies were contradictory in some cases, we could infer reproducible results of a signature microbiota profile in HBV-associated infections characterized by an increase in *p\_Pseudomonadota*, *c\_Bacilli*, *g\_Prevotella*, *g\_Streptococcus*, *g\_Veillonella* and a decrease in *c\_Clostridia*, *f\_Lachnospiraceae* and *g\_Roseburria*. In addition, a high abundance of yeast species *Saccharomyces cerevisiae* and *Candida tropicalis* was a constant finding. These

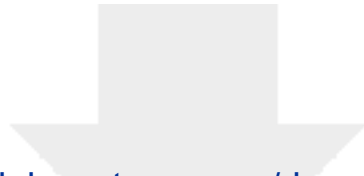
signatures could be used as non-invasive biomarkers for diagnosing HBV-related infections. In addition, it provided insight into the importance of monitoring the prescription of probiotic yeast as *Saccharomyces* species in clinical settings since they have been reported to be associated with HBV-related complications such as liver cirrhosis.

It is worth noting that we searched different databases and AI-powered research tools to retrieve all the related articles that could be missed in a traditional search. In this context, we retrieved *six* studies that did not appear by a standard search. However, we did not employ AI tools or other AI-assisted technologies to replace our primary duty of data interpretation or scientific conclusion formulation.

Thank you for considering our manuscript for publication. Neither the manuscript nor any parts of its content are currently under consideration or published in another journal. All authors have approved and revised the manuscript and agree with its submission to **Microbial Pathogenesis**.

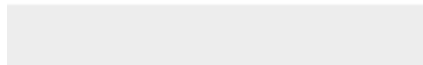
Best regards,

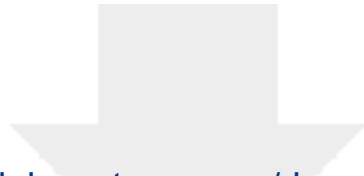
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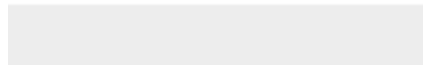
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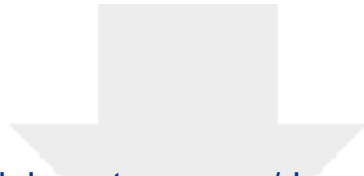


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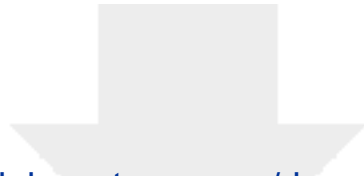




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**Table\_S4.xlsx**



1 **Gut Dysbiosis Signatures Associated with Hepatitis B Virus-Related Diseases: An**  
2 **Insightful Review Using AI-Powered Research Tools**

3 Reham MAGDY WASFY<sup>1,2</sup>, Maryam TIDJANI ALOU<sup>1,2</sup>, Patrick Borentain<sup>3</sup>, Stéphane  
4 Ranque<sup>1,4</sup>, Didier RAOULT<sup>1,2</sup>, René Gérolami<sup>1,2,3</sup>, Matthieu MILLION<sup>1,2,5\*</sup>

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18 **ABSTRACT**

19 **Background:** Hepatitis B virus (HBV) has threatened public health, mainly resulting in liver  
20 fibrosis, cirrhosis, and even damage due to hepatic carcinoma. Growing evidence has  
21 supported the role of gut microbiota in different stages of liver disease. However, the  
22 mechanisms of hepatic injury from gut microbiota dysbiosis are not fully understood.  
23 Moreover, no consensus has been reached on the microbial signatures across different disease  
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41 indicate a signature microbial composition in HBV-associated infection despite the  
42 challenging microbiota composition detected in different disease subgroups. These taxa

43 showed diagnostic power between health and most disease stages, implicating the potential of  
44 the gut microbial as non-invasive biomarkers for diagnosing pan-HBV-induced liver disease.

45 **KEYWORDS**

46 Hepatitis B virus infection, Gut Dysbiosis, Fecal Microbiota, Mycobiota, Systematic Review,  
47 Artificial Intelligence.

## 48 INTRODUCTION

49 About 296 million individuals are chronically infected with hepatitis B virus (HBV)  
50 worldwide, with 1.5 million new infections yearly [1]. Despite the World Health  
51 Organization's objective of eliminating viral hepatitis as a public health hazard by 2030,  
52 annual global HBV mortality is expected to rise by 39% from 2015 to 2030 if current trends  
53 continue [2]. Different disease stages are diagnosed by hepatitis B-e antigen (HBeAg), HBV  
54 DNA levels, alanine aminotransferase (ALT) values, and liver inflammation. Balancing the  
55 pathogenic ability and immunity defense, some patients could be carriers or experience  
56 chronic hepatitis B (CHB), characterized by elevated ALT levels and moderate/severe liver  
57 diseases [3]. Although the CHB disease course has been described in the literature, the  
58 pathophysiology of HBV disease progression is not entirely understood [4,5]. Many studies  
59 highlighted the role of different factors, such as HBV viral load, HBV mutations, and host  
60 immunity, in the disease progression [6–8]. CHB infection tends to be asymptomatic;  
61 however, tissue repair against inflammation makes CHB patients at high risk of manifesting  
62 fibrosis, cirrhosis as well as fatal complications, such as portal hypertension, spontaneous  
63 bacterial peritonitis (SBP) [9], hepatic encephalopathy (HE) [10], and hepatocellular  
64 carcinoma (HCC) [11].

65 'Gut-Liver-Axis' describes the bidirectional interconnecting relationship between the  
66 gut and liver where bile, nutrients, and other metabolites are exchanged [12]. Typically, the  
67 gut hosts a myriad of microbiota, such as bacteria [13], fungal species [14], and  
68 bacteriophages [15], described as a superorganism [16], that play different complex  
69 immunological and nutritional roles in both the gastrointestinal tract and the liver [17,18].  
70 Predominant strains in the adult intestine belong to the major phyla of *Bacteroidota* (formerly  
71 *Bacteroidetes*), *Bacillota* (formerly *Firmicutes*), and *Proteobacteria* (formerly  
72 *Pseudomonadota*) containing gram-negative bacteria and gram-positive *Clostridia* [19]. A

73 growing body of literature suggests a connection between gut microbiota dysbiosis and CHB  
74 [20–22], in addition to significant differences in gut microbial composition between CHB or  
75 HBV-related liver cirrhosis (LC) and healthy controls have been widely reported [23,24].  
76 However, no agreement has been reached on distinctive microbial signatures across different  
77 disease stages.

78         Advancements in therapeutic options, including interferon and antiviral treatment  
79 (AVT), can only suppress HBV DNA replication or reduce cirrhosis and HCC complications  
80 but cannot cure CHB [25,26]. Therefore, further development of new treatment methods is  
81 critical for the global eradication of HBV. Additionally, emerging data reveals that directed  
82 treatment of dysbiosis may have potential therapeutic value in liver disease and CHB  
83 management [27,28]. Therefore, we addressed the cutting edge of the latest microbiota-  
84 targeted therapies in CHB patients with highlights on the future research directions of clinical  
85 trials.

86         Herein, the objectives of the current review will mainly summarize and discuss: (1)  
87 Gut microbiota alteration in different stages of HBV; (2) A distinctive microbiome signature  
88 in HBV-infected patients at various stages of the disease. These profiles might make it  
89 possible to find novel HBV diagnostic and treatment options: (3) Gut microbiota-related  
90 mechanisms of liver fibrosis, and (4) Gut microbiota-targeted treatment for HBV infection  
91 and associated complications.

92

## 93 **1. DATA COLLECTION**

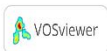
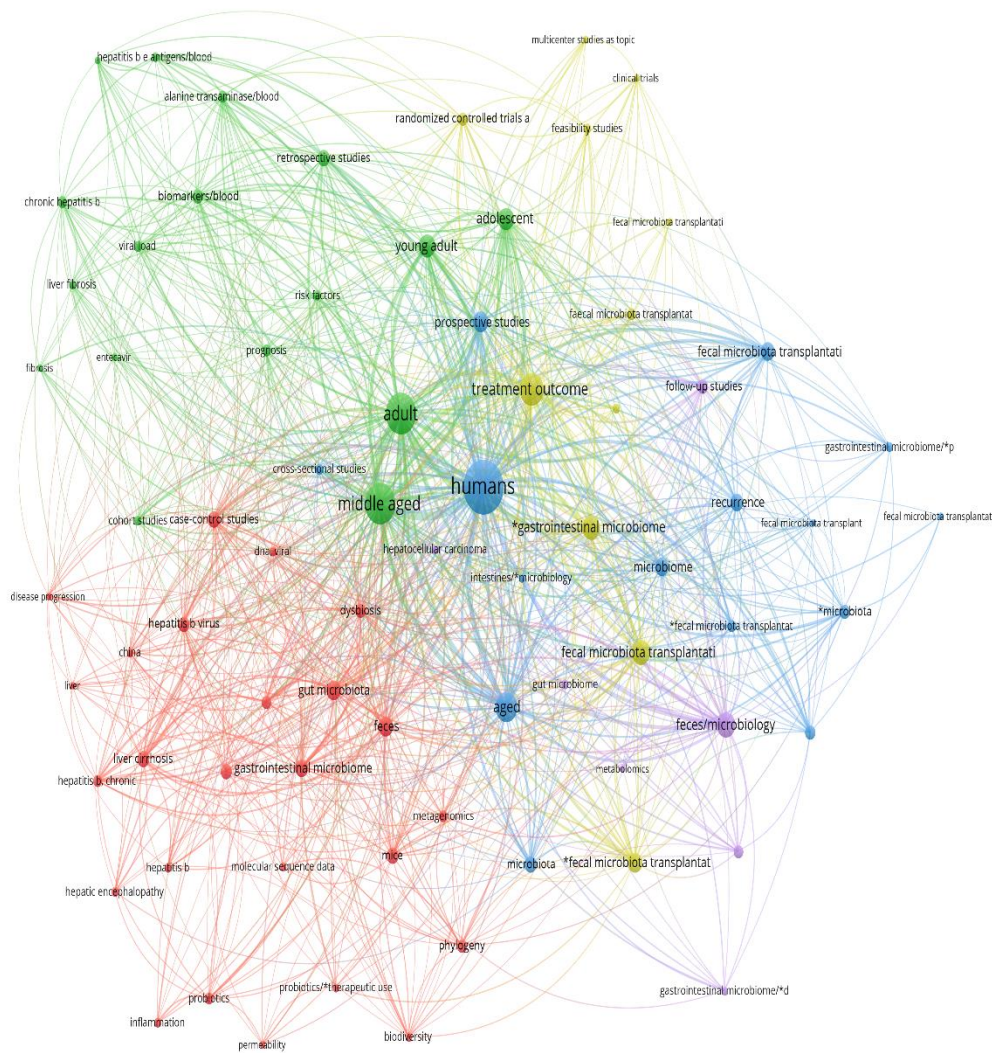
### 94 **1.1 Methods of Bibliographic strategy and selection criteria**

95 A systematic literature search was conducted for case-control studies on CHB and  
96 microbiota. In a comprehensive search, PubMed, Google Scholar, Wiley Library, and Web of  
97 Science (WOS), as well as artificial intelligence (AI)-powered research tools such as

98 Semantic Scholar and Scite, were investigated. Additionally, cross-referencing of all selected  
99 articles was reviewed. The search keywords and their synonyms were identified by MeSH  
100 database. The search was applied from 2000 and included only the articles written in English.  
101 We used Publish or Perish [29] and Zotero [30] software to retrieve and analyze citations.  
102 In order to include all relevant literature and decrease the chances of missing relevant trials,  
103 we generated a broad screening approach with the query using the keywords in the following  
104 syntax ("metagenom\* OR microbio\* OR fecal flora OR intestinal flora OR gut bacteria OR  
105 Human microbio\* OR dysbios\* OR disbios\* OR bacterial translocation OR disbacterios\*"  
106 AND "HBV" OR "chronic hepatitis B" OR hepatitis B AND virus AND infection AND  
107 Chronic OR hepatic cirrhosis OR liver fibrosis" OR "gut liver axis" OR pr?biotic\* OR "fecal  
108 microbiota transplantation" OR "Fecal transplantation"). As detailed in **Supplementary**  
109 **Table S1**, this syntax has been adjusted for every database.

110 A mapping of terms used in the titles/Abstracts of HBV-studied microbiome articles  
111 retrieved from different databases is illustrated in **Fig. 1**. This indicates that further  
112 explorations and more studies and other geographical locations into investigating dysbiosis in  
113 CHB patients are needed.





114

115 **Figure 1: Visualization of Term Mapping using VOS Viewer Software.**

116 The figure provides a visualization of the most common terms in the 334 retrieved articles of this review about  
 117 gut microbiota dysbiosis in HBV-related infection in several screened databases. The terms include all keywords  
 118 or MeSH terms used in the titles/Abstracts of 307 HBV-studied microbiome articles between 2000 and 2023.

119 The figure illustrates the mapping of terms using VOS Viewer software, showcasing the relationships and  
 120 connections between different terms. Terms in this network are connected by co-occurrence (based on the  
 121 documents' terms occurring together) to discover the best search keywords and define the popularity, age, and  
 122 impact of topics in the detected studies. The minimum number of occurrences of a keyword is *five*. Out of 1825  
 123 keywords, 110 met the threshold. For every 110 keywords, the total strength of the co-occurrence links with  
 124 other keywords was calculated. The keywords with the greatest total link strength were selected.

125 The color-coded nodes and edges represent clusters and associations, and terms – are located relative to each  
126 other so that the distance between two objects is an approximate measure of their similarity within the set,  
127 providing insights into the semantic landscape of the analyzed articles. The terms are grouped in colored  
128 clusters. Cluster 1 (**Red**; 25 items) as China, metagenomics, gut microbiota, hepatitis B, chronic, case-control  
129 studies; Cluster 2 (**Green**, 17 items) as adult, prognosis, fibrosis; Cluster 3 (**Blue**, 15 items) as fecal microbiota  
130 transplant, humans, microbiome, microbiota, prospective studies; Cluster 4 (**Yellow**, *eleven* items) as fecal  
131 microbiota transplants, clinical trials; Cluster 5 (**Purple**, *seven* items) as gut microbiome, metabolomics.

132 The Preferred Reporting Items for Systemic Reviews and Meta-Analysis Statement  
133 (PRISMA) guidelines were followed (**Fig. 2A**). The following inclusion criteria were  
134 considered: (1) Study design: peer-reviewed and published cross-sectional, case-control,  
135 cohort studies and interventional studies reporting the effects of HBV on human gut  
136 microbiota composition in adults, (2) Participants: case of patients diagnosed with HBV  
137 carriers, CHB and HBV-related complications such as LC, HCC, or liver transplantation  
138 (LT). Controls are population-based or hospital-based controls without related liver diseases.  
139 (3) Main outcome reported different taxa abundance as phylum, class, order, family, genus,  
140 and species of fecal microbiota or well-defined clinical outcome parameters associated with  
141 HBV-associated dysbiosis. (4) Publishing time cutoff is June 2023. The exclusion criteria  
142 included studies without a control group, cirrhosis studies not related to HBV infection, *in*  
143 *vitro* studies, studies without data on gut microbiome composition, studies reported in  
144 languages other than English, and gray literature

145

#### 146 **Study Selection and Data Extraction**

147 A total of 1888 articles were retrieved from PubMed, 1183 articles from Google Scholar, 588  
148 from Wiley Library, 1497 from WOS, 379 from Semantic Scholar, and 194 from Scite, for a  
149 total of 5729. The first selection based on titles and abstracts yielded 334 articles, of which 70  
150 duplicates were eliminated. After removing duplicate results, a list of 86 eligible publications

151 was generated, screening titles and abstracts. Each publication on this list was further  
152 reviewed independently to confirm adherence to the inclusion/exclusion criteria. Afterward,  
153 the full texts of relevant articles were retrieved and thoroughly inspected. Extracted data  
154 included study design, populations, sample size, study country, and gut microbiome analysis  
155 methodology. Extracted outcomes included HBV-related dysbiosis and assessment of hepatic  
156 markers, metabolites, immunological markers, and bile acids.

157

## 158 **1.2 Results of Bibliographic Search**

159 This systematic review yielded a total of 61 articles (**Fig. 2A**), including (25 eligible human  
160 case-control microbiome studies, *three* mycobiome studies, 17 microbiota-targeted  
161 therapeutic studies, *eight* mouse-model studies, and *nine* other studies (virome, probiotics  
162 studies)). AI tools such as Semantic Scholar shared the identification of 39.2% (22/56) of the  
163 studies found by manual citation searching with *six* studies that other databases haven't  
164 retrieved (**Fig. 2B**). Indicating that compared to traditional approaches, AI-powered research  
165 tools may be effective in decreasing the time required for literature search. However, we did  
166 not employ these AI tools or other AI-assisted technologies to replace our primary duty of  
167 data interpretation or scientific conclusion formulation. The included studies evaluated HBV-  
168 related dysbiosis in different patient subgroups. All the selected studies met the quality  
169 assessment requirements based on the modified STROBE checklist. A total of 24 papers were  
170 removed, with the reasons given in the **Supplementary Table. S2**.

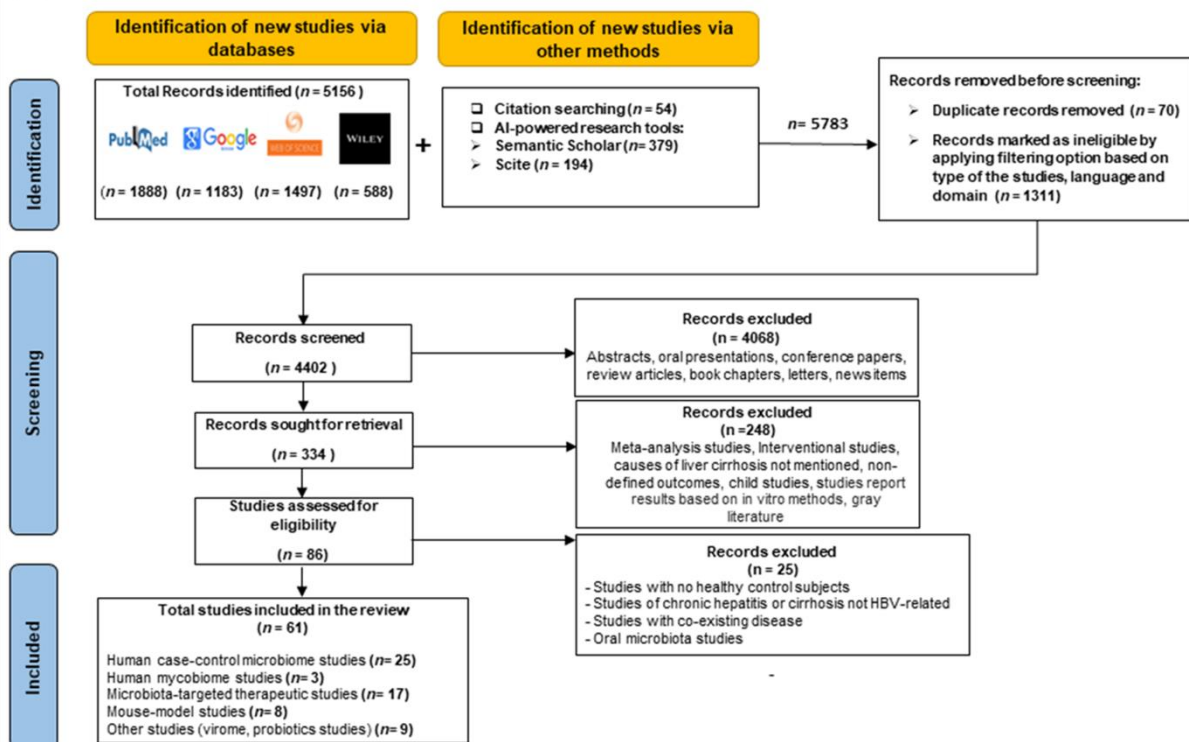
171 Study characteristics are demonstrated in detail in the **Supplementary Table. S3**. Different  
172 molecular methods were used to characterize gut microbiota, including denaturing gradient  
173 gel electrophoresis (DGGE), Quantitative PCR (qPCR), and sequencing of the 16s rRNA  
174 genes. All microbiota analyses were investigated in adults. All microbiome studies were

175 performed in Asia (22 studies were performed in China, *two* in South Korea, and *one* in  
 176 Taiwan).

177 Regarding human microbiome studies, *seven* reported a correlation between gut dysbiosis and  
 178 hepatic markers, *three* reported changes in bile acids, *three* reported changes in  
 179 immunological markers, and *six* showed changes related to metabolic markers. Most studies  
 180 ( $n=23$ ) reported changes in microbiota profile to the genus level, while only seven reported  
 181 the species level. Different studies have reported either no change in beta microbial diversity  
 182 or difference between HBV-infected subgroups.

183

(A)



184



185

186 **Figure. 2 Data search and Collection process.** (A) A flowchart of how articles were identified and filtered to  
 187 be included in the systematic review based on the PRISMA statement. (B) A chart of the detailed number of  
 188 studies detected in all included databases applying different filters: Filter\_1 (language/ year/domain); Filter\_2  
 189 (exclusion of poster, review articles, book chapters, editorial material, letter abstract, early access); Filter\_3  
 190 (suitable title/abstract); Filter\_4 (inclusion full appropriate articles and applying manual citation searching).  
 191 Tools such as Semantic Scholar allowed the identification of 22 studies (the same studies found by manual  
 192 citation searching) with *six* studies that other databases haven't retrieved.

193 **2. GUT DYSBIOSIS CONTRIBUTES TO LIVER INJURY THROUGH GUT-LIVER**  
194 **AXIS**

195 The individual's microbiota profile vastly differs between geographic regions [31]. In  
196 addition to harboring other fungi and bacteria, the gut is also a binding site for the induction  
197 of host immunity, affecting the gut mucosal barrier, lymphoid tissues, and regulatory T cells.  
198 Host immunity can be promoted when epithelial cells express pattern recognition receptors as  
199 toll-like receptors (TLR) specific to microbial particles like lipopolysaccharides (LPS) or  
200 peptidoglycans [32,33].

201 Usually, the intestinal microbiota and the liver coexist separately; however, the liver  
202 can be exposed to intestinal microbes during gut dysbiosis or liver cirrhosis [34]. Several  
203 studies have investigated the relationship between gut dysbiosis and cirrhosis of different  
204 aetiologies, such as cholangitis, metabolic liver disease, and alcoholic liver disease [35–38],  
205 with growing evidence suggesting that gut dysbiosis may accelerate simple steatosis to  
206 steatohepatitis [37]. So, the common mechanisms of gut dysbiosis-mediated liver injury  
207 could be endotoxemia, intestinal barrier dysfunction, and bile acid fluctuations [36]. Growing  
208 evidence suggests that gut dysbiosis and CHB infection may interact similarly to other liver  
209 diseases [39]. Therefore, this study summarizes the most recent available data about HBV-  
210 associated dysbiosis and the pathophysiology of related liver injury.

211

212 **3. CHARACTERISTICS OF HEPATITIS B PATIENTS WITH DYSBIOSIS**

213 Studies have shown that HBV-infected patients tend to have different gut microbiota profiles  
214 than healthy controls (**Supplementary Table. S3**). Patients with HBV infection may  
215 experience different disease stages from HBV carriers, acute HBV infection to CHB without  
216 liver injury until HCC, depending on the host and viral variables [3]. In this part, HBV-  
217 related different stages of disease will be discussed concerning gut dysbiosis.

### 218 3.1 Patients with no/mild liver injury (compensated patients)

219 Patients in the compensated stage are often asymptomatic [40] or have treatable liver disease  
220 and, therefore, we considered a) HBV carriers, b) acute HBV infection, c) CHB or cirrhosis  
221 with Child-Turcotte-Pugh (CTP)- class A, are compensated patients with no/mild liver injury.

222

#### 223 a) Hepatitis B virus (HBV) carriers

224 After the infection, HBV may be spontaneously cleared or cause chronic disease in different  
225 individuals depending on factors such as age and immunity [41]. The same phenomenon has  
226 been observed in animal experiments, in which hepatitis B surface antigen (HBsAg) of  
227 immature mice remained positive [42]. A study by Lu et al. showed a decrease in the  
228 *Bifidobacteria/Enterobacteriaceae* (B/E) ratio in HBV carriers compared to controls [43].  
229 However, HBV carriers with normal ALT were somewhat similar to the control group but  
230 significantly different from those with high ALT, and the B/E ratio was not different between  
231 the groups [44]. They indicated that HBV carriers could have a similar microbiota profile as  
232 controls.

233 In addition, Chen et al. reported that HBV carriers had the highest observed species as  
234 controls but the lowest *Fusobacteria*, *BetaPseudomonadota*, and stable *Bacteroides*  
235 compared to controls [45]. Interestingly, Yang et al. suggested that HBV carriers might be the  
236 most suitable donors for fecal microbiota transplantation (FMT) for higher  $\alpha$  diversity and  
237 abundance of potentially beneficial *Lachnospira* genera [46]. Moreover, a recent study  
238 reported that gut microbiome in HBV carriers was dominated by taxa involved in fatty acid  
239 and lipid metabolism, such as *Eubacterium coprostanoligenes*, contributing to the  
240 cholesterol-lowering effects in HBV patients and *Alloprevotella* producing short-chain fatty  
241 acids (SCFAs) [47].

242 **b) Acute HBV infection**

243 Due to the difficulty of studying acute HBV infection in humans, animal studies have been  
244 used. By using a mouse model of both acute and CHB infection, Zhu et al. reported that  
245 *Bifidobacterium* and *Lactobacillus* peaked in both disease groups compared to controls at day  
246 14 and the ratio of *Firmicutes/Bacteroides* increased in the early stages of infection (Day 14)  
247 and decreased significantly over time (Day 49) [48]. A similar study on HBV-infected and  
248 ETV-treated mice also reported significant changes in genera, but found increases in  
249 *Butyricoccus* and decreases in *Clostridium* instead [39].

250

251 **c) Chronic hepatitis B (CHB): Non-end stage**

252 In humans, CHB is significantly correlated with changes to gut microbiota even with no/mild  
253 liver injury. CHB patients with CTP scores <9 (classes A and B) reported a significant  
254 increase in the *Veillonellaceae* family and a decrease in the *Alistipes*, *Asaccharobacter*,  
255 *Bacteroides*, *Butyricimonas*, *Parabacteroides*, and *Ruminococcus* genera [24]. Another study  
256 in CHB with fibrosis observed an increase in *Prevotella* and a decrease in the same genera as  
257 the previous study, responsible for bile acid metabolism, such as *Bacteroides*, *Clostridium*,  
258 *Lactobacillus*, *Ruminococcus* and *Bifidobacterium* [49]. These data demonstrate that  
259 compensated CHB infection with no/mild liver injury is associated with significant changes  
260 in the gut microbiota.

261 To further understand the gut microbiota dynamics in CHB, some studies investigated  
262 clinical indicators reflecting liver function and infection state. A recent study showed that  
263 *Lactobacillus*, *Clostridium*, and *Bifidobacterium* abundance was lower in patients with  
264 normal ALT than in controls [50]. In addition, *Bacteroides fragilis*, bile salt hydrolase (BSH)  
265 containing species, and the only member of archaeon harboring BSH activity,  
266 *Methanobrevibacter smithii*, was significantly lower in patients with normal ALT group [50].



267 Moreover, *Streptococcus*, *Veillonella*, and *Haemophilus* showed positive correlations with  
268 aromatic amino acids (such as phenylalanine and tyrosine), which are assumed to play roles  
269 in CHB progression [24]. In addition, *Dialister*, unclassified *Clostridiales* and  
270 *Ruminococcaceae* were positively associated with some hepatic inflammatory markers and  
271 enzymes [24].

272

### 273 **3.2 Patients with advanced stages of liver injury (decompensated patients)**

274 A growing number of studies have also revealed a correlation between the gut microbiota and  
275 advanced stages of HBV infection characterized by a) CHB-associated liver cirrhosis (CHB-  
276 LC), b) HCC, and c) HE. Liver cirrhosis is a dangerous premalignant condition with an  
277 increasing incidence of genetic aberrations and an elevated risk of HCC [51,52]. The  
278 diagnosis of decompensated cirrhosis is based on the presence of ascites, variceal bleeding or  
279 HE, and liver failure (LF) in the context of massive infiltration from a tumor [53].

280

#### 281 **a) Chronic hepatitis B-associated liver cirrhosis (CHB-LC): End-stage CHB**

282 Patients with CHB-LC showed lower diversity with the severity of the disease [45,46]. In a  
283 study by Deng et al., *Veillonellaceae* and *Lachnospiraceae* families were depleted, and  
284 *Megamonas* was enriched in patients with CHB-LC compared to controls [54]. Additionally,  
285 *Enterobacteriaceae* increased while *Bifidobacteria* and lactic acid bacteria (*Lactobacillus*,  
286 *Pediococcus*, *Leuconostoc*, and *Weissella*) showed a marked decrease in CHB-LC [43].  
287 Moreover, the B/E ratio can be used for tracing the progression of liver disease and was  
288 found to be decreased in CHB-LC [43].

289 According to the combined results of multiple studies, there is a well-acknowledged  
290 decrease in *Firmicutes* abundance and an increase in *Pseudomonadota* during the progression  
291 of CHB-LC [55]. However, a previous study reported a reduction in both *Bacteroidetes* and

292 *Firmicutes* from healthy to cirrhotics, while *Pseudomonadota* and *Actinobacteria* increased  
293 stepwise from healthy to cirrhotics [45]. *Bacteroides* were similar across all tested groups at  
294 the genus level, while *Haemophilus*, *Fusobacterium*, *Veillonella*, *Streptococcus*, and  
295 *Ruminococcus* increased in later disease stages. Additionally, two species were reported  
296 *Dialister succinatiphilus* and *Alistipes onderdonkii*, to be abundant in controls and decrease  
297 significantly with disease progression [45].

298 Additionally, dynamic alteration of gut microbiota is a valuable indicator to predict the  
299 prognosis of end-stage liver disease. *Enterococcus* was significantly higher, as mentioned in  
300 in the HBV-related LF (progression group), while abundant *Faecalibacterium* was associated  
301 with the regression group [56]. Moreover, a higher abundance of *E. coli* is consistent with an  
302 increasing level of LPS in the circulation of patients with end-stage liver disease [57–59].  
303 Interestingly, the microbiota profile of cirrhotics appeared to be more oriented toward  
304 methanethiol (CH<sub>3</sub>SH) production, whereas the control group resembled hydrogen sulfide  
305 (H<sub>2</sub>S) production. Serum methanethiol levels are linked to complications such as HE,  
306 implying a solid connection between dysbiosis and the severity of liver diseases [60].  
307 Furthermore, compared to compensated cirrhosis, the gut microbiota composition in the  
308 decompensation stage is characterized by an increase in the abundance of potentially  
309 pathogenic bacteria, particularly *Alcaligenaceae*, *Porphyromonadaceae*, *Veillonellaceae*, and  
310 *Enterobacteriaceae* [61].

311

#### 312 **b) Hepatocellular carcinoma (HCC)**

313 Patients with HCC tend to present a distempered gut microbiota and abnormal metabolites  
314 [62]. Zheng et al. reported a depletion in butyrate-producing genera and an increase in LPS-  
315 producing genera; moreover, *Clostridioides* was positively related to the tumor size of HCC  
316 [22]. This finding was supported by another study that reported *Bacteroides*, *Lachnospiraceae*,

317 and *Clostridium XIVa* to be enriched in HCC patients, with a positive correlation with the  
318 tumor burden [63]. Additionally, *Parabacteroides* were significantly increased in the non-  
319 small group compared to the small HCC group [63]. By integrating the clinical characteristics  
320 and database analysis, serum bile acids as metabolites of the previous three genera could be  
321 essential mediators in promoting liver carcinogenesis.

322 Moreover, the abundance of *Prevotella* was much more significant in HBV-related HCC  
323 compared to non-HBV-HCC [62]. Interestingly, a recent study investigated gut microbiota  
324 profiles in HBV-related HCC patients with post-hepatectomy liver failure (PHLF) [64].  
325 *Bacteroides*, *Faecalibacterium*, and *Pantoea* abundances were higher post-operatively, while  
326 *Faecalibacterium* and *Subdoligranulum* were abundant pre-operative. However, the bacteria  
327 with significant postoperative differences showed no significant differences preoperatively,  
328 except for *Faecalibacterium* [64], which could be used as a potential marker of disease  
329 diagnosis and follow-up.

330

### 331 c) Hepatic encephalopathy (HE)

332 Hepatic encephalopathy (HE) is a fatal central nervous system complication caused by acute  
333 or chronic hepatitis or decompensated cirrhosis [65]; characterized by ammonia-related  
334 cerebral edema and consciousness disturbance [66].

335 Interestingly, research has shown a significant increase  
336 in *Veillonellaceae*, *Enterococcus*, *Megasphaera*, and *Burkholderia* in cirrhotic patients with  
337 HE. This taxonomic shift was associated with hyperammonaemia and systemic inflammation,  
338 contributing to worsening HE symptoms [67]. Other studies showed that cirrhosis-related  
339 cognitive dysfunction is associated with decreased abundance of autochthonous families and  
340 increased *Alcaligenaceae* and *Porphyromonadaceae* [68,69]. Even though the exact  
341 underlying mechanisms of HE in patients with cirrhosis remain unclear, the accumulation of

342 microbe-derived products such as ammonia, mercaptans, and benzodiazepine-like substances  
343 [61], as well as neurotransmitters produced by the microbiota, including serotonin dopamine  
344 [70] are believed to play a critical role. These substances can cross the blood-brain barrier  
345 and affect astrocyte function, likely to result in neurotransmission disorders [65]. Those  
346 findings suggest the impact of the network between the enteric and autonomic nervous  
347 systems on gut microbiota.

348

#### 349 **4. Intestinal microbiota signatures) in HBV-associated dysbiosis and related** 350 **pathophysiology**

351 It is essential to consider regional and geographical differences using gut microbiota profiles  
352 as diagnostic tools. The presence and accuracy of biomarkers found in one study may be  
353 completely different in another [31,71]. Therefore, we summarized the abundant taxa  
354 reported in various HBV-related diseases (**Fig. 3; Supplementary Table. S4**) based on the  
355 qualitative analysis of the 25 human case-control microbiome studies included in this review  
356 (**Supplementary Table. S3**). Notably, the mentioned abundance in each group was compared  
357 to the controls. Consistently dysregulated microbiota in at least two datasets were  
358 highlighted, denoting reproducible results, as depicted in **Fig. 3**. However, the CHB-LT  
359 group showed no reproducible results (**Supplementary Table. S4**).

Abundant Taxa	HBV-Related Diseases				
	HBV-Carriers	CHB	CHB-LF	CHB-LC	CHB-HCC
<b>Phylum</b>					
<i>Bacteroidota</i> (formerly <i>Bacteroidetes</i> )	↓ (3)	↑ (4)	↓ (1)	↓ (5)	↑ (3)
<i>Bacillota</i> (formerly <i>Firmicutes</i> )	↓ (2)	↓ (4)	↑ (1)	↓ (7)	↓ (3)
<i>Pseudomonadota</i> (formerly <i>Proteobacteria</i> )	↑ (1)	↑ (4)	↑ (1)	↑ (7)	↑ (4)
<i>Actinomycetota</i> (formerly <i>Actinobacteria</i> )	↑ (1)	1:1	↑ (1)	↑ (4)	↑ (2)
<i>Fusobacteriota</i>	↑ (1)	↑ (1)	NA	↑ (2)	↑ (2)
<b>Class</b>					
<i>Clostridia</i>	neutral* (1)	↓ (2)	NA	↓ (3)	↓ (1)
<i>Bacteroidia</i>	NA	↑ (3)	NA	1:1	↑ (1)
<i>Gammaproteobacteria</i>	neutral* (1)	↑ (2)	NA	↑ (3)	NA
<i>Actinobacteria</i>	↑ (1)	1:1	NA	↑ (2)	↓ (1)
<i>Bacilli</i>	↑ (1)	1:1	NA	↑ (3)	↑ (2)
<b>Order</b>					
<i>Eubacteriales</i> (formerly <i>Clostridiales</i> )	↑ (1)	↓ (1)	NA	↓ (3)	1:1
<i>Bacteroidales</i>	NA	↑ (3)	NA	1:1	↑ (2)
<i>Enterobacteriales</i>	NA	↑ (1)	NA	↑ (2)	1:1
<i>Lactobacillales</i>	NA	neutral* (1)	NA	↑ (2)	↑ (2)
<b>Family</b>					
<i>Bifidobacteriaceae</i>	↓ (1)	↓ (2)	NA	↓ (2)	↓ (1)
<i>Enterobacteriaceae</i>	↓ (1)	1:1	NA	↑ (6)	↑ (2)
<i>Lachnospiraceae</i>	NA	1:1	NA	↓ (6)	↓ (2)
<i>Veillonellaceae</i>	NA	↑ (1)	NA	↑ (2)	NA
<i>Rikenellaceae</i>	NA	↑ (1)	NA	↓ (3)	1:1
<i>Ruminococcaceae</i>	NA	↓ (2)	NA	↓ (6)	↓ (2)
<i>Coriobacteriaceae</i>	NA	1:1	NA	↓ (2)	↓ (1)
<i>Clostridiaceae</i>	NA	↓ (1)	NA	↓ (4)	↓ (2)
<i>Bacteroidaceae</i>	NA	↑ (1)	NA	↓ (2)	1:1
<i>Streptococcaceae</i>	NA	neutral* (1)	NA	↑ (4)	↑ (2)
<i>Prevotellaceae</i>	NA	↑ (2)	NA	1:1	1:1
<b>Genus</b>					
<i>Clostridium</i>	1:1	↓ (2)	1:1	↓ (4)	↑ (1)
<i>Bifidobacterium</i>	neutral* (1)	↓ (3)	NA	↓ (3)	1:1
<i>Lactobacillus</i>	1:1	↓ (2)	↑ (2)	2:2	↑ (1)
<i>Veillonella</i>	1:1	↑ (3)	↑ (1)	↑ (4)	↑ (4)
<i>Megamonas</i>	↑ (1)	↑ (2)	↓ (1)	1:1	↓ (1)
<i>Desulfovibrio</i>	↑ (2)	↑ (1)	NA	↑ (1)	NA
<i>Prevotella</i>	↑ (1)	↑ (4)	↓ (2)	↓ (3)	↑ (2)
<i>Bacteroides</i>	↓ (1)	↑ (3)	↓ (1)	↓ (4)	↑ (2)
<i>Ruminococcus</i>	↑ (1)	↑ (2)	NA	3:3	↓ (2)
<i>Escherichia/Shigella</i>	↑ (1)	1:1	↑ (1)	↑ (2)	1:1
<i>Parabacteroides</i>	NA	↑ (1)	NA	1:1	↑ (2)
<i>Enterococcus</i>	↑ (1)	↑ (1)	↑ (3)	↑ (2)	1:1
<i>Faecalibacterium</i>	NA	↑ (2)	↑ (1)	1:1	↓ (2)
<i>Akkermansia</i>	↑ (1)	↑ (1)	NA	↑ (2)	NA
<i>Streptococcus</i>	↑ (1)	↑ (5)	↑ (2)	↑ (6)	↑ (3)
<i>Blautia</i>	↑ (1)	↑ (2)	↑ (1)	2:2	↑ (2)
<i>Roseburia</i>	↓ (1)	↓ (2)	↓ (1)	↓ (2)	↓ (1)
<i>Haemophilus</i>	NA	↑ (2)	NA	↑ (1)	NA
<i>Fusobacterium</i>	1:1	↑ (2)	↓ (1)	1:1	NA
<i>Sutterella</i>	↑ (1)	↑ (3)	NA	↑ (1)	NA
<i>Proteus</i>	NA	NA	NA	↑ (1)	↑ (2)
<i>Lachnospira</i>	↑ (1)	↓ (2)	↓ (1)	↓ (3)	↓ (3)
<i>Klebsiella</i>	↑ (1)	↑ (1)	↑ (2)	↑ (2)	↑ (1)
<b>Species</b>					
<i>Bacteroides eggerthi</i>	NA	↓ (1)	NA	↓ (2)	NA
<i>Escherichia coli</i>	NA	NA	NA	↑ (2)	NA

Reproducible results in at least 2 studies  
 Reproducible results in > 2 studies  
 Contradicting results (same number of studies ↑ vs. ↓)

360

361

**Figure. 3** A chart summarizing consistently dysregulated gut microbiota taxa in at least two studies. Data was

362

collected from 25 human case-control studies involved in this review (**Supplementary Table. S4**). The results

363

represent the comparison of each group vs. the control group. Results in gray are non-reproducible.

364 \*: Neutral means no difference in abundance compared to the controls. Numbers between brackets denote the  
365 number of studies. ↑, increase in abundance reported; ↓, decrease in abundance reported  
366 **HBV**: Hepatitis B virus; **CHB**: Chronic hepatitis B infection; **CHB-LF**: Chronic hepatitis B-associated liver  
367 failure; **CHB-LC**: Chronic hepatitis-associated liver cirrhosis; **CHB-HCC**: Chronic hepatitis B-associated  
368 hepatocellular carcinoma; **NA**: Not available.

369         Studies have shown that HBV-infected patients tend to have different abundance of  
370 gut microbiota profiles (**Fig. 3**). This section will cover the previously described gut  
371 microbiota with consistent dysregulated patterns (**Fig. 3**). In addition, the mechanisms by  
372 which microbiota-mediated liver injury occurs will be discussed.

373

#### 374 *Pseudomonadota*

375 Many human studies have reported a consistent increase in *Pseudomonadota* abundance  
376 [45,50,55,57]. *Pseudomonadota* is divided into *six* classes; among them is the  
377 *Gammaproteobacteria* class, harboring human pathogens as *Escherichia* species [72].  
378 Interestingly, those species were also highly abundant in HBV-related diseases [45,50,55,57].

379         *Pseudomonadota* was reported to cause hepatic injury via LPS-produced endotoxemia  
380 [73]. Moreover, LPS increases blood-brain barrier permeability and stimulates tumor necrosis  
381 factor (TNF), causing HE [74]. Interestingly, significantly abnormal LPS distributions were  
382 observed in CHB patients with the highest levels at the peak of liver failure. Besides, LPS  
383 changes were related to disease severity, implying that LPS causes secondary hepatic injury  
384 [75].

385

#### 386 **The *Bifidobacteria/Enterobacteriaceae* (B/E) ratio**

387 Several studies have used the shift in microbiota taxa to create unique heuristics that may  
388 distinguish between distinct disease progression stages [43,45,55]. One is the  
389 *Bifidobacteria/Enterobacteriaceae* (B/E) ratio, initially discussed in the HBV study by Lu et

390 al. 2011 [43]. The study reported low *Bifidobacteria* abundance in CHB patients and even  
391 lower in cirrhotic patients [43]. Moreover, *Enterobacteriaceae* family (mainly of Gram-  
392 negative bacteria with LPS [76]) was reported to be highly abundant in LC more than CHB  
393 [22,45,55,57]. However, other studies have found no difference in the B/E ratio between  
394 disease groups [24,44]. This suggests the B/E ratio is disturbed only in cirrhosis; however,  
395 those conclusions require further research on a larger population to be confirmed.

396

### 397 **The *Bacteroidetes/Firmicutes* (B/F) ratio**

398 The increased ratio of B/F was associated with inflammatory disorders in CHB-related  
399 complications [55,74,77]. Hence, many *Bacteroidetes* are Gram-negative bacteria that  
400 produce LPS, whereas *Firmicutes* are Gram-positive bacteria; an increased B/F ratio means a  
401 higher burden of LPS exposure [54]. As previously mentioned, evidence is emerging that  
402 CHB patients were often accompanied by metabolic endotoxemia, producing pro-  
403 inflammatory cytokines such as TNF- $\alpha$  and reactive oxygen species (ROS), promoting  
404 pathogenesis in liver diseases [78].

405

### 406 **Lactic acid-producing bacteria (LAB)**

407 Intestinal LAB bacteria include species belonging to genera such as *Lactobacillus*,  
408 *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, and  
409 *Weissella* [79]. Interestingly, one study has explicitly investigated the protective role of the  
410 total *Lactobacillus* species by quantitative PCR (qPCR) technique [80]. Wu et al. reported  
411 that *Lactobacillus* diversity was lowest in CHB-LC compared to the CHB-LT and control  
412 group. CHB-LC group showed an increase in *L. gasseri* and a decrease in *L. acidophilus* and  
413 *L. reuteri* [80]. Those *lactobacilli* may be involved in intestinal homeostasis. A previous  
414 study reported that *L. gasseri* and *L. acidophilus* produce gassericin A and acidocin B

415 bacteriocins with antibacterial activities [81]. Moreover, *L. reuteri* inhibited TNF- $\alpha$  induction  
416 by inducing various cytokines and may inhibit the actions of other *Lactobacillus* species,  
417 such as *L. rhamnosus* and *L. fermentum* [82].

418 Nevertheless, some studies reported increased abundance in the *Lactobacillales* order  
419 in CHB-LC [55,57] and the *Lactobacillaceae* family in CHB-HCC [45,83]. One study  
420 reported a marked decrease in *Lactobacillus* in CHB and CHB-LC [43]. Conversely,  
421 beneficial *Lactobacillus* was higher in patients with decompensated LC than those with  
422 compensated LC [84]. This finding suggests that *lactobacillus* in decompensated LC  
423 contradicted its probiotic function [74]. Therefore, further *Lactobacillus* investigation to the  
424 species level is recommended in HBV-related diseases.

425 Additionally, genera such as *Streptococcus* [45,46,55,85] and *Enterococcus*  
426 [22,46,56] showed high abundance in CHB, CHB-LC, and CHB-HCC. However,  
427 *Pediococcus*, *Leuconostoc*, *Weissella* [43], and *Bifidobacterium* [43,50,55,74] showed a  
428 marked decrease in CHB and CHB-LC.

429 Regarding *Bifidobacterium* species, one study reported marked alteration in CHB and  
430 CHB-LC groups by qPCR, shifting from beneficial to pathogenic species. The abundance of  
431 *B. catenulatum*/*B.pseudocatenulatum* group and *B. longum* was decreased, and *B. dentium*  
432 was increased in patient groups. However, no significant differences were detected for *B.*  
433 *bifidum* and *B. infantis* [85]. The protective role of *B. catenulatum* is to prevent bacteria  
434 translocation and decrease the release of local gut cytokine and endotoxins [86]. Clinical  
435 trials and animal experiments indicated *B. longum* effectively reduced serum ammonia levels  
436 in minimal HE [87]. Therefore, a supplement of probiotics for the above-mentioned species  
437 (except for *B. dentium*), may be helpful in patients with CHB-LC against liver damage.  
438 Indeed, *B. dentium* was considered to be an opportunistic pathogen reported to mainly



439 survive in the oral cavity [88]; however, it is still unknown whether any causal relation exists  
440 between a higher level of *B. dentium* in the intestine and liver injury,

441           Nevertheless, this contributory role is still an emerging issue. Therefore, the  
442 relationships between hepatic histopathological changes and the commonest beneficial gut  
443 microbiota, namely LAB, should be investigated further with more focus on the species level.

444

#### 445 **Sulfate-reducing bacteria (SRB)**

446 *The Desulfovibrio* genus is a dominant population of human intestinal SRB that widely exists  
447 in the anaerobic intestinal tract [89]. Different studies have shown that *Desulfovibrio*  
448 increases significantly in HBV carriers, CHB and CHB-LC [44,45], but there were  
449 differences at the species level. In addition to the direct influence of *Desulfovibrio*, its  
450 metabolites lead to the release of inflammatory cytokines, such as Interleukin-6 (IL-6), and  
451 Interleukin-8 (IL-8) [90]. Hydrogen sulfide (H<sub>2</sub>S), one of its primary metabolites, causes  
452 cytotoxicity [91], a proliferation of hepatic stellate cells (HSC), and the expression of hepatic  
453 fibrosis markers [92]. Interestingly, LC-derived *Desulfovibrio* showed a stronger H<sub>2</sub>S  
454 production ability and a higher antibiotic resistance compared to healthy-  
455 derived *Desulfovibrio* [93]. Hence, *Desulfovibrio* species could be used as a non-invasive  
456 biomarker in diagnosing and following CHB-LC, and therefore, they should be further  
457 investigated in larger study populations.

458           *Bilophila*, another SRB [94], showed increased abundance in the CHB-LF group of  
459 patients, associated with changes in hepatic biomarkers such as ALT and aspartate  
460 aminotransferase (AST) [56], while another study reported its decrease [95]. However, in a  
461 mouse model, *Bilophila* was increased in Entecavir (ETV)- treated HBV group [39].  
462 Therefore, further exploration of the relationship between SRB and the development of HBV-  
463 related complications is recommended.

464 **Bacteria associated with bile acids (BAs) metabolism**

465 Bile acids are produced in the liver and enter the small intestine to aid in the digestion of fats  
466 and fat-soluble vitamins [96]. Recent research has discovered a significant positive  
467 association between serum levels of primary BAs, and the advancement of liver fibrosis  
468 [49,60]. Moreover, several studies have indicated that CHB patients without liver cirrhosis  
469 showed a decreasing ratio of unconjugated/conjugated BAs [50]. Interestingly, genera that  
470 harbor bile salt hydrolase (BSH) enzymes used for BAs deconjugation, such as *Lactobacillus*,  
471 *Clostridium*, *Bifidobacterium*, *Enterobacteriaceae* were suppressed in CHB patients [50].  
472 Additionally, 7-alpha-hydroxysteroid dehydrogenase (hdhA) enzyme that is specifically  
473 responsible for secondary BA generation was impaired in CHB patients [97]. Notably, this  
474 enzyme is majorly found in *Clostridia*, *Ruminococcus* spp. [97]. *Bacteroides* also harbors  
475 hdhA enzyme, especially *B. fragilis* [98], which was significantly lower in CHB patients  
476 [50].

477

478 **Anti-inflammatory and butyrate-producing bacteria**

479 The anti-inflammatory short-chain fatty acids (SCFAs) activity and anti-carcinogenic  
480 potential roles of *Prevotella*, *Alloprevotella*, *Bacteroides*, *Ruminococcus*, *Faecalibacterium*,  
481 *and Phascolarctobacterium* have been reported [62]. CHB infection showed a progressive  
482 decline of *Phascolarctobacterium* [77] *and Bacteroides* [24,46,49,57] compared to healthy  
483 subjects; however, *Prevotella* [49,55,62,83] *and Alloprevotella* [47,62] were highly abundant  
484 in CHB-HCC. Additionally, several studies reported a decrease in *Faecalibacterium* in CHB  
485 [55,64,95,99,100], while others reported an increase in CHB-LC and HCC [56,62,74,99].  
486 Interestingly, *Faecalibacterium* were noted to be possible indicators of gut health [101];  
487 when *Faecalibacterium* levels were low, inflammatory processes were stimulated, resulting  
488 in the development of disease [102]. Furthermore, some studies reported an increased

489 abundance of *Ruminococcus* in CHB-LC and HCC [45,55], while others reported a decrease  
490 [77,83]. However, the precise taxonomic classification of *Ruminococcus* is misleading as it is  
491 continuously reclassified [103].

492 In this context, the mechanism of liver injury is the disruption of SCFAs production.  
493 The human microbiome degrades incomplete host digestion products and produces SCFAs  
494 products such as acetate, propionate, and butyrate. Butyrate has been identified as the primary  
495 energy source of enterocytes, and a lack of butyrate has been linked to decreased intestinal  
496 barrier integrity [96]. Moreover, butyrate is known as anticarcinogenic and anti-inflammatory  
497 and plays a role in oxidative stress [104].

498 *Anaerostipes*, producing SCFAs-genus, were also highly abundant in HBV carriers  
499 with normal than elevated ALT levels [44]. Moreover, *Anaerostipes* were more abundant in  
500 CHB [44,54] but declined in CHB-HCC patients [105]. Hence, the gut microbial composition  
501 varied according to HBV-induced serum ALT levels; these findings suggested a potential link  
502 between gut and liver inflammation [44].

503

#### 504 **Other CHB-associated taxa**

505 **Pro-inflammatory bacteria** such as *Enterococcus* [22,46,56] and *Escherichia Coli* [22,57]  
506 were reported to be highly abundant in CHB-LC. The liver injury could be explained through  
507 the activation of cytokines attracting inflammatory cells and enhancing myofibroblast  
508 formation [106]. Myofibroblasts, which produce collagen, are not found in healthy livers  
509 [107]; however, in response to toxic liver injury, myofibroblasts are primarily transformed  
510 from activated HSCs [108] and subsequently activated HSCs migrate and secrete  
511 extracellular matrix (ECM) to form a fibrous scar [109]. Moreover, several studies in CHB  
512 patients have postulated immunological links between gut dysbiosis and disease progression.  
513 [42,43,62]. For example, *Enterobacteriaceae* were positively correlated with TNF- $\alpha$  and

514 reported a higher abundance in CHB-LC than in HBV carriers and control [43]. Moreover,  
515 patients with cirrhosis showed weakened intestinal tight junctions and reduced tight junction  
516 protein expression [110] assessed by zonulin, a robust tight junction regulator [111]. Patients  
517 with CHB-LC and HCC have significantly higher serum zonulin levels, which were  
518 proportional to HBV stages [112]. Interestingly, a correlation between zonulin levels and the  
519 abundance of certain bacterial species, such as pro-inflammatory *E. coli*, has been  
520 demonstrated [113].

521         However, another study reported an unpredicted decrease in pro-inflammatory  
522 bacteria like *Escherichia-shigella* in CHB-HCC patients compared to non-hepatitis-related  
523 HCC [62]. Both studies emphasize the role of gut microbiota in HBV infection and disease  
524 progression, where the authors attributed this phenomenon to a lack of protective memory T  
525 cells due to CHB infection.

526 **Beneficial genera:** *Lachnospira* was reported to consistently decrease in CHB, LC, and  
527 HCC in several studies [22,46,74,77,83]. *Lachnospiraceae* were found to play a role in the  
528 management of CHB via the reduction in LPS secretion and bacterial translocation [105,114].  
529 *Akkermansia*, was reported to increase in resolved CHB infection [115], but another study  
530 showed its increase in CHB-LC [77]. In addition, a study showed a restoration of  
531 *Akkermansia* in a mouse model receiving AVT [39]. These conflicting results indicate that  
532 additional research is required to determine how *Akkermansia* contributes to HBV-related  
533 infections compared to controls.

534 *Veillonella* and *Megamonas* genera were identified to be significantly contributed to the  
535 pathogenesis of liver disease in different studies. *Veillonella* increased in CHB [45,55,84],  
536 CHB-LC, and HCC [54,57,62,77], while *Megamonas* showed high abundance in CHB  
537 [24,52,100], but it declined in CHB-LC and HCC [64,70]. Both genera are known to produce  
538 butyrate and SCFAs [116], contributing to anti-inflammatory response in the host [117].

539 Additional *in vitro* evidence suggested that *Veillonella* LPS stimulated the release of  
540 inflammatory cytokines like IL-6, IL-1 $\beta$ , IL-10, and tumor TNF- $\alpha$  [118]. These results  
541 suggested intestinal *Veillonella* metabolites affect liver pathology and inflammation [119].  
542 Interestingly, a previous study found that the level of *Veillonella* was positively correlated  
543 with CTP score in patients with CHB-LC [54]. However, the elevated *Veillonella* and  
544 decreased *Megamonas* genera might suggest the unbalanced inflammatory status in patients  
545 with CHB-LC.

546 In summary, and according to the previous findings, we can propose a typical  
547 microbiota signature profile in HBV-associated infections based on the most reproducible  
548 results of reported microbiota changes, as demonstrated in (**Fig. 4**). This signature is  
549 characterized by a consistent increase along *Pseudomonadota* phylum including  
550 (*Gammaproteobacteria* class, *Enterobacteriales* order, *Enterobacteriaceae* family, *Klebsiella*,  
551 *proteus* and *Sutterella* genera and *Escherichia coli* species). However, Members of *Bacillota*  
552 (formerly *Firmicutes*) phylum showed a constant decrease among class *Clostridia* (except  
553 *Blautia* genus increased). Still, a consistent increase in class *Bacilli* members (including  
554 *Lactobacillales* order harboring *Streptococcus* and *Enterococcus* genera and *Veillonellaceae*  
555 family having *Veillonella* genus) (**Fig. 4**). Interestingly, *Bacteriodes eggerthi* decreased,  
556 unlike the constant increase in members of *Bacteroidia* class. This finding supports the  
557 importance of investigating HBV-associated dysbiosis to the species level that could show  
558 different abundance. In conclusion, the mechanisms by which the previous microbiota  
559 signature induces liver injury in HBV-associated dysbiosis are summarized in (**Fig. 5**).

560

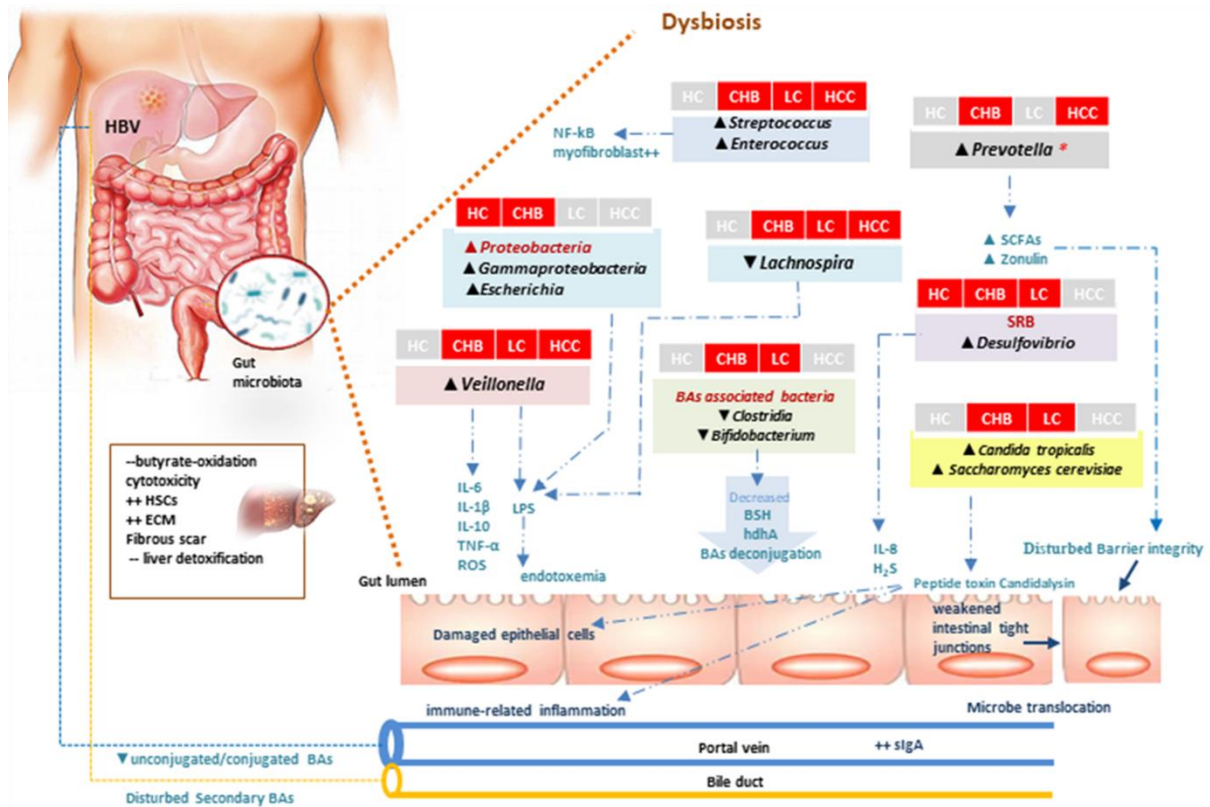
PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	
Pseudomonadota	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia	Escherichia coli	
			Klebsiella			
	Betaproteobacteria	Burkholderiales	Morganellaceae	Proteus		
			Sutterellaceae	Sutterella		
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae			
			Prevotellaceae	Prevotella		
			Bacteroidaceae	Bacteroides	Bacteroides eggerthi	
Bacillota	Clostridia	Eubacteriales	Lachnospiraceae	Lachnospira		
				Roseburia		
				Blautia		
				Clostridiaceae	Clostridium	
	Bacilli	Lactobacillales	Lactobacillaceae			
			Streptococcaceae	Streptococcus		
			Enterococcaceae	Enterococcus		
Negativicutes	Veillonellales	Veillonellaceae	Veillonella			
Actinomycetota	Actinomycetes	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium		
		Coriobacteriales	Coriobacteriaceae			
Thermodesulfobacteriota	Desulfovibrionia	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio		

561

562

563 **Figure. 4 Identified microbiota signatures in HBV-associated gut dysbiosis.**

564 Pathobiont taxa with a high abundance are highlighted in red, while symbiont species with a low abundance are  
 565 marked in green. Taxa highlighted in grey are either not reported in the reviewed publications but included in  
 566 the graphic to better represent the taxonomy profile, or the reported results were inconclusive.



567

568 **Figure. 5** Possible pathophysiological mechanisms induced by identified signature microbiota in HBV-  
 569 associated diseases. Gradually decreased *Lachnospira* and increased *Streptococcus*, *Enterococcus*, *Prevotella*,  
 570 and *Veillonella* seem to play essential roles in the progression from LC to HCC, which could be considered the  
 571 potential microbial markers for the early diagnosis of HCC. \* *Prevotella* produces SCFAs that maintain gut  
 572 barrier integrity; however, it also correlates to increased zonulin serum levels [113], causing weakened intestinal  
 573 tight junctions. **NF-kB**: Nuclear factor-kB; **SRB**: Sulfate-reducing bacteria; **SCFAs**: Short chains fatty acids;  
 574 **LPS**: Lipopolysaccharides; **IL**: Interleukin; **TNF**: Tumor necrosis factor; **ROS**: Reactive oxygen species; **H<sub>2</sub>S**:  
 575 Hydrogen sulfide; **BSH**: Bile salt hydrolase; **hdhA**: 7- $\alpha$ -hydroxysteroid dehydrogenase; **BAs**: Bile acids;  
 576 **HSCs**: hepatic stellate cells; **ECM**: Extracellular matrix; **sIgA**: Secretory immunoglobulin A. ++: proliferation;  
 577 --: Inhibition. Predominant taxa in different disease stages are illustrated (red squares) as follows: **HC**: HBV-  
 578 carriers, **CHB**: Chronic HBV infection, **LC**: liver cirrhosis, **HCC**: hepatocellular carcinoma.

## 579 5 NON-BACTERIAL MICROBIOTA IN HBV-ASSOCIATED DYSBIOSIS

580

### 581 5.1 Gut virome

582 In the human body, the gastrointestinal system is home to most viruses [120], forming viral  
583 fingerprints unique for each individual and primarily comprising bacteriophages [120].

584 Viruses, in general, and phages, specifically, are modulated through the presence and  
585 abundance of their bacterial hosts [121].

586 Phages can control bacterial population density and facilitate the lysis of bacterial  
587 cells. According to a recent study, *Streptococcus* species were the primary interface between  
588 bacteria and phages in patients with LC and HE [122]. Since *Streptococcus* species have been  
589 reported to be consistently highly abundant in CHB and HBV-related complications  
590 [45,46,55,99], this could suggest that HBV infection could be related to gut viruses.

591 However, the precise role of gut virome in the etiology of HBV infection remains  
592 unknown, owing to the complex pathogenesis of HBV and a lack of relevant studies. A pilot  
593 study was conducted to investigate the frequency of Human herpesvirus-6 (HHV-6)  
594 reactivation in viral HBV and its impacts on the course of the primary disease. According to  
595 the serological and immunohistochemical findings, HBV infection has higher autoantibody  
596 formation once HHV-6 is present [123].

597 Interestingly, two studies have investigated gut virome as a potential therapeutic  
598 target in cirrhosis. One study reported that phages linked with bacterial species such as  
599 *Escherichia* and *Lactobacillus* increased with LC progression, while those related to health,  
600 such as crAssphages were lower [122]. In addition, there were stronger positive linkages  
601 between *Faecalibacterium* phage and *Microviridae* spp and potentially beneficial SCFA-  
602 producing taxa after rifaximin treatment [122]. Moreover, *Microviridae*, predominantly  
603 *Enterobacteriaceae* phages, increased in patients with LC receiving rifaximin. Their positive



604 linkages with otherwise beneficial bacterial species may suggest a proper post-rifaximin  
605 context associated with lower *Enterobacteriaceae* [124].

606 In conclusion, it is challenging to determine whether the changes in the gut virome are  
607 a cause or a result of the disease, especially since LC severity is more correlated with bacteria  
608 than viruses. However, modulating LC progression as a complication of CHB infection by  
609 affecting bacteria through phages or modified medication is essential to analyze.

610

## 611 **5.2 Gut mycobiome**

612 Fungi are believed to be a crucial part of the human microbiota, interacting with other  
613 microorganisms in the gut [125]. However, due to their low abundance in the human body,  
614 research studies on mycobiota have been far fewer than those on microbiota for the past two  
615 decades [126]. The gut fungi in adults mainly comprise three phyla: *Ascomycetes* (harboring  
616 *Aspergillus*, *Penicillium*, and *Candida*), *Basidiomycetes*, and *Zygomycetes* [127].

617 There has been little research on the relationship between gut mycobiota and HBV  
618 infection, with changes in the mycobiome linked to the progression of HBV infection in  
619 patients with and without LC. Only *three* studies, all from China, have been reported and  
620 discussed in this review (**Table 1**). One study reported a higher richness of fungal species in  
621 patients with CHB-LC than in CHB [114]. Only *Candida* spp. and *Saccharomyces*  
622 *cerevisiae* were obtained by culture-dependent approach. Enteric fungal diversity showed  
623 little difference between HBV carriers and controls [114]. Another study reported a high  
624 prevalence of *Aspergillus*, *Candida*, *Galactomyces*, *Saccharomyces*, and *Chaetomium* in  
625 patients with CHB-LC [128]. Moreover, Guo et al. noted a high abundance of *C.*  
626 *parapsilosis*, *C. glabrata*, *C. tropicalis*, and *S. cerevisiae* in patients with CHB-LC [129].  
627 Those findings show that the diversity of enteric fungi is positively related to HBV disease  
628 progression.

629           Recent studies have proven that *C. albicans* could secrete a peptide toxin called  
630 Candidalysin, which directly damages epithelial membranes and activates host immune  
631 responses [130]. Since *Candida* species were highly abundant in CHB, this mechanism could  
632 have a role in HBV-related liver injury (**Fig. 5**).

633           Interestingly, a prior study conducted on patients with LC (cause of cirrhosis not  
634 mentioned) reported that *Ascomycota* was negatively correlated with *Bacteroidetes*;  
635 moreover, a significant reduction of *Basidiomycota/Ascomycota* ratio (used to define the  
636 fungal dysbiosis) was reported in LC compared to controls [131]. In addition, the worsening  
637 of the Model for End-Stage Liver Disease (MELD) score (used as a predictor of survival in  
638 LC) was correlated negatively with *Basidiomycota/Ascomycota* ratio [131]. This implies that  
639 both mycobiome and bacterial microbiome need to be considered to improve prognostication.

640           In conclusion, the high abundance of *C. tropicalis* and *S. cerevisiae* was a  
641 reproducible finding in HBV-related infections (detected in at least two studies (**Table. 1**)).  
642 However, a recent study revealed that most strains of *Saccharomyces* and *non-*  
643 *Saccharomyces* yeasts are safe microorganisms, and could be regarded as probiotic yeast  
644 [132]. Therefore, prescribing such yeast species as probiotics should be cautiously applied,  
645 especially in HBV-infected patients.

646           The above findings demonstrate that fungi are significant modulators of the overall  
647 gut microbial dysbiosis in LC. However, the studies of interacting microorganisms are  
648 limited. Hence, more studies are needed to determine such interactions to identify better the  
649 role of bacterial-fungal correlation networks in disease pathogenesis and progression.

650

651 **Table 1.** Mycobiota changes in human HBV-related diseases

Author	Cohort (n)	Methodology	Mycobiota changes	Relevant findings
<b>Chen et al., 2011</b> [114]	HBV carriers (n = 33) CHB (n = 35) CHB-LC (n = 38) Healthy controls (n = 55)	-Culture -PCR-RFLP -18S rDNA clones and sequencing	<i>Aspergillus versicolor</i> ↑ <i>Aspergillus penicillioides</i> ↑ <i>Candida solani</i> ↑ <i>Candida albicans</i> ↑ <i>Candida austromarina</i> ↑ <i>Candida intermedia</i> ↑ <i>Candida milleri</i> ↑ <i>Candida tropicalis</i> ↑ <i>Saccharomyces paradoxus</i> ↑ <i>Saccharomyces cerevisiae</i> ↑ <i>Galactomyces geotrichum</i> ↑ <i>Simplicillium obclavatum</i> ↑ <i>Simplicillium lanosoniveum</i> ↑ <i>Chaetomium globosum</i> ↑ <i>Hyphozyma variabilis</i> ↑ <i>Aureobasidium pullulans</i> ↑ <i>Doratomyces stemonitis</i> ↑ <i>Wallemia muriae</i> ↑ <i>Asterotremella albida</i> ↑ <i>Cryptococcus fragicola</i> ↑ <i>Rhizopus microsporus var.</i> ↑ <i>Malassezia pachydermatis</i> ↓ <i>Penicillium freii</i> ↓ <i>Candida krissii</i> ↓	<ul style="list-style-type: none"> <li>➤ Both fungal abundance and fungal diversity ↑</li> <li>➤ All OTUs belonged to the <i>three</i> major fungal taxa: <i>Zygomycetes</i> (2.7%), <i>Ascomycetes</i> (78.4%), and <i>Basidiomycetes</i> (18.9%).</li> <li>➤ Only <i>five</i> species were identified by the culture-dependent method: <i>C. albicans</i> and <i>C. krusei</i> were detected in all groups, <i>C. glabrata</i> detected in HBV-LC, CHB, and control groups; <i>C. tropicalis</i> was detected in the HBV-LC group; <i>S. cerevisiae</i> was detected in CHB group.</li> <li>➤ The culture-independent method did not identify <i>C. krusei</i> and <i>C. glabrata</i> from fecal samples, which was found by the culture-dependent method.</li> <li>➤ Patients with CHB-LC and CHB shared greater fungal diversity than HBV carriers and controls and patients with CHB-LC had the highest fungal diversity and composition among all groups.</li> </ul>
<b>Mou et al., 2018</b> [128]	CHB (n = 52) HBV-LC (n = 52) Healthy controls (n = 40)	Culture	<b>Genus:</b> <i>Saccharomyce</i> ↑	<ul style="list-style-type: none"> <li>➤ <i>Saccharomyces</i> in the CHB and CHB-LC groups were significantly greater than in the control group (<math>P &lt; 0.05</math>).</li> <li>➤ <i>Saccharomyces</i> showed a weak correlation with serum IL-17A and a negative correlation with vitamin D levels in patient groups.</li> </ul>
<b>Guo et al., 2010</b> [129]	HBV carriers (n = 66) CHB (n = 68) CHB-LC (n = 80) Healthy controls (n = 84)	qPCR	<b>Species: (Prevalence)</b> <i>Candida parapsilosis</i> ↑ <i>Candida glabrata</i> ↑ <i>Candida tropicalis</i> ↑ <i>Candida krusei</i> ↑ <i>Saccharomyces cerevisiae</i> ↑	<ul style="list-style-type: none"> <li>➤ The sensitivity of the qPCR assays is significantly higher than the traditional isolation and culture method (<math>P &lt; 0.001</math>).</li> <li>➤ Frequency of fungal species (DNA copy numbers) <ul style="list-style-type: none"> <li>▪ <i>C. glabrata</i>, <i>C. tropicalis</i>, and <i>S. cerevisiae</i> were more frequently found in HBV carriers than controls (<math>P &lt; 0.05</math>).</li> <li>▪ Although copies of target DNA for <i>C. albicans</i> were significantly</li> </ul> </li> </ul>

different among the groups (especially LC  $P<0.01$ ), the prevalence values were not significantly different

- For *C. glabrata* and *C. tropicalis*, no significant difference in DNA copy numbers was observed among four groups,

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652

653 **HBV:** Hepatitis B virus; **CHB:** Chronic hepatitis B virus infection; **CHB-LC:** Chronic hepatitis B-associated liver cirrhosis; **PCR:** Polymerase chain reaction; **RFLP:**

654 Restriction fragment length polymorphism; **qPCR:** Quantitative polymerase chain reaction; **OTUs:** Operational taxonomic units and **IL:** Interleukin.

## 655 6. GUT MICROBIOTA-TARGETED TREATMENT

656 Antiviral medication has been acknowledged as an efficient method for treating and even  
657 reversing liver fibrosis and cirrhosis [133]. In addition, host health is dependent on the  
658 balance of the composition of the entire microbial community rather than one or a few  
659 dominant organisms [134]. Hence, with increasing evidence of the link between HBV  
660 infection and gut dysbiosis, several studies focused on manipulating the gut microbiota to  
661 treat CHB or slow disease progression by applying different treatment options (**Table. 2**).

662

### 663 6.1 Reverse HBV-associated dysbiosis with medications

#### 664 A. Antiviral treatment (AVT)

665 The main goal of all current treatment strategies is to suppress HBV replication [3].  
666 Interferon alpha and nucleoside analogs (NAs), such as ETV, are the principal AVT choices  
667 with low resistance and long-acting effects [135].

668 A previous study reported that ETV therapy could reverse gut microbiota in HBV-  
669 associated dysbiosis in a mouse model [39]. Reduced HBV DNA levels were documented in  
670 addition to restoration of specific beneficial bacteria such as *Akkermansia*, *Lacnospiracea*,  
671 and *Marvinbryantia* to proportions similar to controls [39]. Moreover, a recent study reported  
672 a marked increase of *Clostridium* and *Erysipelotrichaceae* after *eight* weeks of ETV  
673 treatment, while *Streptococcus*, *Atopobium*, and *Murdochiella* were markedly decreased  
674 [136]. Although adding *Clostridium butyricum* (CB) to ETV did not improve serum  
675 biochemical, immunologic, or virologic variables, it did affect the gut microbiota in CHB  
676 patients treated with ETV [137]. At the phylum level, significant differences in abundance  
677 between the untreated group and the ETV + CB group included *Firmicutes*, *Actinobacteria*,  
678 *Cyanobacteria*, *Euryarchaeota*, and *Synergistetes* [137] (**Table 2**). However, dynamic and

679 synergistic research on liver fibrosis outcomes and gut microbiota changes during AVT is  
680 still limited and needs further investigation.

681

## 682 **B. Antibiotics**

683 Rifaximin is a rifamycin-based nonsystemic antibiotic with low gastrointestinal absorption  
684 and good antibacterial activity [138]. Rifaximin has been used to prevent and treat HE [139].  
685 Current research investigates how rifaximin could treat liver complications by acting on gut  
686 microbiota (**Table 2**).

687         In a randomized trial, patients with LC and ascites were assigned to one of two  
688 groups: rifaximin or placebo for *four* weeks. Rifaximin group showed a decrease in gut  
689 microbiota abundance with a remarkable difference in *Pseudomonadales* abundance [140].  
690 Another two studies found that rifaximin also had some effect on gut microbiota abundance,  
691 where a slight decrease in *Veillonellaceae* and an increase in *Eubacteriaceae* were noticed  
692 [141]. Yet, the metabolite levels changed, endotoxemia was relieved, and serum fatty acid  
693 levels were increased significantly [142]. Conversely, prophylactic rifaximin did not improve  
694 SBP and was associated with *Escherichia coli*, *Enterococci*, and *Klebsiella* species in  
695 cirrhotic CHB patients with ascites [143]. In conclusion, rifaximin appears to directly impact  
696 bacterial function and pathogenicity rather than having a bactericidal effect.

697

## 698 **6.2 Bacterial probiotics**

699 Probiotics are nonpathogenic microorganisms that live in the gut, and when applied at  
700 treatment doses (at least 10<sup>6</sup> viable CFU/g), they may help to balance the gut microbiota  
701 [144]. *Lactobacillus* and *Bifidobacterium* genera are commonly reported as clinically  
702 available probiotics [145]. The protective mechanisms include reduced endotoxin levels,

703 antimicrobial production, improved gut barrier function, and modulation of local and  
704 systemic immunity [55].

705         The role of probiotics in HBV-related complications has been established in several  
706 studies. Probiotic therapy by *Lactobacillus acidophilus* was reported to improve blood  
707 ammonia and psychometric tests in HE [146]. Moreover, after *three* months of probiotic  
708 administration of *Clostridium butyricum* mixed with *Bifidobacterium infantis*, a previous  
709 study showed a significant improvement in venous ammonia and cognition levels. In  
710 addition, the abundance of pathogenic *Enterococcus* and *Enterobacteriaceae* was reduced  
711 [147] (**Table 2**). Interestingly, probiotics were effective in primary HE prevention in 26.3%  
712 of cirrhotic patients via replacing the pathogenic pro-inflammatory  
713 (*Veillonellaceae*, *Enterococcus*, and *Megasphaera*) and urease-producing organisms  
714 (*Alcaligenaceae* and *Porphyromonadaceae*) [148]. Moreover, a randomized controlled study  
715 showed that the combination of probiotics and lactulose was helpful for the prevention of HE  
716 patients with CHB-LC [149]. However, CHB patients treated with a probiotic containing  
717 LAB, *three* strains of *B. lactis*, *L. rhamnosus*, and *L. acidophilus* showed a significant  
718 increase in the treated group ( $p = 0.001$ ). However, CTP scores and liver functions did not  
719 significantly change [150].

720         Notably, due to recent advancements in synthetic biology, further engineered  
721 microorganisms, such as engineered *Escherichia coli* Nissle 1917 (Ecn), have been developed  
722 to lower blood ammonia levels and eventually reduce the incidence of HE [151,152].  
723 However, these strains have not progressed to clinical studies in hyperammonemia patients,  
724 and the clinical effects need further investigation.

725         In conclusion, probiotics could present potential therapeutic approaches for modifying  
726 HBV-associated dysbiosis. However, some studies have shown inconsistent results,

727 indicating the need for more clinical studies in HBV-infected patients with larger  
728 populations.

729

### 730 **6.3 Probiotic fungi**

731 Despite some intriguing recent discoveries about how the gut mycobiota contributes to the  
732 development of liver disease, there have been few promising studies of fungal novel  
733 therapeutic options for liver disease.

734 *Saccharomyces boulardii* is probably the most commonly used probiotic fungi.

735 Animal studies have indicated the protective and therapeutic role of *S. boulardii* in liver  
736 diseases via gut microbiota change and attenuating liver inflammation [153]. As mentioned  
737 earlier, most *Saccharomyces* and non-*Saccharomyces* yeasts strains were reported to be safe  
738 microorganisms and could be applied as an alternative to the probiotic yeast *S. boulardii*  
739 [132]. However, some side effects of *S. boulardii*, such as fungemia, were documented in  
740 clinical settings with immunosuppressed patients [154]. Therefore, probiotic fungi should be  
741 prescribed with caution.

742 Interestingly, macrofungi, known as mushrooms, such as *Agaricus bisporus* [155]  
743 and *Pleurotus ostreatus* [156], have shown their medicinal properties in proving  
744 hepatoprotective activity in mice models.

745 In conclusion, the probiotic effects of some fungal species have yet to be fully  
746 explored. Furthermore, probiotic fungus supplementation to prevent or treat HBV-related  
747 diseases can be anticipated; nevertheless, their medical application needs to be carefully and  
748 thoroughly investigated.



#### 749 **6.4 Faecal microbiota transplantation (FMT)**

750 Fecal microbiota transplantation is an emerging treatment method that transfers the gut  
751 microbiota from a healthy donor to a patient [157]. It is one of the most promising therapies  
752 in many diseases because it reshapes gut microbial communities [117].

753 Studies of FMT on animal models show promising results. Neuroinflammation was  
754 reduced in cirrhotic mice model treated with FMT [158]. Moreover, human studies on  
755 cirrhotic patients have shown promising FMT results in improving HE [159]. Several studies  
756 evaluating FMT in CHB patients are summarized in **Table 2**.

757 In a pilot study on treated CHB patients with HBeAg-positive status, the FMT arm  
758 experienced a significant decline in HBeAg titer compared to controls [160]. The previous  
759 findings imply that FMT can induce HBeAg seroclearance with high efficacy. The previous  
760 results were consistent with another clinical trial on HBeA-positive patients receiving AVT  
761 for over a year. The results showed that 16.7% of the FMT arm patients achieved HBeAg  
762 seroclearance [161].

763 Interestingly, a case report evaluated FMT in HBsAg-positive woman with LC  
764 reported a decrease in CTP score with an increase in beneficial bacteria like  
765 *Faecalibacterium* after FMT [162]. Surprisingly, some researchers have proposed that FMT  
766 of some potential beneficial bacteria can change the occurrence of disease, and HBV carriers  
767 might be the most suitable donors for slightly higher microbiota abundance [56]. However,  
768 such finding needs to be further validated.

769 Furthermore, in a randomized clinical trial, FMT helped reduce hospitalizations and  
770 improve cognition and dysbiosis for LC with recurrent HE [159]. Additionally, the same  
771 authors later verified the safety of FMT capsules through a phase 1, randomized, and  
772 placebo-controlled clinical trial [163].

773 Overall, using FMT as a therapy for HBV-associated dysbiosis showed promising  
774 results. Further research into this technique is already currently in progress, with the hope of  
775 providing future guidance in its clinical application. However, due to the limitations of a  
776 small number of participants and a lack of randomized clinical trials, further extensive, well-  
777 designed studies and trials are warranted to confirm the initial assumptions and promote the  
778 clinical practicability of such therapeutic options. The clinical trials conducted to date on  
779 FMT effectiveness in CHB have been summarized in **Table 3**.

780 **Table. 2** Microbiota-targeted treatment in HBV-associated dysbiosis

Study type	Reference	Cohort (n)	Treatment protocol	Relevant findings
<b>AVT</b>				
Human study	Lu et al., 2021 [136]	CHB ( <i>n</i> = 30); Healthy controls ( <i>n</i> =30).	Eight weeks of daily ETV; ( <i>n</i> = 30).	<ul style="list-style-type: none"> <li>➤ After ETV treatment, gut microbiota abundance significantly increased.</li> <li>➤ Significant improvement in ALT, IL-6, IL-8, TNF-<math>\alpha</math>, and HBV DNA levels.</li> </ul>
Human study	Lu et al., 2021 [137]	CHB ( <i>n</i> = 60); Healthy controls ( <i>n</i> =30).	Eight weeks of daily ETV + CB; ETV ( <i>n</i> = 30), ETV + CB ( <i>n</i> =30).	<ul style="list-style-type: none"> <li>➤ Marked differences in <i>Firmicutes</i>, <i>Actinobacteria</i>, <i>Cyanobacteria</i>, <i>Euryarchaeota</i>, and <i>Synergistetes</i> before initial treatment and after ETV + CB treatment.</li> <li>➤ Significant differences in <i>Actinobacteria</i>, <i>Fusobacteria</i>, <i>Pseudomonadota</i>, <i>Saccharibacteria</i>, and <i>Synergistetes</i> between the ETV group and ETV + CB group.</li> <li>➤ No improvement in blood biochemical, immunological, and virological parameters.</li> </ul>
Mouse model	Li et al., 2020 [39]	HBV-infected mice at six weeks old (total <i>n</i> = 10); HBV infected ( <i>n</i> = 5), HBV + ETV ( <i>n</i> = 5).	35 days after HBV infection, four weeks of daily ETV, ETV ( <i>n</i> = 5).	<ul style="list-style-type: none"> <li>➤ ETV reversed dysbiosis.</li> <li>➤ Changes in bacteria proportions of <i>Akkermansia</i>, <i>Lacnospiraceae</i>, and <i>Marvinbryantia</i> in the ETV-treated group.</li> </ul>
<b>Antibiotics</b>				
Randomized trial	Kimer et al., 2018 [140]	LC ( <i>n</i> = 54); CHB (not mentioned).	Four weeks of rifaximin 550-mg BD or placebo BD; Rifaximin ( <i>n</i> = 36), Placebo ( <i>n</i> = 18).	<ul style="list-style-type: none"> <li>➤ Rifaximin had minor effects on gut microbiota.</li> <li>➤ Rifaximin showed little impact on the inflammatory markers TNF-<math>\alpha</math>, IL-6, IL-10, IL-18, and CRP.</li> </ul>
Human study	Kaji et al., 2020 [141]	LC ( <i>n</i> = 30).	Four weeks of rifaximin 1200 mg/d, rifaximin ( <i>n</i> = 30)	<ul style="list-style-type: none"> <li>➤ Rifaximin relieved HE endotoxemia with improved intestinal hyperpermeability.</li> <li>➤ Rifaximin didn't affect serum proinflammatory cytokine levels (TNF-<math>\alpha</math>, IL-6, IFN-<math>\gamma</math>, IL-10).</li> </ul>
Human study	Bajaj et al., 2013 [142]	LC patients with MHE ( <i>n</i> =20).	Eight weeks of rifaximin 550-mg BD, rifaximin ( <i>n</i> = 20)	<ul style="list-style-type: none"> <li>➤ Minor effect on gut microbiota with little decrease in <i>Veillonellaceae</i> and increase in <i>Eubacteriaceae</i>.</li> <li>➤ Rifaximin significantly improved cognition and endotoxemia.</li> <li>➤ Increase in serum fatty acids.</li> </ul>
Human study	Lutz et al., 2014 [143]	LC with ascites ( <i>n</i> =152).	Prophylactic antibiotic treatment before the time of paracentesis; rifaximin ( <i>n</i> =27), other systemic antibiotics ( <i>n</i>	<ul style="list-style-type: none"> <li>➤ Prophylactic rifaximin did not improve SBP.</li> <li>➤ Prophylactic rifaximin was associated with the dominant bacteria in ascites: <i>Escherichia coli</i> and <i>Enterococcus</i>.</li> <li>➤ <i>Klebsiella</i> species were mainly recovered from the rifaximin group.</li> </ul>

= 17).

**Probiotics**

Human study	Xia et al., 2018 [147]	CHB-LC with MHE (n=30).	Three months of probiotics 1500-mg TD, which contained CB combined with <i>Bifidobacterium infantis</i> . Probiotics group (n =30).	<ul style="list-style-type: none"> <li>➤ Significant improvements in ALT, AST, bilirubin, albumin, and decreased ammonia levels.</li> <li>➤ Considerable improvement in psychometric tests.</li> <li>➤ Significant decrease in venous ammonia, LPS, D-lactate, and diamine oxidase.</li> <li>➤ The beneficial bacteria were significantly enriched, while <i>Enterococcus</i> and <i>Enterobacteriaceae</i> were decreased.</li> </ul>
Human study	Kwak et al., 2014 [150]	Chronic liver disease patients: HBV (8/25), ALD (14/25), HCV (2/25), Cryptogenic (1/25).	<p>- Probiotic containing six LAB species once per day for four weeks.</p> <p>- Six bacterial species included: <i>Bifidobacterium bifidum</i>, <i>Bifidobacterium lactis</i>, <i>Bifidobacterium longum</i>, <i>Lactobacillus acidophilus</i>, <i>Lactobacillus rhamnosus</i>, <i>Streptococcus thermophilus</i>.</p>	<ul style="list-style-type: none"> <li>➤ Small intestinal bacterial overgrowth, measured by hydrogen breath test, was present in 26% of the patients and improved in 24% of the probiotic group, while 16% worsened in the placebo group.</li> <li>➤ Liver chemistry, CTP scores, and intestinal permeability did not significantly change between groups.</li> </ul>
Human study	Agrawal et al., 2012 [149]	LC patients with recovered HE (n = 235).	Three months of lactulose 30–60 mL/d, or three probiotics capsules per day, contained four strains of <i>Lactobacillus</i> ; Lactulose (n = 80), Probiotics (n = 77).	<ul style="list-style-type: none"> <li>➤ Lactulose and probiotics were effective for secondary prophylaxis of HE in cirrhotic patients.</li> </ul>
Human study	Ziada et al., 2013 [146]	LC patients with MHE (n =90); CHB (not mentioned).	Four weeks of lactulose 30–60 mL/day, or three probiotics capsules per day, contained <i>Lactobacillus acidophilus</i> ; Lactulose (n = 30), Probiotics (n = 30).	<ul style="list-style-type: none"> <li>➤ Probiotic was more tolerated than lactulose.</li> <li>➤ Both of them can improve blood ammonia and psychometric tests and reduce the risk of developing HE.</li> <li>➤ The levels of brain neurometabolites were improved in the probiotic group.</li> </ul>
Animal model	Nicaise et al., 2008 [164]	Ornithine-transcarbamoylase deficient Sparse-fur mice; Carbon-tetrachloride-rats;	NCIMB8826 (wild-type strain <i>Lactobacillus plantarum</i> ),	<ul style="list-style-type: none"> <li>➤ EV101 administration was effective in controlling hyperammonemia in constitutive animal models with a significant effect on survival.</li> </ul>

		Thioacetamide-induced acute liver failure mice	or EV101 (engineered <i>Lactobacillus plantarum</i> ) oral and intrarectal administration	
Mouse model	Kurtz et al., 2019 [151]	Ornithine transcarbamylase-deficient <i>spfash</i> mice; Thioacetamide-induced acute liver failure mice; Healthy human controls ( $n = 52$ ) for phase 1 trial.	Non-modified <i>Escherichia coli</i> Nissle 1917 (EcN), SYNBI020 administration	<ul style="list-style-type: none"> <li>➤ SYNBI020 reduced systemic hyperammonemia and improved survival in mouse models.</li> <li>➤ SYNBI020 was well tolerated in healthy controls.</li> </ul>
Mouse model	Ochoa-Sanchez et al., 2021 [152]	Bile-duct ligated rats.	Non-modified EcN, S-ARG, or S-ARG <sup>+</sup> administration	<ul style="list-style-type: none"> <li>➤ S-ARG was further modified to synthesize butyrate, which had the potential to prevent HE.</li> </ul>
<b>FMT</b>				
Human study	Ren et al., 2017 [160]	CHB patients ( $n = 18$ ) with positive HBeAg status for less than three years were treated with ETV or TDF. <i>Five</i> patients elected to receive FMT, and <i>four</i> finished the trial. The other 13 who refused FMT were enrolled as a Control group ( $n = 13$ ).	FMT was performed by gastroscopy into the duodenum every <i>four</i> weeks until HBeAg clearance.	<ul style="list-style-type: none"> <li>➤ HBeAg titer declined after each round compared to controls in 75% (3/4) of patients.</li> <li>➤ FMT was effective in HBeAg clearance in CHB, especially in patients who could not cease the oral AVT even after long-term treatment.</li> <li>➤ Gut microbiota revealed some changes with each FMT, but no clear pattern was observed among all patients.</li> </ul>
Human study	Sun et al., 2019 [162]	One 45-year-old woman with CHB-LC with hepatic myelopathy.	The patient underwent <i>three</i> rounds of FMT from <i>four</i> healthy donors, the 1st by colonoscopy, and the last two by gastroscopy	<ul style="list-style-type: none"> <li>➤ Improvement of hepatic myelopathy score.</li> <li>➤ CTP score decreased from 8 to 6, and the patient showed marked improvement in her paraparesis.</li> <li>➤ Increase in beneficial genera like <i>Faecalibacterium</i> and decrease in harmful bacteria like <i>Fusobacterium</i>.</li> </ul>
Human study	Chauhan et al., 2021 [161]	HBeAg(+) CHB patients on oral AVT for >1 year ( $n = 12$ ).	<i>Six</i> FMTs by gastroscopy with antivirals every <i>four</i> weeks ( $n = 12$ )	<ul style="list-style-type: none"> <li>➤ 16.7% (2/12) of patients developed HBeAg clearance compared to none in the AVT arm.</li> <li>➤ <i>Six</i> patients reported one minor adverse event, while one was hospitalized for severe abdominal pain.</li> </ul>
Human study	Bajaj et al., 2017 [159]	DC patients with recurrent HE ( $n = 20$ ); CHB (not mentioned).	After <i>five</i> days of antibiotics, FMT was performed by enema, or SOC, (rifaximin/lactulose) was applied; FMT ( $n = 10$ ),	<ul style="list-style-type: none"> <li>➤ FMT increased diversity and beneficial gut microbiota taxa, improved cognition, and showed better tolerance than SOC.</li> </ul>

Human study	Guo et al., 2021 [165]	CHB (n=18); CHB-LC (n=17).	SOC (n = 10). All patients underwent gastroscopy FMT treatment using a freshly prepared bacterial solution.	<ul style="list-style-type: none"> <li>➤ FMT treatment improved liver function tests, controlled the replication of HBV-DNA, relieved liver fibrosis, and postponed the progression of CHB.</li> <li>➤ The fecal microbiota of the patients gradually approached the composition of the controls.</li> </ul>
Randomized controlled trial	Bajaj et al., 2019 [163]	DC patients with recurrent HE (n = 20); CHB (not mentioned).	FMT was performed by enema, or SOC, (rifaximin/lactulose) was applied; FMT (n= 10), SOC (n = 10).	<ul style="list-style-type: none"> <li>➤ Oral FMT capsules are safe and well tolerated.</li> <li>➤ The diversity in post-FMT increased with higher <i>Ruminococcaceae</i> and <i>Bifidobacteriaceae</i> and lower <i>Streptococcaceae</i> and <i>Veillonellaceae</i>.</li> </ul>
Mouse model	Liu et al., 2020 [158]	Germ-free mice.	Sterile supernatant or entire stool from pre-FMT and post-FMT cirrhotic patients with HE was transplanted to germ-free mice.	<ul style="list-style-type: none"> <li>➤ In mice, fecal microbial colonization from LC patients resulted in greater levels of neuroinflammation and neuronal activation regardless of LC. Reducing neuroinflammation by colonizing germ-free mice with samples from post-FMT patients revealed a direct effect of fecal microbiota irrespective of ongoing hepatic inflammation or injury.</li> </ul>
Mouse model	Wang et al., 2022 [166]	Six to eight-week-old male BALB/c and C57BL/6 mice.	The gut microbiota in BALB/c mice was depleted using broad-spectrum antibiotics and then reconstituted with fecal microbiota from BALB/c or C57BL/6 mice to evaluate the effect of FMT on the outcomes of the immune response to HBV infection.	<ul style="list-style-type: none"> <li>➤ Mice receiving fecal microbiota from BALB/c or C57BL/6 mice displayed different HBV infection outcomes.</li> <li>➤ The fecal microbiota from C57BL/6 mice induced immune tolerance in the liver and prolonged HBV infection.</li> <li>➤ The host genetic background shapes the gut microbiota composition, and commensal bacteria from FMT do not entirely but selectively colonize the gut of the new host.</li> <li>➤ FMT can modulate the host immune response and alter host susceptibility to HBV infection.</li> </ul>

781 **HBV:** Hepatitis B virus; **CHB:** Chronic hepatitis B infection; **LC:** Liver cirrhosis; **CHB-LC:** Chronic hepatitis B-associated liver cirrhosis; **MHE:** Minimal hepatic  
782 encephalopathy; **ALD:** Alcohol-related liver disease; **HCV:** Hepatitis C virus; **FMT:** Fecal microbiota transplantation; **HBeAg:** Hepatitis B e antigen; **CB:** **AVT:** Antiviral  
783 treatment; **DC:** Decompensated cirrhosis; **ETV:** Entecavir; **CB:** *Clostridium butyricum*; **BD:** Twice a day; **TD:** Three times daily; **LAB:** Lactic acid bacteria; **SOC:** standard  
784 of care; **ALT:** Alanine transaminase; **IL:** Interleukin; **TNF:** Tumor necrosis factor; **CRP:** C-reactive protein; **SBP:** Spontaneous bacterial peritonitis; **AST:** Aspartate  
785 transferase; **LPS:** Lipopolysaccharides; **CTP:** Child-Turcotte-Pugh, and **HE:** Hepatic encephalopathy.

786 **Table. 3** Clinical trials evaluating FMT efficacy in CHB patients

Study title	Status	Country	Identifier/ Completion Date	Study Population (n)	Intervention	Evaluation
Study on Gut Microbiota in Chronic HBV Infected Patients	NA	China	NCT03587467 (December, 2020)	CHB (n=90)	16S rRNA gene sequencing of fecal samples.	<b>Primary outcome measure:</b> Gut microbiota shift of patients in different stages.
Efficacy of Addition of Fecal Microbiota Transplant (FMT) and Plasma Exchange to Tenofovir in Comparison to Monotherapy With Tenofovir in ACLF-HBV	Recruiting	India	NCT04431375 (June 10, 2022)	ACLF-HBV (n=70)	Study whether adding plasma exchange & FMT adds benefit compared to tenofovir treatment alone.	<b>Primary outcome measure:</b> 28-day overall survival. <b>Secondary outcome measures:</b> <i>Three</i> -month survival. Reduction in HBV DNA level. Reduction in CTP and MELD scores.
Study on Effect of Intestinal Microbiota Transplantation in Chronic Hepatitis B (CHB)	NA	China	NCT03429439 (November 2020)	CHB (n=60)	Proposing IMT combined with AVT over 26 weeks.	<b>Primary outcome measure:</b> Changes in serum HBsAg, <b>Secondary outcome measures:</b> Changes in anti-hepatitis B antigens. Relief of gastrointestinal symptoms. Fecal microbiota before and after IMT.
Study on Effect of Intestinal Microbiota Transplantation in Hepatitis B Virus Induced Cirrhosis	NA	China	NCT03437876 (December 2020)	HBV-LC (n=60)	Patients will be randomized to either a control or IMT group over 12 months, applying four IMT at 2-week intervals for all patients.	<b>Primary outcome measure:</b> Change of liver Fibroscan score <b>Secondary outcome measures:</b> Relief of gastrointestinal symptoms. Changes in gut microbiota at <i>three</i> , <i>six</i> , and <i>twelve</i> months.
Randomized Controlled Trial Comparing the Efficacy and Safety of FMT in Hepatitis B Reactivation Leads to Acute on Chronic Liver Failure.	Completed	India	NCT02689245 (March 13, 2018)	ACLF-HBV (n=64)	Group 1:Tenofovir Group 2:Tenofovir with FMT  Tenofovir would be given 300 mg once daily; FMT through a naso-jejunal tube for <i>seven</i> days.	<b>Primary outcome measure:</b> 3-month Transplant free survival <b>Secondary outcome measures:</b> Reduction in HBV DNA level $\geq 2$ log. Improvement in MELD and CTP scores. Improvement in clinical parameters. Change in gut microbiome in both. Assessment of organ failures.

787 **HBV:** Hepatitis B virus; **CHB:** Chronic hepatitis B; **ACLF:** Acute-on-chronic liver failure; **CHB-LC:** Chronic hepatitis B-associated liver cirrhosis; **FMT:**  
788 Fecal microbiota transplantation; **IMT:** Intestinal microbiota transplant; **AVT:** Antiviral treatment; **MELD:** Model for End Stage Liver Disease; **CTP:** Child-  
789 Pugh Turcotte, and **HBsAg:** Hepatitis B surface antigen. Data collected from the official website <https://ClinicalTrials.gov> (Accessed June, 2023).  
790



## 791 7. CONCLUSIONS AND PERSPECTIVES

792 Hepatitis B is one of the most common liver diseases worldwide, and studies  
793 describing HBV-associated dysbiosis lack consensus on a signature microbiota profile in  
794 most disease stages. Therefore, this review provided a comprehensive overview of the most  
795 recent research on HBV-associated dysbiosis investigating microbiome, mycobiome, and  
796 virome studies. We used traditional databases and AI tools to retrieve all the available  
797 studies. We did not consider using other AI-assisted technology or tools to retrieve the key  
798 results of the articles as they could produce authoritative-sounding output that is incorrect or  
799 incomplete in many situations compared to our manual analysis. Denoting that applying the  
800 technology with human oversight and control is mandatory and indispensable.

801 Our manual analysis showed that the results were heterogeneous in some cases,  
802 particularly in patient subgroups. This could be due to factors influencing gut microbiota,  
803 such as genetics, age, environmental exposures, and diet, which were not adjusted in many  
804 cases.

805 Eventually, reproducible results of a signature microbiota profile in most HBV-related  
806 diseases were obtained. An increase in *Pseudomonadota*, *Bacilli*, *Prevotella*, *Streptococcus*,  
807 *Veillonella* and a decrease in *Clostridia*, *Lachnospiraceae*, and *Roseburria* characterizes this  
808 signature. High abundance of *Saccharomyces cerevisiae*, and *Candida tropicalis* was a  
809 constant finding. Notably, dysbiosis was observed in all groups except for HBV carriers, who  
810 seemed similar to the control group. This indicates the role of microbiota in the pathogenesis  
811 of HBV infection.

812 It is challenging to determine whether the changes in gut microbiota are a cause or a  
813 result of HBV infection or which is the initiating factor between gut microbiota alteration and  
814 HBV disease progression to LC and HCC. Hence, well-established animal models may help  
815 elucidate the causality effect, especially in the acute stage. Moreover, modulating CHB-LC

816 progression affecting bacteria through phages or fungi is essential to analyze. Therefore,  
817 more studies investigating microbiota changes to the species level are needed in a more  
818 geographically diverse population.

819 Probiotics and FMT are promising gut microbiota-targeted therapies. However,  
820 probiotic fungi should be carefully prescribed, and more extensive FMT randomized clinical  
821 trials are needed to confirm the practicability in clinical settings. Hence, synthetic probiotics  
822 and selective microbiota transplantation may make these therapies more precise and bring  
823 fewer side effects.

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#### 828 **AUTHOR CONTRIBUTIONS**

829 R.M.W contributed to independent literature search and review, analysis, original draft  
830 writing, and visualization. M.T.A reviewed the final version. S.R reviewed the mycobome  
831 studies. P.B and R.G performed a critical review of the manuscript. D.R contributed to  
832 conceptualization and project administration. M.M supervised the study, performed the  
833 methodological quality assessment for the studies, and revised the results. All authors  
834 reviewed and approved the final version of the manuscript.

#### 835 **Declaration of generative AI and AI-assisted technologies in the writing process**

836 During the preparation of this work, the authors declare that no AI tools or any AI-assisted  
837 technologies have been used in the writing process or data extraction/analysis for the  
838 included studies in this review. The only AI tools used are [Semantic Scholar] and [Scite] in  
839 order to deeply investigate our systematic review by searching broad non-traditional

840 databases to retrieve all the related studies for our topic. The authors reviewed the content  
841 and take full responsibility for the content of the publication.

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## **CHAPTER II**

### **Gut Microbiota Characterization in HBV-Infected Patients Using Culturomics and Metagenomic Approaches**

# Preface

The link between gut microbiota and its derivatives in HBV infection has attracted considerable interest [6,7], mainly when the disease progression was found to occur even when a viral load is undetectable [43]. Bacterial products from the gut microflora have been associated with systemic inflammation and severe liver diseases [37,86]. One of those products is ethanol, known for its damaging effect on the liver [87].

Different studies reported significant changes in gut microbiota composition in HBV-infected patients based on metagenomics analysis, characterized by high abundance in *Proteobacteria* phylum [28] and genera such as *Streptococcus* [29], *Prevotella* [44], and *Veillonella* [46] and low abundance of *Clostridium* [28]. However, no study has investigated gut microbiota in HBV-infected patients based on cultivation techniques. Unlike molecular approaches, the high-throughput culturomics approach has been documented in studying gut microbiota, providing viable pure cultures [63]. Moreover, to date, culturomics has enabled the isolation of more than 300 new bacterial species in the human gut [62,63]. Therefore, our objective was to describe a culturomics-based microbiome profile in addition to metagenomic in HBV-infected patients compared to control. The species identified by both approaches were compared to identify new species or missing repertoire of probiotics, highlighting the complementarity of these two approaches.

Moreover, *Enterocloster bolteae* (formerly known as *Clostridium bolteae*) is an emerging gastro-intestinal pathogen [48], with significantly higher abundance in autistic children [49]. *E. bolteae* species showed high abundance in patients with fatty liver disease [50] and are known to produce ethanol [52]. However, its role or abundance profile hasn't been reported in HBV infection. Hence, reporting *E. bolteae* and possible endogenous ethanol production by bacterial strains enriched in HBV-infected patients is essential to our study.

## **Article 2**

### **Ethanol-Producing *Enterocloster bolteae* is enriched in Hepatitis B Virus-Associated Gut Dysbiosis: A Case-Control Culturomics Study**

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## Article

# Ethanol-Producing *Enterocloster bolteae* Is Enriched in Chronic Hepatitis B-Associated Gut Dysbiosis: A Case–Control Culturomics Study

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**Abstract:** Background: Hepatitis B virus (HBV) infection is a global health epidemic that causes fatal complications, leading to liver cirrhosis and hepatocellular carcinoma. The link between HBV-related dysbiosis and specific bacterial taxa is still under investigation. *Enterocloster* is emerging as a new genus (formerly *Clostridium*), including *Enterocloster bolteae*, a gut pathogen previously associated with dysbiosis and human diseases such as autism, multiple sclerosis, and inflammatory bowel diseases. Its role in liver diseases, especially HBV infection, is not reported. Methods: The fecal samples of eight patients with chronic HBV infection and ten healthy individuals were analyzed using the high-throughput culturomics approach and compared to 16S rRNA sequencing. Quantification of ethanol, known for its damaging effect on the liver, produced from bacterial strains enriched in chronic HBV was carried out by gas chromatography–mass spectrometry. Results: Using culturomics, 29,120 isolated colonies were analyzed by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI–TOF); 340 species were identified (240 species in chronic HBV samples, 254 species in control samples) belonging to 169 genera and 6 phyla. In the chronic HBV group, 65 species were already known in the literature; 48 were associated with humans but had not been previously found in the gut, and 17 had never been associated with humans previously. Six species were newly isolated in our study. By comparing bacterial species frequency, three bacterial genera were serendipitously found with significantly enriched bacterial diversity in patients with chronic HBV: *Enterocloster*, *Clostridium*, and *Streptococcus* ( $p = 0.0016$ ,  $p = 0.041$ ,  $p = 0.053$ , respectively). However, metagenomics could not identify this enrichment, possibly concerning its insufficient taxonomical resolution (equivocal assignment of operational taxonomic units). At the species level, the significantly enriched species in the chronic HBV group almost all belonged to class *Clostridia*, such as *Clostridium perfringens*, *Clostridium sporogenes*, *Enterocloster aldenensis*, *Enterocloster bolteae*, *Enterocloster clostridioformis*, and *Clostridium innocuum*. Two *E. bolteae* strains, isolated from two patients with chronic HBV infection, showed high ethanol production (27 and 200 mM). Conclusions: Culturomics allowed us to identify *Enterocloster* species, specifically, *E. bolteae*, enriched in the gut microbiota of patients with chronic HBV. These species had never been isolated in chronic HBV infection before. Moreover, ethanol production by *E. bolteae* strains isolated from the chronic HBV group could contribute to liver disease progression. Additionally, culturomics might be critical for better elucidating the relationship between dysbiosis and chronic HBV infection in the future.

**Keywords:** dysbiosis; gut microbiota; chronic hepatitis B virus infection; *Enterocloster bolteae*; culturomics; metagenomics



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## 1. Introduction

Hepatitis B virus (HBV) infection is a global health burden. Around 257 million people are HBV-positive worldwide [1]. HBV infection constitutes the primary driver of severe complications such as cirrhosis and hepatocellular carcinoma (HCC) [2]. Antiviral treatment has proven to slow HBV progression; however, the disease occurs in a small percentage of people even when a viral load is undetectable (50 IU/mL) [3]. Therefore, the pathophysiologic mechanisms underlying the evolution of chronic infection with HBV are still incompletely understood and deserve further investigation.

Gut microbiota has a complicated and mutually beneficial relationship with the host and plays a vital role in the host's metabolism, nutrition, pathological processes, and immune function [4]. The link between gut microbiota, its derivatives, and the pathophysiology of the liver has attracted considerable interest [5,6]. The liver receives various gut-derived substances (bacterial products, environmental toxins, and food antigens) via the biliary tract, portal vein, and systemic circulation due to the structural link to the intestine known as the gut–liver axis [7]. Bacteria and bacterial products from the gut microflora have been associated with systemic inflammation and severe liver diseases [8,9]. One of those products is ethanol, which is known to damage liver sinusoidal endothelial cells and induces centrilobular sinusoidal collapse, which reduces blood flow and impairs the microcirculatory exchange of nutrients and clearance of waste products [10].

In fact, different studies reported significant changes in gut microbiota composition in patients with chronic HBV infection based on metagenomics analysis, characterized by high abundance in *Proteobacteria* phylum [11] and genera such as *Streptococcus* [12], *Prevotella* [13], *Ruminococcus*, and *Veillonella* [14,15] and low abundance of *Clostridium* [11,16,17]. However, no study has investigated gut microbiota in chronic HBV infection based on cultivation techniques. The high-throughput culturomics approach has been documented in studying gut microbiota [18]. Culturomics provides viable pure cultures, unlike molecular approaches that only give information on the genus level (species-level assignment is uncertain for polyphyletic groups such as *Enterobacteria*), without any information regarding their viability status. In addition, microbial culture using selective media has made it possible to detect and cultivate minority bacterial populations that may pass undetected by genomic technologies [19]. By achieving this, culturomics could eliminate different limitations of metagenomics, such as extraction and bioinformatic bias, yielding difficult discrimination between species owing to short amplicon length [19]. Metagenome-assembled genomes (MAGs) [20] are a new trend that is aimed at overcoming these limitations. However, MAGs are artificial because they represent a bio-informatic reconstruction and may be biased by chimeric and repeat contigs [21]. Moreover, to date, culturomics has enabled the isolation of more than 300 new bacterial species in the human gut that were previously believed to be uncultured by traditional culture methods [18,19].

A previous study has reported the value of culturomics, performed in a case–control study, in defining the missing repertoire of probiotics and beneficial species that could be used in fecal microbiota transplantation (FMT), which requires viable isolated strains [22]. Interestingly, patients with chronic HBV infection who underwent FMT have achieved hepatitis B e-antigen (HBeAg) clearance in different studies [23–25]. Additionally, probiotics were reported to function by preventing liver inflammation from impeding the advancement of HCC via antioxidant and anti-metastatic activities [26]. In addition, the role of probiotics, particularly in chronic HBV-related complications such as hepatic encephalopathy, has been established in different studies [27–30]. As a result, it is critical to distinguish between culturomics, which specifically detects live organisms, and metagenomics, which yields DNA sequences most likely derived from already-dead species and cannot be isolated or multiplied [31]. To date, not all bacteria can grow, even in culturomics [31].

Moreover, among the genus *Clostridium*, a new genus of anaerobic bacteria, namely *Enterocloster*, has emerged as a reclassification due to advances in phylogenetics [32]. *Enterocloster bolteae* (formerly known as *Clostridium bolteae*) is an obligately anaerobic, Gram-positive, rod-shaped, and spore-forming gastrointestinal pathogenic bacterium [33].

*E. bolteae* was reported to produce butyrate, propionate, and acetate, which alter the motility and contraction rate of the gastrointestinal tract attributed to chronic diarrheal episodes [34]. *E. bolteae* appears to produce *beta*-lactamases with resistance to  $\beta$ -lactam antibiotics such as penicillin G, ticarcillin, and piperacillin–tazobactam [35]. It is known to be present in the stools of most children, with a significantly higher abundance in autistic children [36]. *E. bolteae* showed high abundance in patients with fatty liver disease [37]; however, its role or abundance profile has not been reported in chronic HBV infection. *E. bolteae* was reported to excrete metabolites that are thought to act as neurotoxins [38] and is known to produce ethanol [39]. In patients with chronic HBV infection, alcohol intake is associated with more severe liver disease [40]. Hence, reporting *E. bolteae* in chronic HBV infection is an interesting aspect of our study.

In this small study, we are the first to describe a culturomics-based microbiome profile to the species level in patients with chronic HBV infection and healthy individuals. The species identified by both culturomics and metagenomics for each group of samples were compared, highlighting the complementarity of these two approaches. Our study aims to detect possible endogenous ethanol production by specific bacterial strains enriched in patients with chronic HBV infection.

## 2. Material and Methods

### 2.1. Study Design

A case–control study was carried out in the Hepatology Department of Marseille University Hospital (south-eastern France), Marseille, France, according to STROBE statement guidelines [41] from January to June 2022. The HEPATGUT study was approved by the local ethics committee of l’Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France (IHUMI, 2020-004), approved by the Protection of Persons Committee (Approval No. CPP: 21.04391.000046—21075), and carried out according to the 2013 Declaration of Helsinki (1975; World Medical Association, 2013) [42]. Informed consent was obtained from the study subjects before their enrolment.

### 2.2. Study Population

The HEPATGUT study included 48 people with HBV and controls, intending to analyze 10 HBV and 10 control samples using culturomics and metagenomics. However, only eight HBV and ten controls have been studied and are reported in the present article after the exclusion of two HBV samples due to misclassification, in order to ascertain perfectly defined cases and controls. The HBV patients were recruited from Marseilles University Hospital. Healthy controls without chronic diseases or on regular medications were recruited using a snowball approach [43]. Data on age, gender, weight, height, and dietary habits were collected. Patients with chronic HBV infection were confirmed to be positive for hepatitis B surface antigen for at least six months; the diagnosis was made according to European Association for the Study of the Liver (EASL) guidelines [44].

Alcoholism and probiotics/antibiotics intake in the previous month were exclusion criteria for both cases and controls. In addition, vegetarians (those who eat only vegetables, fruits, grains, nuts, and occasionally eggs or dairy products) were excluded, as they represent a minority, and this dietary habit has been associated with a distinct gut microbiota [45]. The other exclusion criteria for all patients were as follows: patients with viral hepatitis other than HBV, solid organ transplantation, smoking, immunosuppressive drugs treatment within six months, acute or chronic infectious diseases, other liver disease, metabolic disease, malignancy, and autoimmune diseases. The fecal samples from all participants were collected and kept in sterile plastic tubes containing 1000  $\mu$ L transport medium; they were immediately (within 15 min) stored at  $-80$  °C until used. The transport medium consisted of 3 g/L NaCl, 0.2 g/L KCl, 0.1 g/L CaCl<sub>2</sub>, 0.1 g/L MgCl<sub>2</sub>, 0.2 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.25 g/L Na<sub>2</sub>S, 0.1 g/L glutathione, and 0.1 g/L uric acid. The pH was adjusted to 7.5, and the medium was filtered at 0.2  $\mu$ m.

### 2.3. High-Throughput Culturomics Approach

The stool samples (eight samples from patients with chronic HBV infection and ten samples from controls) were cultured according to the culturomics approach previously established in our laboratory [46]. Fast culturomics was applied using four culture conditions, as comprehensively detailed in a previous publication by Naud et al. [47]. Sampling of specimens was carried out in anaerobic conditions. For this purpose, the stool sample was placed in a half-open jar, itself placed under anaerobic conditions in a zip bag (Oxoid, Dardilly, France) complemented by an anaerobic GasPak (Becton Dickinson, Le Pont de Claix, France). The stool samples were aliquoted and frozen at  $-80^{\circ}\text{C}$  until use. One gram of stool sample was diluted in one millileter of phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Illkirch, France), and the diluted samples were directly inoculated on agar plates and also introduced with a syringe for pre-incubation into aerobic and anaerobic blood culture bottles. For direct inoculation, the stool sample was carried out on 5% sheep blood enriched Columbia (COS) agar (BioMérieux, Craponne, France) and inhouse YCFA (yeast extract, casein hydrolysate, fatty acids) agar. The sample was also pre-incubated in two liquid culture media at  $37^{\circ}\text{C}$ : the commercially available Biomerieux medium and the YCFA medium, supplemented with 2 mL defibrinated sterile sheep blood (BioMérieux, Marcy l'Etoile, France), and 2 mL active rumen filtered at  $0.22\ \mu\text{m}$ . Then, the blood culture vials and the directly inoculated agar plates were incubated at  $37^{\circ}\text{C}$ .

Inoculation on blood agar of the preincubated samples in blood culture bottles was performed for one month on different incubation days: Day 1, Day 3, Day 7, Day 10, Day 15, Day 21, and Day 30 [48]. To this end, the preincubated stool samples were diluted from  $10^{-1}$  to  $10^{-10}$  in PBS to dilute the sample and identify the maximum number of bacterial species. Then,  $50\ \mu\text{L}$  of diluted sample was deposited and homogenized onto COS, but only the YCFA + rumen + blood culture condition was also deposited onto YCFA agar. Each agar plate was incubated for 24 h for aerobic conditions or 48 h for anaerobic conditions at  $37^{\circ}\text{C}$ , mimicked with a zip bag containing an anaerobic GasPak. Bacterial colonies different in appearance, size, or color were subcultured on COS agar. These agars were then incubated according to the culture condition previously described (direct inoculation, aerobic bottle + rumen + blood, anaerobic bottle + rumen + blood, and anaerobic bottle + YCFA + rumen + blood).

The purified resulting colonies were then identified using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF/MS) according to the manufacturer's instructions [49,50]. Each colony was deposited in duplicate on a 96 MSP microplate and covered with  $2\ \mu\text{L}$  matrix solution. The solution was made of saturated  $\alpha$ -cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 2.5% trifluoroacetic acid. Measurements were performed with a MicroFlex LT/SH (Bruker, Bremen, Germany). Spectra were recorded in the positive linear mode. Data were automatically acquired using FlexControl v.3.4 and MALDI Biotyper Compass v4.1 software for the run preparation and bio-typing analysis. The spectra of different species were compared to MBT Compass BDAL Library v.11 (Bruker) containing 10,833 references of spectra (dispatched between 3893 species), as well as our laboratory database Culturomics containing 9973 references of spectra (dispatched between 2186 bacterial species) (accessed in October 2022). A score  $> 1.9$  allowed identification at the species level. In the case of unidentified colonies by MALDI-TOF (score  $< 1.9$ ), DNA from the unidentified colonies was extracted, amplified, purified, and analyzed, as described in [22]. The sequences with a similarity percentage under 98.65 or 95% [51] were identified as new species or new genera, respectively, and described according to the taxonogenomics concept [52]. If the bacterial species identification was not interpretable, 16S ribosomal DNA gene sequencing was performed.

The isolated species were compared to a recently published cultured human bacteria repertoire describing the bacterial species that have been cultured at least once from humans [53].

#### 2.4. Method of 16S Ribosomal DNA Gene Amplification and Sequencing

A mechanical treatment was first performed on samples with powder glass beads acid washed (G4649-500 g Sigma, St. Louis, MO, USA) and 0.5 mm glass beads cell disruption media (Scientific Industries, Bohemia, NY, USA) using a FastPrep-24™ 5G Grinder (mpBio) at maximum speed (6.5 m/s) for 90 s. In the previous study [54], Method 1 is associated with classical lysis and protease treatment, the samples were treated through two kinds of lysis methods, namely, method 1 and method 5, according to a prease step, followed by purification with E.Z.N.A Tissue DNA Kit (Omega bio-tek, Norcross, GA, USA), while method 5 is associated with using a deglycosylation step and purification on the EZ1 Advanced XL device with the EZ1 Qiagen tissue kit (Qiagen, Courtaboeuf, France). The libraries were constructed according to the protocol: “16S Metagenomic Sequencing Library Preparation” (Illumina Inc, San Diego, CA, USA). For each extraction protocol, the 16S “V3-V4” region of DNA samples was amplified by a PCR of 45 cycles using the Kapa HiFi Hotstart ReadyMix 2x (Roche, Basel, Switzerland). The primers used contain a conserved V3-V4 region with overhang Illumina adapters (FwOvAd\_341F: 5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3’; RevOvAd\_785R: 5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTA TCTAATCC3’). The same probes used to extract the samples were used as a negative control in addition to a blank of PCR containing the water used to prepare the mix. After purification on AMPure beads (Beckman Coulter Inc, Fullerton, CA, USA), concentration was measured using high-sensitivity Qubit technology (Beckman Coulter Inc., Fullerton, CA, USA), and dilution to 3.5 ng/μL was performed. At this step, the library of method 1 was pooled, volume to volume, to the library of method 5, and Illumina sequencing adapters and dual-index barcodes were added to the amplicon. After purification on AMPure beads (Beckman Coulter, Brea, CA, USA), this library was pooled with other multiplexed samples. The concentration of the pooled libraries was quantified by a Qubit assay with the high sensitivity kit (Life Technologies, Carlsbad, CA, USA) and diluted to 4 nM. Before loading for sequencing on MiSeq (Illumina, San Diego, CA, USA), the pool was denatured and diluted at 8 pM. Automated cluster generation and paired-end sequencing with dual-index reads were performed in a single 39 h run in a 2 × 250 bp. The paired reads were filtered according to the read qualities. The raw data were configured in fastq files for R1 and R2 reads.

#### 2.5. Measurement of Ethanol Production by Strains Enriched in Chronic HBV Samples

The bacteria were grown in a yeast–peptone–glucose (YPG) medium composed of the following components: 10 g yeast extract (BioMérieux, Craponne, France), 20 g peptone (BD Diagnostic, Le Pont de Claix, France), 20 g D-glucose (BD Diagnostic, Le Pont de Claix, France), and 1 L of distilled water. Once all ingredients were thoroughly dissolved, the broth was filtered using the RapidFlow™ filtration system (Thermo Fisher, Illkirch, France) with a 0.22 μm membrane, and 10 mL of filtered broth was poured into Hungate tubes. Each tube was inoculated at a concentration of one McFarland to standardize bacterial concentration. Several colonies were picked and suspended in tubes with sterile 0.85% NaCl medium (BioMérieux, Craponne, France), and turbidity was measured using a McFarland densitometer (BioMérieux, Marcy l’Etoile, France).

The strains were grown in three different experiments, with some variations in the protocol in terms of inoculated bacterial concentration and degassing time. Experiment 1: the bacterial suspension was inoculated at one McFarland, and the tubes were degassed for two minutes. Experiment 2: To promote the growth of *E. bolteae* strains, the concentration of the bacterial suspension was increased to three McFarland, the other strains were grown at one McFarland, and the degassing time was two minutes for all strains. Experiment 3: All strains were grown at one McFarland, and the tubes were degassed for three minutes. *E. bolteae* strain s28/42 was grown on two different media (YPG and BACT/ALERT® FN Plus flask (bioMérieux, Lyon, France)). The BACT/ALERT FN Plus flasks for blood culture contain polymeric beads that adsorb antibiotics with peptones/biological extracts,



anticoagulants, vitamins, amino acids, carbon sources, trace elements, and other complex amino acid and carbohydrate substrates in purified water, in a vacuum atmosphere of N<sub>2</sub> and CO<sub>2</sub>.

Quantification of ethanol produced in each culture was carried out by headspace gas chromatography–mass spectrometry (HS-GC–MS) using a Turbomatrix HS110 sampler connected to a Clarus 690 chromatograph and a SQ8 T single quadrupole mass spectrometer (Perkin Elmer, Waltham, MA, USA). A standard ethanol curve was prepared with concentrations ranging from 0.5 mM to 100 mM spiked with isopropanol as an internal standard (5 mM). Samples were dispensed into headspace (HS) vials using 1 mL aliquot of each culture, then spiked with isopropanol. HS vials were heated at 60° C (for 10 min) and pressurized to 25 psi (1 min). The volatilized content was transferred to the GC (0.03 min, split 10/1). Alcohols were then separated through an Elite BAC2 column (30 m, 0.32 ID, Perkin Elmer) maintained at 70 degrees using helium as carrier gas (19.5 psi). A selected ion recording method was used to measure ethanol (31 *m/z*) and isopropanol (45 *m/z*). The data obtained were analyzed using Turbomass 6.1 software (Perkin Elmer).

### 2.6. Bioinformatic Analysis

The raw sequencing data for all samples were deposited into the NCBI Sequence Read Archive database (accession number: PRJEB62828). Noisy sequencing data were excluded, and chimeric sequences were identified and removed by Chimera Slayer. The clean data were clustered into operational taxonomic units (OTUs) at the 97% similarity threshold using UCLUST algorithm after the removal of singletons. The alpha, beta diversity, and linear discriminant analysis (LDA) were calculated using the Microbiome Analyst Platform (<https://www.microbiomeanalyst.ca/>; accessed on 17 March 2023) [55]. Shared taxa present in all groups were defined as the core microbiota.

### 2.7. Statistical Analysis

The results are expressed as the mean ± standard deviation. The normal distribution was performed using the Kolmogorov–Smirnov test. Two-tailed unpaired Student’s *t*-tests or Mann–Whitney tests were used when the data were normally or not normally distributed, respectively. Moreover, the Chi-squared test was used to compare the proportions of a sufficiently large number of species (>20 in each contingency table cell). In contrast, Fisher’s exact and bilateral Barnard’s exact tests were used if the number of species was small (typically < 20 in one or more cells of the table). For gut microbiota analysis, differences in the relative abundances of OTUs of dominant bacteria were analyzed using Mann–Whitney U-tests. Statistical significance was accepted at *p* < 0.05. All analyses were performed with GraphPad Prism Software for Windows (GraphPad Software, San Diego, CA, USA) (version 9.0).

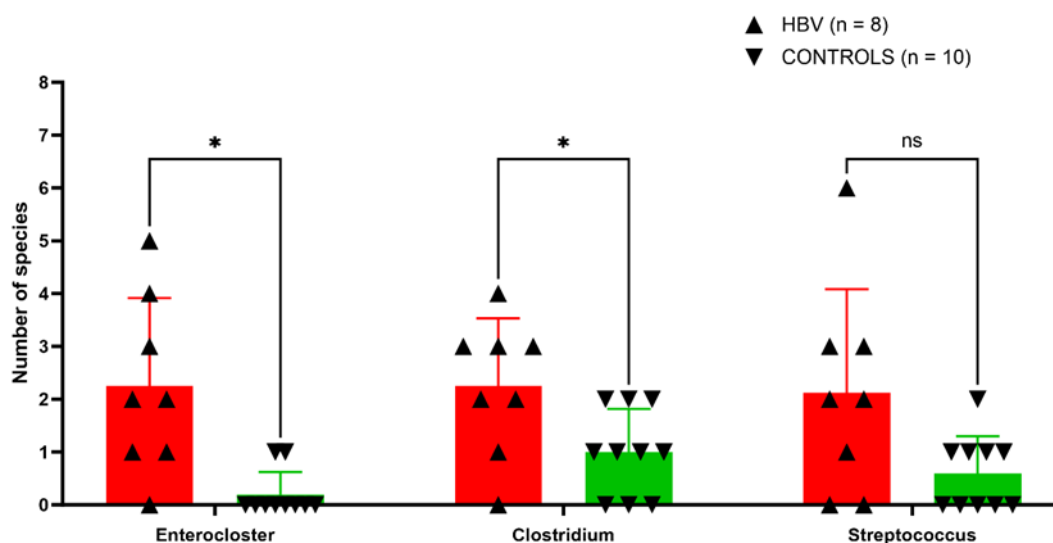
## 3. Results

### 3.1. Altered Diversity in Chronic HBV Samples by Culturomics

Of the 18 samples, 29,120 colonies were analyzed by MALDI–TOF to identify a total of 340 species belonging to 169 genera, and 6 phyla (chronic HBV samples (*n* = 8) yielded 240 species and 14,340 colonies (1793 ± 225 colonies per sample); conversely, control samples (*n* = 10) yielded 254 species, and 14,780 colonies (1478 ± 265 colonies per sample) were isolated (unpaired *t*-test, *p* = 0.0168). In addition, 154 species were shared between chronic HBV and control groups, while 86 species were uniquely detected in the chronic HBV group. A detailed list of the isolated bacterial species is mentioned in Supplementary File S1.

In chronic HBV samples, 5 bacterial phyla were isolated, with mostly *Bacillota* (148 species), followed by 34 *Bacteroidota*, 32 *Actinobacteria*, 16 *Proteobacteria*, and 2 *Synergistetes*. In control samples, 6 phyla were isolated, with a majority of *Bacillota* (143 species), followed by 50 *Bacteroidota*, 40 *Actinobacteria*, 12 *Proteobacteria*, 1 *Synergistetes*, and 1 *Verrucomicrobia* (Table S1).

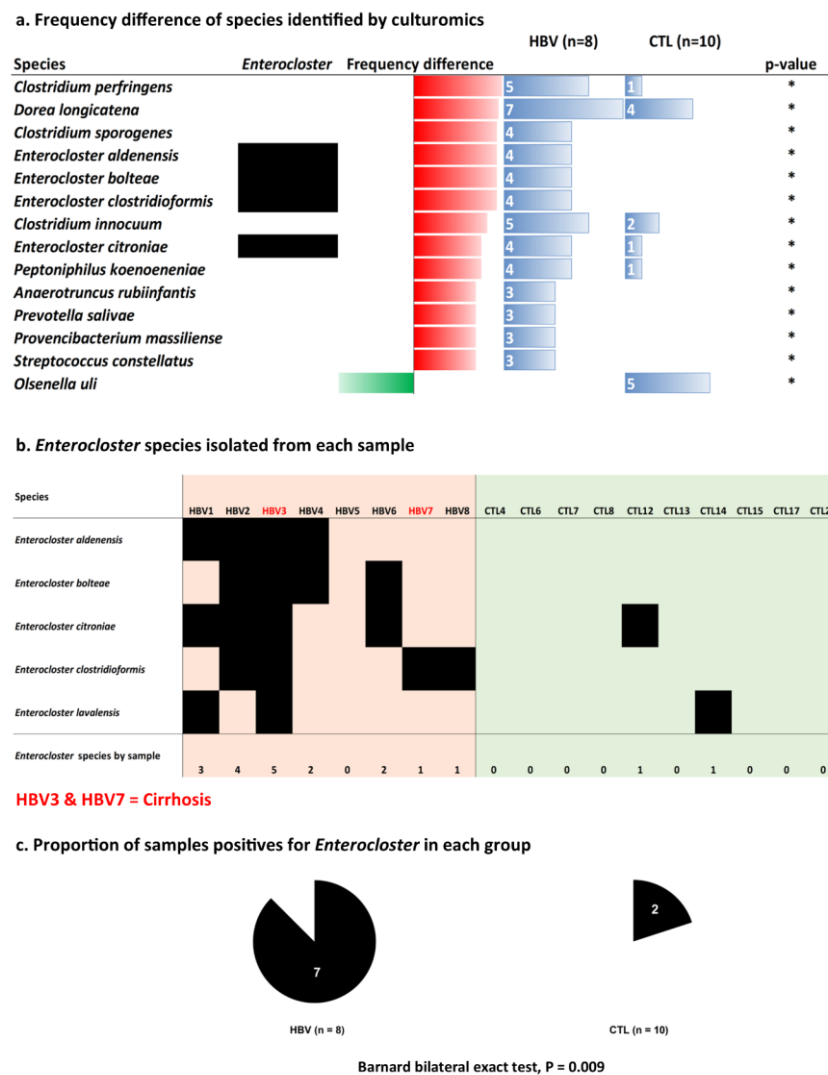
The 240 species isolated from chronic HBV samples belonged to 43 different families and 128 different genera. Among them, nine were the best-represented genera in terms of the diversity in the species, including *Bacteroides* (eleven species), *Enterococcus* (eleven), *Alistipes* (nine), *Streptococcus* (nine), *Clostridium* (eight), *Peptinophilus* (eight), *Enterocloster* (five), and *Prevotella* (five). In control samples, a total of 111 genera were identified, with the 7 most-represented genera including *Bacteroides* (15 species), *Enterococcus* (11), *Alistipes* (12), *Peptinophilus* (9), *Bifidobacterium* (7), *Limosilactobacillus* (7), and *Clostridium* (6) (Table S1). Thirty-four genera were only represented in the chronic HBV group and not in the control group. Three bacterial genera from the *Bacillota* and *Bacilli* phyla were found to have increased diversity (number of species of this genus per sample) in patients with chronic HBV infection, although only two genera, *Enterocloster* ( $p = 0.0016$ ) and *Clostridium* ( $p = 0.041$ ), showed significantly increased diversity (Table S1; Figure 1).



**Figure 1.** Boxplot showing the number of species per sample identified by culturomics in the three genera with a significant frequency difference between patients with chronic hepatitis B virus (HBV) infection (Red) versus the control group (Green). Two-tailed Mann–Whitney test; \*  $p$  value < 0.05. ns: not statistically significant.

At the species level, the species with the highest significant difference in frequency belonging to class *Clostridia* were four *Enterocloster* (*E. bolteae*, *E. aldenensis*, *E. clostridioformis*, and *E. citroniae*) and three *Clostridium* (*C. perfringens*, *C. sporogenes* and *C. innocuum*) in the chronic HBV group. However, *Olsenella uli*, which belongs to Class *Coriobacteriia*, was more frequent in the control group (Figure 2a). The proportion of positive samples for *Enterocloster* was higher in the chronic HBV group (7/8 vs. 2/10,  $p = 0.009$ , bilateral Barnard's exact test). Two patients with chronic HBV infection (HBV3, HBV7) were diagnosed with cirrhosis. HBV3 showed a greater diversity of *Enterocloster* species than the other samples (Figure 2b).

Overall, the hitherto unknown diversity was assessed and defined as the number of new species added to the species not previously known from the human gut by sample for culturomics analysis and as the number of unidentified OTU for metagenomics analysis [22]. The difference was not significant by culturomics for new species ( $0.75 \pm 1.09$  in chronic HBV vs.  $1 \pm 1.6$  in controls,  $p = 0.96$ ), as well as for previously known species which had not been previously found in humans ( $3.5 \pm 1.41$  in HBV vs.  $2.36 \pm 1.07$  for controls,  $p = 0.0763$ ) (Table S2).



**Figure 2.** (a) Species with a significant frequency difference between chronic hepatitis B virus (HBV) and control (CTL) samples by the culturomics approach. (b) Detailed *Enterocloster* species isolated per sample. (c) Proportion of samples positive for *Enterocloster* species. Barnard’s bilateral exact test was used to test the *p*-value. \* *p*-value < 0.01.

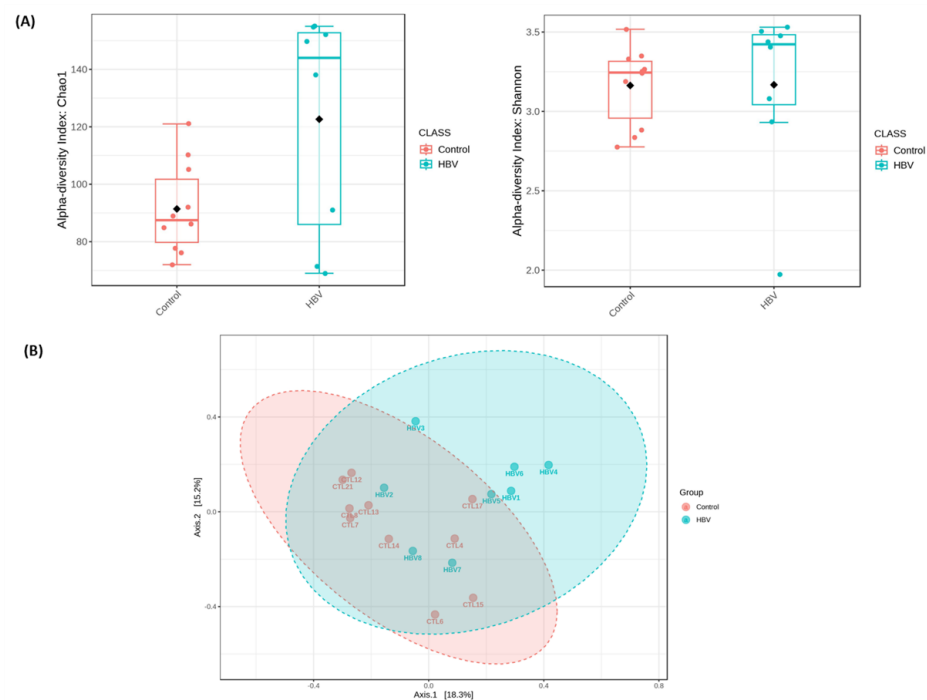
By comparing the species isolated in the chronic HBV group to a recently published cultured human bacteria repertoire describing 3242 bacterial species, 81 shared species, and 5 species were uniquely defined in the HBV group. Moreover, 14 species were found in common between chronic HBV and control groups (Supplementary File S1). In addition, among the 240 species isolated in the chronic HBV group, 175 species were known in the human gut, 48 species were known in humans but not in the gut, 17 species were known but not previously found in humans, and 6 species were identified as new species (Table S3). In the control group, 11 unknown species were isolated from the human gut, among which 4 new genera were identified (Table S3), 13 were known but not previously found in humans, and 44 were already known in humans but not previously found in the gut.

### 3.2. Diversity Assessed by Metagenomics

The sequencing run expressed good quality monitoring parameters, as denoted in a cluster density of 965 K/MM2, passing filters of 53.6%, Q score of 2.3G 83.6%, and PhiX of 15%. Sequencing yielded 1,704,021 good-quality total reads (associated and non-associated reads) for the 18 samples included in our study (1,016,049 for chronic HBV and 687,972 for

controls). The number of reads per sample was significantly higher in the chronic HBV group ( $127,006 \pm 58,171$ ) than in controls ( $68,797 \pm 33,188$ ) ( $p = 0.016$ ).

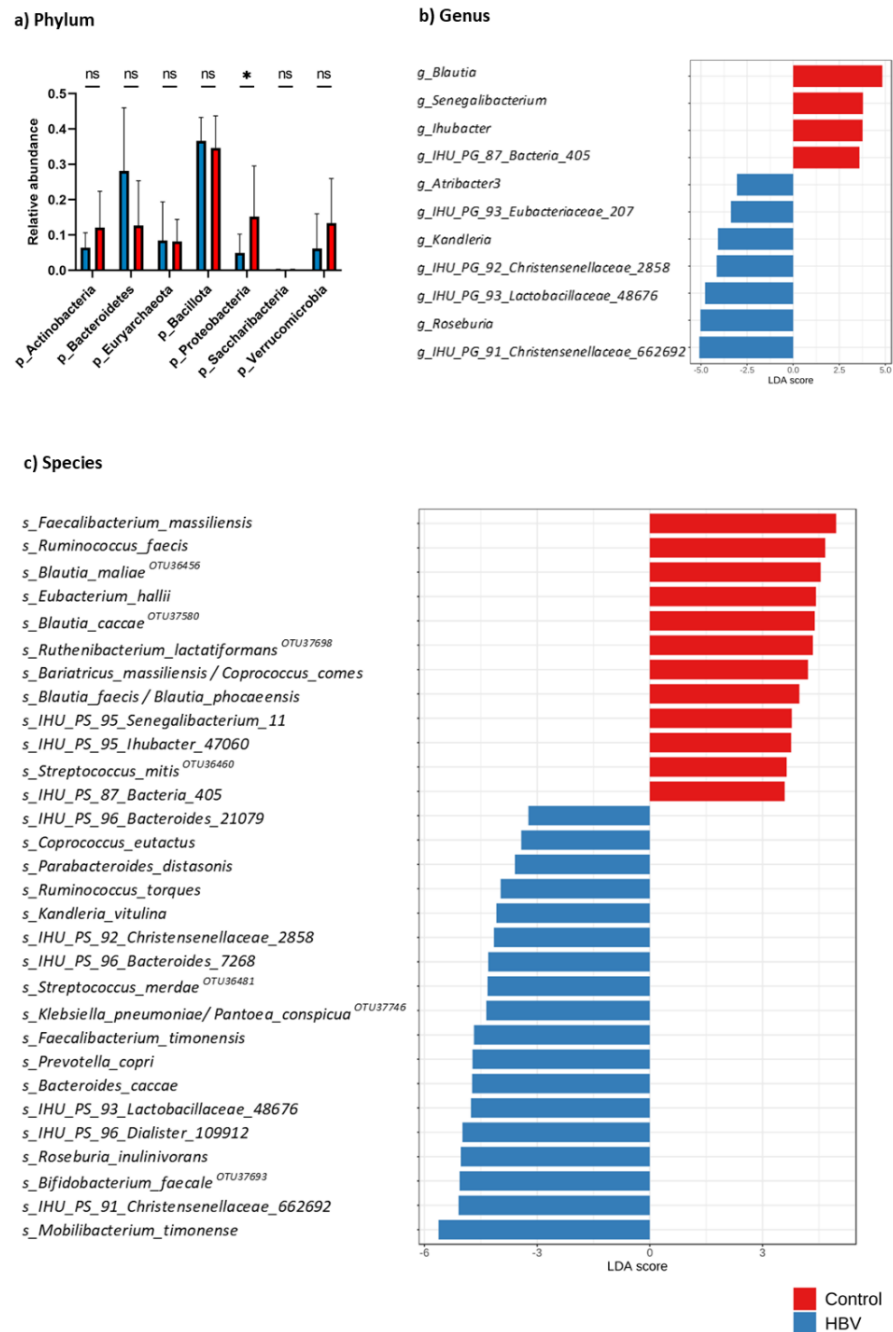
The metagenomics analysis showed that in chronic HBV samples, 826,517 associated reads were dispersed throughout 7 phyla (*Actinobacteria*, *Bacteroidetes*, *Euryarchaeota*, *Bacillota*, *Proteobacteria*, *Candidatus Saccharibacteria*, and *Verrucomicrobia*). Control samples generated 507,865 associated reads and were divided into the same 7 phyla detected in the chronic HBV group. These reads matched 682 species in the HBV group and 715 in the control group. The richness and diversity of both groups were compared through the alpha diversity metrics shown in Figure 3. There was no significant difference in the Chao1 index ( $p = 0.168$ ) or Shannon index ( $p = 0.315$ ). Beta diversity showed significant differences in bacterial communities through principal coordinate analysis (PCoA) ( $R^2 = 0.108$ ,  $p = 0.009$ ) (Figure 3). No significant difference existed between the groups, except for *Proteobacteria*, which showed a significant decrease in patients with chronic HBV infection,  $p = 0.034$  (Figure 4). The chronic HBV group showed 76 genera and the control group showed 74 genera (*g\_IHU\_PG\_93\_Eubacteriaceae\_207* and *g\_Atribacter3* were absent from the control group) (Table S4). Highly significant different genera in both groups with LDA score  $> 2.0$  and  $p < 0.05$  are illustrated in Figure 4. Interestingly, no significant difference was noticed regarding abundance for the same frequent genera detected by culturomics as *Clostridium* ( $p = 0.145$ ) and *Streptococcus* ( $p = 0.260$ ). *Enterocloster* was not recognized as an abundant taxon by LDA. Highly abundant OTUs assigned to the species level with LDA score  $> 2.0$  and  $p < 0.05$  in both groups were shown in Figure 4.



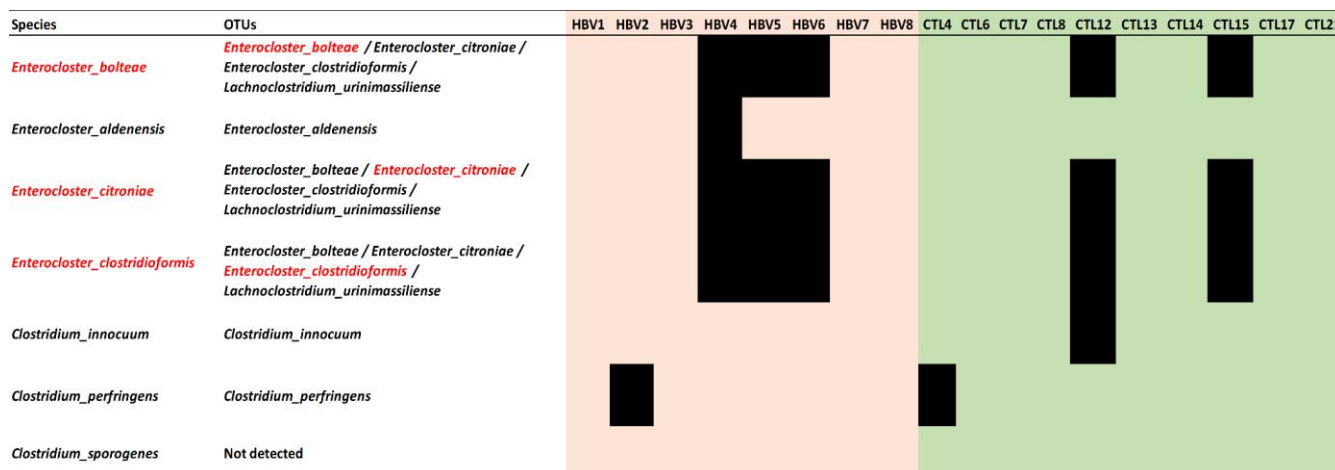
**Figure 3.** Comparison of alpha diversity and beta diversity in patients with chronic hepatitis B virus (HBV) and control groups. (A) Alpha diversity in box plots. Each boxplot represents the data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (black diamond). (B) Principal coordinate analysis (PCoA) plot of beta diversity.

Notably, six out of all seven species belonging to *Enterocloster* and *Clostridium* genera were identified as multi-assigned OTUs in the dataset of 16S ribosomal RNA sequencing results (*Enterocloster\_aldenensis*, *Enterocloster\_bolteae*, *Enterocloster\_citroniae*, *Enterocloster\_clostridioformis*). *Enterocloster\_bolteae* could not be identified as a single OTU. *Clostridium\_innocuum* was identified as a single OTU, while *Clostridium\_perfringens* was identified in single and multi-assigned OTUs. *C. sporogenes* was not identified at all in the

samples. *Clostridium innocuum* and *Clostridium perfringens* species were unequivocally identified (only one species is known for this OTU). Three species were identified equivocally (Figure 5). Overall, none of the seven species with significant differences identified by culturomics had a considerable difference by sequencing. Similarly, no significant differences were noticed regarding the relative abundance of all OTUs attributed to *Enterocloster* or *Clostridium* at the genus level.



**Figure 4.** Relative abundance of gut microbiota in patients with chronic HBV infection (n = 8) and the control group (n = 10). (a) Barplot expressing abundant phyla. (b) Genus-level and (c) species-level were selected via LefSe (LDA score > 2). Some OTUs correspond to several species (see Table S5). For clarity, only one clinically relevant species is represented on the graphic. HBV: Hepatitis B virus; LefSe: linear discriminant analysis effect size; LDA: linear discriminant analysis; OTUs: operational taxonomic units. ns: not statistically significant. \*  $p < 0.05$ .



**Figure 5.** *Enterocloster* and *Clostridium* species detected in 16s RNA sequencing data file per sample. OTUs: Operational Taxonomic Units; HBV: hepatitis B virus infection; CTL: control. *E. bolteae*, *E. citroniae*, and *E. clostridioformis* (red words) were identified equivocally.

The metagenomics analysis showed a decreased aerotolerant alpha diversity ( $1.11 \pm 0.40$  in chronic HBV vs.  $1.21 \pm 0.49$  in controls;  $p = 0.514$ ) in addition to a decreased anerointolerant diversity in the chronic HBV group ( $2.85 \pm 0.53$  in HBV vs.  $3.08 \pm 0.31$  in controls;  $p = 0.359$ ). These results confirmed the specific decrease in anaerobic diversity found by culturomics, indicating loss of anaerobic species in patients with chronic HBV infection. The hitherto unknown diversity (unidentified OTUs) was consistently and significantly increased in the chronic HBV group ( $p = 0.0434$ ). Additionally, at the prokaryotic level, 18.65% of all reads in the chronic HBV group were not assigned vs. 25.67% in control ( $p < 0.0001$ ).

### 3.3. Missing Repertoire in Patients with Chronic HBV Infection

For identification of potential probiotic species, all of the bacterial species that were identified both by culturomics and metagenomics were considered in the control samples but not in HBV samples (Supplementary File S1). The common species between both approaches in the control group were six, including *Alistipes merdae*, *Christensenella massiliensis*, *Dialister succinatiphilus*, *Fenollaria timonensis*, *Mediterranea massiliensis*, and *Metaprevotella massiliensis*. However, the HBV group showed nine shared species between both approaches, including *Clostridium marseillense*, *Mogibacterium neglectum*, *Mogibacterium vescum*, *Pantoea agglomerans*, *Prevotella caccae*, *Prevotella copri*, *Terrisporobacter glycolicus*, *Weissella cibaria*, and *Weissella confusa*.

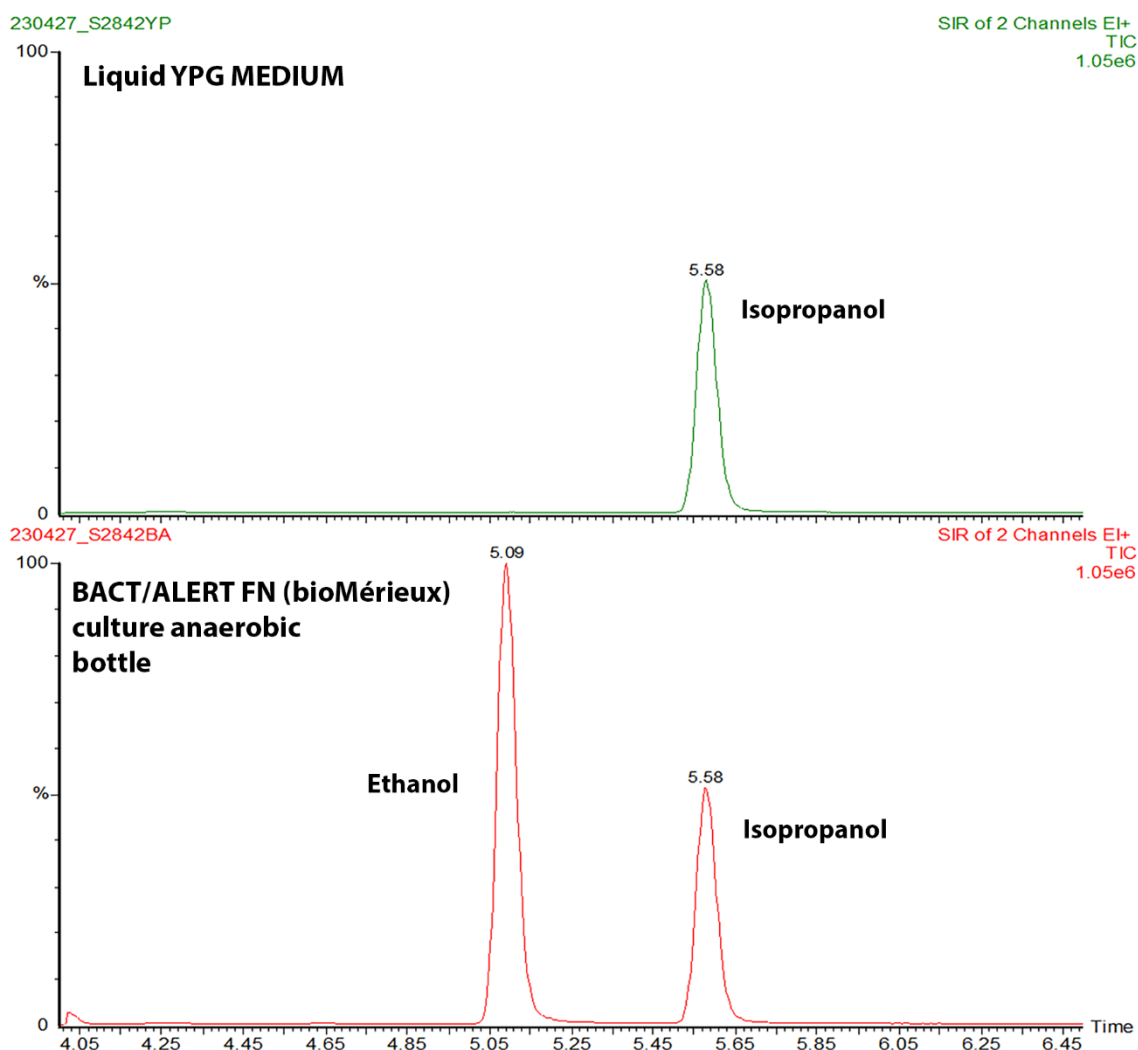
Comparing the species identified by metagenomics and culturomics in chronic HBV and control groups, 213 species were found exclusively in the control samples (Supplementary File S1). These species belonged overwhelmingly to the *Bacillota* phylum (89; 41.75%) and *Proteobacteria* (73; 34.27%), followed by a low number of species from the *Actinobacteria* (22; 10.33%), the *Bacteroidetes* (26; 12.21%), and *Euryarchaeota* (1; 0.47%) phyla. Among the missing repertoire, strikingly, 129 species (60.56%) were strictly anaerobic.

The literature was searched for each species to find a possible probiotic use for humans. Three species, *Limosilactobacillus oris*, *Propionibacterium freudenreichii*, and *Streptococcus oralis*, were found to have possible probiotic features (Table S6). Two of them (*L. oris* Q6189 and *S. oralis* Q4071) were isolated by culturomics in the control samples and are readily available in our laboratory’s Collection de Souches de l’Unité des Rickettsies (CSUR) collection.

### 3.4. Ethanol Quantification Produced by Enterocloster Species

The ethanol production of strains isolated from patients with chronic HBV infection was investigated (Table S7). Following two attempts in YPG and BACT/ALERT FN media,

the third effort using a closed BACT/ALERT FN bottle to establish controlled anaerobiosis with an optimized medium (a technique developed during microbiological culturomics) [48], showed significant ethanol production (Figure 6). No ethanol detection was observed for any of the *E. bolteae* strains or *E. citroniae* in experiment 1 and 2 (Table S7). However, in experiment 3, *E. aldenensis* strain s16/38 and *E. bolteae* strain s28/42 exhibited a weak ethanol production below 0.25 mM on YPG medium. Interestingly, *E. bolteae* strain s28/42 showed significantly higher ethanol production when growing on FN medium (Table S7).



**Figure 6.** Detection of ethanol by gas chromatography–mass spectrometry (GC–MS) produced by isolated *Enterocloster* strain S28/42 in HBV using YPG or BACT/ALERT® FN Plus culture media. The first chromatogram shows the analysis of *E. bolteae* strain s28/42 grown on YPG medium (10% glucose), in which bacterial growth was deficient and no ethanol was detected. The second chromatogram corresponds to the same strain grown on BACT/ALERT® FN Plus, in which bacterial growth was evident within 24 h, and the presence of ethanol was observed at the peak at 5.09. The peak at 5.58 in both graphs corresponds to the isopropanol used as an internal standard.

#### 4. Discussion

In this study, we report four enriched *Enterocloster* species, for the first time, in patients with chronic HBV infection using the culturomics approach rather than metagenomics. To our knowledge, no *Enterocloster* species were linked to chronic HBV infection.

The “culturomics” approach, whose effectiveness in analyzing gut microbiota has not yet been established [18], offers a significant benefit over metagenomics to exclude

the enormous number of ingested bacteria that are killed in the upper gut by the acidic environment and bile salts [56] and to provide live strains on which further analysis can be performed. The popular method for examining the diversity of gut microbiota is metagenomics; however, the results of those studies has shown very low reproducibility, likely due to variations and biases in sampling, DNA extraction methodology, sequencing method, and data analysis technique [57]. The discordance between culturomics and metagenomics results has been a surprising finding since the very first development of this approach in 2009, with less than 20% common species [48]. Interestingly, the metagenomic approach detected a lower number of bacterial species than culturomics in a study conducted on a patient with resistant tuberculosis [58]. This demonstrates the depth bias of metagenomics. Moreover, it is a central point for the culturomics approach developed in our lab ten years ago to consider that all bacteria could grow [48]. Indeed, culturomics aims to reproduce the natural micro-environment and successfully discover hundreds of new bacterial species. However, several species and strains remain uncultured to date. However, the sample's preparation could significantly impact culturability, including the mode of sampling, time of exposure to oxygen, and mode of freezing. For instance, using cryoprotectants could have dramatically improved culturability [59]. In addition, it was reported that culturability depends on the abundance of the bacterial species in the sample [31].

Eventually, both approaches seem to have several biases that could explain the disparities and complementarity [18,19]. Nevertheless, despite the discrepancy between metagenomics and culturomics [60,61], culturomics allowed the extension of gut microbiota's known diversity and functions [62], as we proved by the results of this study. Furthermore, culturomics is a validated technique with consistent species identification that avoids some previously mentioned disadvantages of the metagenomic approach, such as inferior taxonomic resolution [19].

We intended to analyze ten samples per group by culturomics because it is a time-consuming approach in which one sample takes approximately four weeks to be analyzed. In addition, this small study was designed to be an exploratory study typically conducted to gain insights, explore potential relationships, and generate hypotheses for further investigation. Furthermore, exploratory research depends on the topic and the field. However, exploratory studies often come with certain limitations, and one common concern is the small sample size [63]. In our exploratory study, it is not only about the time consumed for every culture condition. It is also about the in-depth and accurate identification of the isolated colonies; as mentioned in the results section, from the 18 samples, 29,120 colonies were analyzed by MALDI-TOF to identify a total of 340 species (HBV samples ( $n = 8$ ) yielded 240 species and 14,340 colonies ( $1793 \pm 225$  colonies per sample) vs. control samples ( $n = 10$ ) yielded 254 species, and 14,780 colonies ( $1478 \pm 265$  colonies per sample) were isolated; unpaired  $t$ -test,  $p = 0.0168$ ) (Supplementary File S1). Since power refers to the likelihood that we will find a significant result (an effect) in a studied sample or population [64]. Accordingly, several colonies and species identified per sample in our study made our results robust. The significant difference with low statistical power corresponded to dramatic and clear-cut differences. Hence, further studies with increased power (from more samples) are encouraged to confirm our results and decipher less important differences.

A total of 240 and 254 living, viable, and cultivable bacterial species were identified in the chronic HBV and control groups, respectively. Fifty-five species have been recognized as core microbiome detected by both approaches. Among them, 213 species formed a missing repertoire in patients with chronic HBV infection since they were found by both techniques exclusively in the healthy control group but not in the chronic HBV group. The global diversity, by culturomics, was significantly decreased alongside the aerointolerant species in the HBV group compared to controls. Metagenomic results showed the same diversity pattern. The hitherto unknown diversity was significantly increased by metagenomics in the chronic HBV group. Nevertheless, some studies using the metagenomic approach showed different results. Joo et al. reported higher alpha diversity in the HBV group [65],



while Zheng et al. reported its decrease [66]. This could be attributed to differences in sample sizes and study populations.

Gut microbiota at the phylum level showed a non-significant increase in *Proteobacteria* and a decrease in *Actinobacteria* and *Bacteroidetes* in the HBV group by culturomics. However, metagenomics showed a significant reduction in *Proteobacteria* and a non-significant increase in *Bacteroidetes*. Indeed, several metagenomic studies supported our culturomics findings, which showed a continuous increase in the abundance of *Proteobacteria* in chronic HBV infection [11,14]. Interestingly, in agreement with Lagier et al. [18], most undetected human gut bacterial species grew in strict anaerobic (44%) or microaerophilic (5%) conditions, and 33% of them belonged to the phylum *Proteobacteria*. In contrast, only 9% belonged to the phylum *Bacteroidetes*. Those findings also support our culturomics results and align with the fact that our developed culturomics culture conditions were applied to cultivate those missing bacteria. However, the discrepancy in *Proteobacteria* abundance results between culturomics and metagenomics approaches could be attributed to the small sample size or other previously mentioned biases in the metagenomic approach. According to further research, *Bacteroidetes* and *Actinobacteria* in the chronic HBV group either increased or decreased [11,14,15]. Interestingly, both approaches reported an increase in *Bacillota*, which was in agreement with the previous studies [14,66]. Therefore, further exploration of gut microbiota dysbiosis in chronic HBV infection is warranted.

Among the top highly represented genera by culturomics, *Enterocloster* and *Clostridium* were significantly increased in the chronic HBV group. However, metagenomics showed a significant abundance of different genera, including *Roseburia*, *Kandleria*, and *Atribacter*, in the HBV group. Our results are different from most previous studies, which reported a decrease in *Clostridium* [11,16,17] and *Roseburia* [15,16] genera in chronic HBV infection. This could be attributed to the different sample size and diagnostic approaches used in this study. *Kandleria*, a genus from the family of *Erysipelotrichidae* [67], and *Atribacter*, a genus of the candidate phylum *Atribacterota* [68], have not been reported before in patients with liver disease, especially with chronic HBV infection. Although non-significantly, the increased abundance of *Streptococcus* in the chronic HBV group agreed with many previous studies [14–16,69].

*Enterocloster* is a genus recently identified in 2019 [32] as a reclassification of *Clostridium* genus, thanks to improved genomics and taxonomy. An unbiased study recently identified this new genus as the main provider of inoviruses [69], potentially pathogenic prophage viruses [70,71]. This genus comprises six validated species [32], including *E. aldenensis*, *E. asparagiformis*, *E. bolteae*, *E. citroniae*, *E. clostridioformis*, and *E. lavalensis*. Five *Enterocloster* species were isolated from chronic HBV samples; only two were in controls. Four species (*E. aldenensis*, *E. bolteae*, *E. citroniae* and *E. clostridioformis*) showed a significantly increased frequency in patients with chronic HBV infection. Notably, both *E. clostridioformis*/*E. bolteae* and *E. asparagiformis*/*E. lavalensis* were closely related based on phylogenetic, phylogenomic, and phenotypic perspectives [32]. However, culturomics alone could separately identify those species that were not distinguishable by sequencing but well-identified by MALDI-TOF, with high identification score > 2. A total of 18 OTUs were identified as *Enterocloster* species, and 448 OTUs were assigned for *Clostridium* species. Among 18 OTUs of *Enterocloster* species, seven OTUs were multi-assigned for *E. asparagiformis* and *E. lavalensis*. *E. bolteae* has been identified in three multi-assigned OTUs.

Species of *Enterocloster* genus have been associated with different diseases and dysbiosis. *Enterocloster aldenensis* was reported in intra-abdominal infections [72]. Additionally, a high carbohydrate-fermenting *Enterocloster* species such as *E. clostridioformis* was documented to be associated with clinical bacteremia cases [73] and was highly abundant in Type II diabetes [74] and Crohn's disease [75]. Interestingly, *E. bolteae* was reported in different neurological diseases such as autism [38,76], multiple sclerosis [77], and neuromyelitis optica spectrum disorders [78]. According to a prior study, *E. bolteae* could produce microbially conjugated bile acids that contribute to the severity of Crohn's disease and irritable bowel syndrome (IBS) [79]. Moreover, *E. bolteae* was identified as a mediator in

fatty acid (FA) acylation to isoBAs (bile acids) [80]. *E. bolteae* has been reported in patients with fatty liver disease [37]. However, *E. bolteae* is not reported in chronic HBV infection; additionally, the role of its produced FA-isoBAs in host physiology or hepatic diseases is under investigation. Surprisingly, among *Enterocloster* species, *E. citroniae* was described as an enriched purine-degrading species and considered a promising therapeutic prebiotic to reduce serum uric acid levels in a clinical trial performed on renal failure patients [81]. Moreover, patients on peritoneal dialysis who experienced a restriction of advanced glycation end products diet (to decrease cardiovascular disease incidence) showed an increase in *E. citroniae*, suggesting its beneficial role [82]. As a result, the increased prevalence of the *Enterocloster* species mentioned in chronic HBV infection necessitates more research into their potential role in liver disease progression, particularly HBV-related complications.

Our culturomics results showed significant abundance in three *Clostridium* species, including *C. perfringens*, *C. innocuum*, and *C. sporogenes* in the HBV group. In fact, the genus *Clostridium* has recently been taxonomically clarified [83], making it possible to specify the associations between this genus and several diseases. In particular, *C. perfringens* is associated with necrotizing enteritis, enterotoxemia, and gas gangrene [84]. Moreover, cirrhotic patients infected with *C. perfringens* manifested poor prognosis [85]. *Clostridium innocuum* was described as an extraintestinal pathogen causing bacteremia, endocarditis, osteomyelitis, and peritonitis and may also cause a *C. difficile*-like antibiotic-associated diarrheal illness [86]. Additionally, *C. sporogenes* was able to transform tryptophan into indole-3-propionic acid affecting intestinal permeability and was found to be negatively correlated with several metabolic diseases [87]. Therefore, the potential role of those particular species deserves further investigation in patients with chronic HBV infection.

Additionally, our study reported different abundant species in the HBV group by metagenomics such as *Coprococcus\_eutactus* (OTU731), *Parabacteroides\_distasonis* (OTU38338), *Ruminococcus\_torques* (OTU38812), *Kandleria\_vitulina* (OTU34133), and multiassigned OTUs for *Streptococcus* (OTU36481) and *Bifidobacterium* (OTU37693) species. Surprisingly, both culturomics and metagenomics approaches identified nine and six species in HBV and control samples, respectively. However, those species were not significantly frequent or abundant, except for *P. copri* (OTU38502), which was significantly abundant in the HBV group by metagenomics. In addition, previous studies had also reported an increase in the *Prevotella* genus in chronic HBV infection [12,15]. *Prevotella* was reported to be related explicitly to the immunotolerant phase of HBV infection [88], indicating that members of *Prevotella* genus could play a vital role in viral escape from the host immune system.

Briefly, the differences between our findings using culturomics and metagenomic approaches and previous HBV-associated gut dysbiosis studies have been summarized in Table S8. Interestingly, most previous studies reported gut dysbiosis to the genus level, and only a few reported the species level. The characteristics of the chronic HBV-associated dysbiosis in our study showed a depletion of aerointolerant diversity and enrichment in potentially pathogenic *Enterocloster* and *Clostridium* species. Unlike earlier research, our study found an increase in *Clostridium* species. However, most previous studies agreed on a significant abundance of *Streptococcus* and *Bacteriodes*. Moreover, one study reported a significant abundance in the same three species reported in this study, including *Dorea longicatena*, *C. citroniae*, and *P. salivae* [89]. Interestingly, no study has reported *E. bolteae* abundance in chronic HBV infection. The discordant results could be related to various factors, including sample size, the study populations, different disease stages, and various diagnostic techniques (culturomics with different culture conditions), while previous studies utilized solely the metagenomic approach. In summary, no method is ideal, and culturomics and metagenomics are necessary and complementary for explaining the dysbiosis associated with chronic HBV infection.

Under anaerobic conditions, it has been reported that *Escherichia*, *Bacteroides*, *Bifidobacterium*, and *Clostridium* genera can produce ethanol by fermentation from consumed carbohydrates [90]. Moreover, *E. bolteae* species was particularly interesting because bacteria from the *Clostridia* class carried genes encoding for ethanol production pathways [37].

Interestingly, *E. bolteae* was described as an opportunistic pathogen in humans [90]. Additionally, it caused liver damage through endogenous ethanol synthesis in patients with non-alcoholic fatty liver disease (NAFLD) by increasing the permeability of the gut epithelial barrier [91]. Consequently, *E. bolteae* strains were re-cultured, but the inoculum concentration increased to three MacFarland (experiment 2) to promote growth. However, no ethanol was detected in any *E. bolteae* strain, but slight bacterial growth was observed due to the turbidity of the medium. We speculated that this might be a problem linked to the tube's anaerobic environment; therefore, the degassing time was increased to three minutes. The results of experiment 3 showed ethanol production from all the strains. Surprisingly, *E. bolteae* strain s28/42, grown in BACT/ALERT® FN, produced the most ethanol. The previous deficit in *E. bolteae* growth could be attributed to the volume of the liquid medium (40 mL in BACT/ALERT® FN vs. 10 mL in YPG medium). The ethanol production in the FN medium was achieved due to its higher nutritional content than the YPG medium.

Notably, intestinal microecological modulators are now used in clinical practice to treat HBV-related liver failure, particularly in patients with hepatic encephalopathy [92,93]. However, the therapeutic efficacy varies considerably due to the differences in gut microbiota composition. FMT is a promising technique to restore healthy microbiota, improve the current treatment protocol, and prevent chronic HBV-associated complications [94], as in cirrhotic patients and hepatic encephalopathy [95]. Tidjani et al. suggested that the ideal situation would be to culture the symbionts absent from patients but present in healthy control to define and accurately replicate a known and efficient mixed fecal microbiota [22]. Interestingly, on comparing the species identified in our study by metagenomics and culturomics in both HBV and control groups, 213 species were found exclusively in the control samples (Supplementary File S1). These species constituted the missing repertoire in patients with chronic HBV infection and could be used for FMT. Nevertheless, due to the limited number of participants and a lack of randomized clinical trials, further well-designed trials are required to confirm the initial assumptions and promote clinical practicability in the future [96].

Furthermore, three species of possible probiotic features were identified in our study, including *L. oris*, *P. freudenreichii*, and *S. oralis*. Some *L. oris* strains were reported as bacteriocin-producing strains used as protective cultures [97]. A probiotic supplement of *P. freudenreichii* was found to reduce the biologically effective dose of aflatoxin exposure and decrease the risk of liver cancer in a previous clinical trial [98]. However, *S. oralis* was involved in oral health by creating a healthy balance of beneficial bacteria while helping reduce its undesirable counterpart [99]. Interestingly, the role of probiotics in chronic HBV-associated complications such as encephalopathy has been proven [27,28]. Since the usage of the missing repertoire of probiotics necessitates the use of viable isolated strains, the ability of the culturomics approach was demonstrated to play a critical role in the cultivation of such gut microbiota.

In this context, the present results suggest endogenous alcohol production by gut microbiota might participate in chronic HBV infection, as recently described in non-alcoholic steato-hepatitis (NASH) patients [100]. Additionally, microbial culturomics allowed us to obtain live bacterial species that could contribute to the pathophysiology of the disease.

Nevertheless, it is essential to acknowledge the limitations of our study in terms of small sample size and sample pre-freezing. The low number of samples in our exploratory study is compensated by the huge number of colonies by sample, allowing us to have a deep and robust characterization of each sample. Unfortunately, we did not use cryoprotectant for sample pre-freezing in this study, which is a possible limitation. Cryoprotectants such as trehalose, glycerol, and/or skimmed milk are proven to improve the culturability of any frozen fecal sample [59]. However, it cannot be ruled out that cryoprotectants may alter the microbial profile of any sample. Moreover, among the transport medium's constitutions used for the samples of this study, two antioxidants, namely glutathione and uric acid, were included to maintain the cultivability of fecal anaerobes [101]. Therefore, our results

are still relevant since the anaerobic spore-forming *Clostridium* species were recovered and isolated successfully from the frozen samples.

Future research should consider more diverse samples and focus on elucidating the mechanisms by which *E. bolteae* might contribute to liver inflammation and HBV disease progression and on exploring interventions to restore healthy gut microbiota in chronic HBV infection.

Finally, our results open new insight into microbiota's potential role in the pathophysiology of chronic HBV infection, paving the way for further research regarding microbiome-targeted therapeutic options such as probiotics and FMT.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms11102437/s1>. References [102–130] are cited in the supplementary materials. Table S1: Bacterial diversity by culturomics. Table S2: Comparison of the cultured gut bacterial global and hitherto diversity between chronic HBV patients and control. Table S3: Unique and putative new species isolated in chronic HBV patients and control groups. Table S4: Relative genus abundance of gut microbiota in chronic HBV patients and control groups. Table S5: OTUs corresponding to species level. Table S6: Potential probiotics identified by culturomics and metagenomics in the control group and their possible effects. Table S7: Concentrations of ethanol produced by different *Enterocloster* species in the three tested conditions using gas chromatography-mass spectrometry (GC-MS). Table S8: Microbiota changes in previous case-control studies conducted on HBV-related diseases. File S1: Culturomics results.

**Author Contributions:** R.M.W. contributed to the investigation, formal analysis, original draft writing, and visualization. B.M. performed culturomics of the control samples. P.B. contributed to the study design, review, and editing. M.T.A. contributed to the review and editing. M.L.M.R. performed ethanol measurement. A.C. contributed to bioinformatic analysis. C.A. contributed to 16S RNA sequencing. N.A. contributed to the formal analysis of ethanol. M.M. contributed to conceptualization, supervision, formal analysis, writing, review and editing, project administration, and visualization. R.G. contributed to the study design, review, and editing. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The metagenomic files are available online under bio project number PRJEB62828. All the cultured species in this study are preserved and available for further investigation. They are deposited in the Collection de Souches de l’Unité des Rickettsies and (CSUR) collection numbers as *Enterocloster bolteae* Q5636, *Clostridium sporogenes* Q5441, and *Clostridium sporogenes* Q5652. Probiotic species are available and deposited in our CSUR collection: *Limosilactobacillus oris* Q6189, *Propionibacterium freudenreichii* Q7880, and *Streptococcus oralis* Q4071.

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## **Article 3**

### ***Agathobaculum massiliense* sp. nov., a new bacterial species prevalent in the human gut and predicted to produce indole and tryptophan based on genomic analysis**

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# Description of *Agathobaculum massiliense* sp. nov., a new bacterial species prevalent in the human gut and predicted to produce indole and tryptophan based on genomic analysis

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**Abstract** The novel bacterial strain Marseille-P4005<sup>T</sup> was isolated from the stool sample of a healthy donor. It is a Gram-stain negative, non-motile, non-spore-forming rod. It grew optimally at 37 °C and at pH 7.0 on 5% sheep blood-enriched Columbia agar after preincubation in a blood-culture bottle supplemented with rumen and blood. This strain does not ferment monosaccharides (except D-tagatose),

disaccharides, or polymeric carbohydrates. The major cellular fatty acids were hexadecenoic (24.6%), octadecanoic (22.8%), and tetradecanoic (20.1%) acids. Next-generation sequencing revealed a genome size of 3.2 Mbp with a 56.4 mol% G+C. Phylogenetic analysis based on the 16S rRNA gene highlighted *Agathobaculum desmolans* strain ATCC 43058<sup>T</sup> as the closest related strain. The OrthoANI, AAI, and digital DNA-DNA hybridization values were below the critical thresholds of 95%, 95–96%, and 70%, respectively, to define a novel bacterial species. Antibiotic resistance genes APH(3′)-IIIa, *erm*(B), and *tet*(W) were detected with high identity percentages of 100%, 98.78%, and 97.18% for each gene, respectively. The APH(3′)-IIIa gene confers resistance to amikacin, *erm*(B) gene confers resistance to erythromycin, lincomycin, and clindamycin, while *tet*(W) gene confers resistance to doxycycline and tetracycline. Based on KEGG BlastKOALA analyses, the annotation results showed that our strain could use glucose to produce L-lactate and pyruvate but not acetate or ethanol. Also, strain Marseille-P4005<sup>T</sup> was predicted to use phenylalanine to produce indole, a major intercellular signal molecule within the gut microbial ecosystem. Through having a gene coding for tryptophan synthase beta chain (*trpB*), strain Marseille-P4005<sup>T</sup> could produce L-tryptophan (an essential amino acid) from indole. Strain Marseille-P4005<sup>T</sup> showed its highest prevalence in the human gut (34.19%), followed by the reproductive system (17.98%), according to a query carried out on the

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Integrated Microbial NGS (IMNGS) platform. Based on phylogenetic, phenotypic, and genomic analyses, we classify strain Marseille-P4005<sup>T</sup> (=CSUR P4005=CECT 9669), a novel species within the genus *Agathobaculum*, for which the name of *Agathobaculum massiliense* sp. nov. is proposed.

**Keywords** Culturomics · Human gut · *Agathobaculum massiliense* · Microbiota · Taxonomics

## Introduction

The human microbiota is a very diverse and complex ecosystem which diversity has been highlighted owing to next generation sequencing (NGS). NGS methods also highlighted a high number of uncultivated species including hitherto unknown species as revealed by the high proportion of unassigned sequences. This led to new advances in the field of bacterial culture to isolate and identify previously unknown species (Fontaine et al. 2012; Lagier et al. 2012, 2015). Culturomics, a high throughout culture approach which was described in 2012, contributed in the identification of a plethora of new bacterial species from human samples (Lagier et al. 2012, 2016; Diakite et al. 2021). In a culturomics project aimed at studying the composition of the human gut microbiota (Diakite et al. 2019), we isolated strain Marseille-P4005<sup>T</sup> from a human stool sample which was classified as a member of the *Agathobaculum* genus.

The *Agathobaculum* genus was created in 2016 with the reclassification and description of *Agathobaculum desmolans* initially described as *Eubacterium desmolans* in 1986 by Morris et al. (Ahn et al. 2016). This genus is classified within the Firmicutes phylum and the *Oscillospiraceae* family, which includes 56 other validly published genera according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al. 2020). The *Agathobaculum* genus currently includes four validly published species including the type species *Agathobaculum butyriciproducens*.

Members of the *Agathobaculum* genus are generally Gram-stain-positive, motile or non-motile, non-spore forming, obligate anaerobes, oxidase- and catalase-negative. They have an optimum growth temperature ranging from 30 to 40 °C and the major

fermentation end product is butyrate or acetate (Ahn et al. 2016). *A. desmolans* has been first isolated from cat faeces (Morris et al. 1986). However, members as *A. butyriciproducens* and *A. ammoniilyticum* were first isolated from healthy human faeces (Ahn et al. 2016; Hitch et al. 2021).

In this study, we aim at classifying and describing strain Marseille-P4005<sup>T</sup>, a putative novel species using the taxonogenomics approach (Ramasamy et al. 2014) which uses phenotypic, proteomic, phylogenetic and genomic characteristics to classify new species. We also sought to identify the most striking gene clusters and genome features and to connect them to the ecophysiology of strain Marseille-P4005<sup>T</sup> and its fitness in its natural environment.

## Material and methods

### Sample collection and bacterial identification

We sought and obtained the free and informed consent of the donor, after having given clear, fair and appropriate information and having respected the legal time limit required before the research began. Then a stool sample was collected and the person's data followed an anonymisation process. The study protocol on the description of the viable human microbiota using the culturomics approach was approved by the ethics committee of the Institut Hospitalo-Universitaire Méditerranée Infection (IHUMI), under agreement number 16-011. This study was performed following the last revision of the Declarations of Helsinki in 2013 (World Medical Association 2013) and in strict compliance with the French legal obligations (French ordinance of 16 June 2016 on research involving the human person amending the public health code resulting from the law of 5 March 2012 to take account of the European regulation EU 536/2014).

The stool samples were cultured according to the culturomics approach previously established in our laboratory (Lagier et al. 2015). Different bacterial colonies were isolated on 5% sheep blood enriched Columbia (COS) agar (BioMérieux, Craponne, France) after preincubation in a blood-culture bottle supplemented with rumen and sheep blood. Isolation was achieved after two days of incubation in an anaerobic atmosphere generated using anaeroGEN

generators (Beckton Dickinson, Arcueil, FRANCE). The bacterial colonies were identified using Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) with the Microflex LT instrument (Bruker Daltonics, Bremen, Germany) as previously reported (Seng et al. 2009). Genome sequencing was used to identify the purified colony in the case of a failed MALDI-TOF MS identification.

### 16S rRNA gene phylogeny

The 16S rRNA gene sequences of strain Marseille-P4005<sup>T</sup> was extracted from its genome using ConEst16S available online ([www.ezbiocloud.net](http://www.ezbiocloud.net), last accessed October 2022). The sequence was compared using BLASTn to the NCBI nucleotide database with the NCBI server. A phylogenetic tree was constructed based on the 16S rRNA gene sequences using MEGA 7 version 7.0.26 with the maximum likelihood (ML) method and 1000 bootstraps replications (Kumar et al. 2018).

### Phenotypic and biochemical characterization

The optimal culture conditions have been determined by attempted growth of strain Marseille-P4005<sup>T</sup> on COS agar at different temperatures (25, 28, 37, 45, and 56 °C) in three atmospheres (aerobic, anaerobic and microaerophilic). Growth was also tested in different NaCl concentrations (5, 5.5, 7.5, 10, 15, and 20%). The salt tolerance of the studied strain was estimated using a medium containing per liter Columbia agar (Thermo Fisher Scientific, Waltham, Massachusetts, US) 42 g, yeast extract 5 g, glucose 2 g, MgCl<sub>2</sub> 10 g, MgSO<sub>4</sub> 10 g, KCl 4 g, CaCl<sub>2</sub> 1 g, NaHCO<sub>3</sub> 0.5 g. Different pH levels were also tested (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5) using a solid medium consisting of per liter Columbia agar 42 g, glucose (MP biomedical, Irvine, California, US) 2 g, NaCl 10 g, MgCl<sub>2</sub> 5 g, MgSO<sub>4</sub> 5 g, KCl 2 g, CaCl<sub>2</sub> (VWR, Radnor, Pennsylvania, US) 1 g. The addition of NaOH or HCl solution was used to adjust the pH of the medium.

The morphologic features of strain Marseille-P4005<sup>T</sup> was described using scanning electron microscopy (SEM) (SU5000, Hitachi, Tokyo, Japan) as previously described (Bellali et al. 2022). Bacterial length and width were measured from sets of SEM micrographs using ImageJ (Schneider et al.

2012). Furthermore, biochemical characteristics were assessed using different API strips: API 50CH, API ZYM and API 20A (bioMérieux, Craponne, France) according to bioMérieux instructions (BioMérieux France 2022).

The fatty acid methyl ester (FAME) analysis was done using gas chromatography/mass spectrometry (GC/MS), as previously reported (Sasser 2006). FAMES were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500- SQ 8 S, Perkin Elmer, Courtaboeuf, France). The spectral profile was compared with those in repertory databases using MS Search 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg, USA) and the FAMES mass spectral database (Wiley, Chichester, UK).

The antibiotic susceptibility of the described strain was assessed through the Minimum Inhibitory Concentration (MIC) determination method. Antibiotics were selected based on the recommendations of European Committee on Antimicrobial Susceptibility Testing (Matuschek et al. 2014). The susceptibility to antimicrobial agents of the strain was tested on 5% sheep blood-enriched Mueller Hinton agar (bioMérieux) using the optimal growth condition. The following antibiotics were tested using E-TEST<sup>®</sup> strips (bioMérieux): daptomycin, imipenem, rifampicin, vancomycin, benzylpenicillin, amikacin, ceftriaxone, amoxicillin, tobramycin, linezolid, clindamycin, oxacillin, ceftazidime, ciprofloxacin, trimetho-sulfamethoxazole, doxycycline and colistin.

### Genome sequencing and analyses

The genomic DNA was extracted using the EZ1 DNeasy Blood Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions (Anani et al. 2019). The whole-genome sequencing was performed using the Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) as previously described (Caporaso et al. 2012). The genome of strain Marseille-P4005<sup>T</sup> was assembled using Spades 3.5.0 software (Prjibelski et al. 2020) and annotated using Prokka version 1.14.5 (Seemann 2014). A map of the circular genome was created using CGview to display the genomic features of our strain (Petkau et al. 2010). The genomic similarities between the studied species and their closely related taxa were calculated using the OrthoANI tool (OAT software) version 0.93.1 (Lee

et al. 2016). OrthoAAI (Average Amino-acid Identity) was also determined using AAI-profiler online tool (Medlar et al. 2018). Moreover, the digital DNA-DNA Hybridization (dDDH) values were calculated using the DSMZ database on the Type (Strain) Genome Server (TYGS) (<https://tygs.dsmz.de>, last accessed January 2023) (Auch et al. 2010).

Pangenome analysis was inferred using the Roary software (Page et al. 2015). A genome-based phylogenetic tree was generated with FastME 2.1.6.1 software (Meier-Kolthoff & Göker 2019).

The metabolic properties of our strain were also predicted from genomic sequencing using different analyses. A BLASTp analysis was performed for our strain and closely related species genomes and matched against the clusters of orthologous groups (COG) database with a minimum identity of 30%, a minimum coverage of 70%, and a maximum E-value of  $1e^{-03}$ . All sequences shorter than 80 amino acids in size were removed. For the metabolic pathway detection, BLASTp Koala against the KEGG prokaryotic database was performed (Kanehisa et al. 2016). Additionally, antimicrobial resistance genes were screened using ABRicate through the analysis of nucleotide sequences against ResFinder and CARD (Comprehensive Antibiotic Resistance Database) (Bortolaia et al. 2020; Alcock et al. 2020). AntiSMASH, a web-based tool designed for detection of biosynthetic gene clusters (BGCs) of secondary metabolites such as PolyKetides (PKs), Non-Ribosomal Peptides (NRPS), was also used (Seemann 2014). In addition, we used ToxFinder-1.0 bioinformatic tool for toxins detection (ResFinder, 2022).

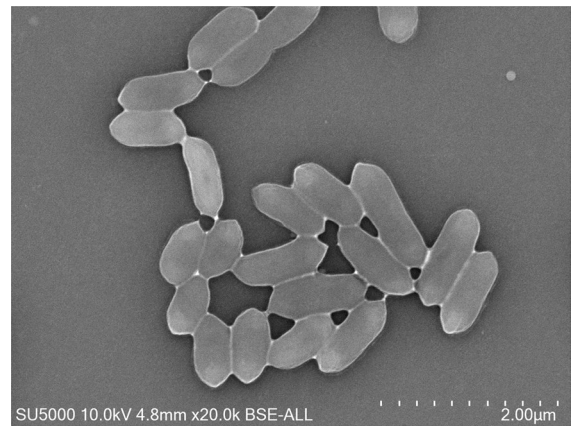
Prevalence and relative abundance in human microbiomes

Finally, we screened public metagenomes of the sequence read archive using the Integrated Microbial NGS (IMNGS) platform (Lagkouvardos et al. 2016) to determine the prevalence of strain Marseille-P4005<sup>T</sup> in the human microbiome.

## Results and discussion

### Identification and 16S rRNA phylogenies

Strain Marseille-P4005<sup>T</sup> was unidentified using Maldi-TOF MS and its spectra were uploaded to the



**Fig. 1** Scanning electron micrographs of *Agathobaculum massiliense* Marseille-P4005<sup>T</sup> recorded on the SU5000 scanning electron microscope. Scale bars and acquisition settings are displayed on the figure

local MALDI-TOF MS database. The 16S rRNA gene was extracted from its sequenced genome. BLASTn of the 16S rRNA gene sequence (Accession number: LT985392.1) showed that strain Marseille-P4005<sup>T</sup> shared its highest sequence similarity (96.74%) with *Agathobaculum desmolans* ATCC 43058<sup>T</sup> (Fig. S1). This similarity level was below the 98.65% threshold set to delineate bacterial species (Kim et al. 2014).

### Phenotypic and biochemical characterizations

Bacterial colonies of strain Marseille-P4005<sup>T</sup> were observed on COS agar (bioMérieux) at temperatures ranging from 28 to 45 °C with the optimal growth occurring at 37 °C after 48 h of incubation. The strain was able to grow in microaerophilic and anaerobic atmospheres with the optimal growth observed in an anaerobic atmosphere. No growth was observed in aerobic atmosphere at different temperature ranges. In the tested pH range, growth was only observed at pH 7. Moreover, strain Marseille-P4005<sup>T</sup> showed no salt tolerance. After 48 h of incubation, bacterial colonies on COS agar were white, punctiform, slightly convex and non-hemolytic. On COS agar, they had a diameter ranging from 0.1 to 0.15 mm. Cells were rod-shaped as revealed by the SEM micrographs and measured  $1.03 \pm 0.21 \mu\text{m}$  in length and  $0.4 \pm 0.1 \mu\text{m}$  in width (Fig. 1).

The strain was Gram-negative, rod-shaped and presented no catalase or oxidase activities. The

phenotypic characteristics of strain Marseille-P4005<sup>T</sup> compared with those of their closest relatives are summarized in Table 1. Using an API<sup>®</sup> 50CH strip, positive reactions were obtained with glycerol, erythritol, dulcitol, D-mannitol, D-tagatose and potassium 5-cetogluconate (Table S1). Using an API<sup>®</sup>

ZYM strip, activities for the following enzymes were observed: alkaline phosphatase, lipase (C14), leucine arylamidase, trypsin, acid phosphatase, naphthol phosphohydrolase and β-galactosidase (Table S2). The presence of a protease was also detected using the API<sup>®</sup> 20A strip (Table S3).

**Table 1** Phenotypic characteristics of strain Marseille-P4005<sup>T</sup> compared with other closely related species

Properties	P4005	AD	AB	BP	BF	IM*
Gram stain	–	+	+	+	+	–
Cell shape	Rods	Rods	Short rod	Cocci in pairs	Coccioid-shaped	Rods
Motility	–	+	–	–	–	+
Cell diameter(μm)	0.8–1.3×0.3–0.4	NA	0.6–0.7×0.9–1.1	1.2–1.5	1.3×0.7	0.5–0.8×1.9–5
Endospore	–	–	–	NA	NA	–
Temperature	28–37 °C	37 °C	37 °C	42° C	37 °C	28–37 °C
Oxygen tolerance	Anaerobic	Strictly anaerobic	Strictly anaerobic	Anaerobic	Strictly anaerobic	Anaerobic
Incubation (days)	2–3	2–3	3	1–2	3	2–3
Salt tolerance	–	–	0.5%	NA	NA	–
Optimum pH	7	7	7	7	7	7
Alkaline phosphatase	+	NA	NA	NA	+	+
Acid phosphatase	+	NA	NA	NA	+	+
Catalase	–	–	–	NA	NA	–
Oxidase	–	–	–	NA	NA	–
Indole	–	NA	NA	NA	+	–
Urease	–	–	–	–	–	–
α-galactosidase	–	NA	NA	NA	–	–
β-galactosidase	+	NA	NA	NA	–	–
Ribose	V	NA	NA	NA	NA	–
Mannose	–	+	+	+	–	–
Mannitol	+	+	+	–	–	–
Sucrose	–	+	+	+	–	–
Glucose	–	+	+	+	+	–
Fructose	–	NA	NA	NA	–	–
Maltose	–	+	+	+	–	–
Sorbitol	–	–	–	–	–	–
Starch	–	NA	NA	+	NA	–
Lactose	–	+	+	–	–	–
Habitat	Human stool	Cat faeces	Korean faecal sample	Chicken caecal content	Human faecal sample	Human left colon
Reference	–	Ahn et al. (2016)	Ahn et al. (2016)	Eeckhaut et al. (2008)	Takada et al. (2016)	Ricaboni et al. (2017)

+ Positive, – Negative, V Variable, NA Not available

P4005 Strain Marseille-P4005<sup>T</sup>; AD *Agathobaculum desmolans* ATCC 43058<sup>T</sup>; AB *Agathobaculum butyriciproducens* NB2B-10-MRS; BP *Butyricoccus pullicaecorum* DSM 23266<sup>T</sup>; BF *Butyricoccus faecihominis* JCM 31056<sup>T</sup>; IM *Intestinibacillus massiliensis* Marseille-P3216<sup>T</sup>. \* API<sup>®</sup> 50CH, ZYM and 20A were performed in our lab

According to the proteomic analysis, the major fatty acids of strain Marseille-P4005<sup>T</sup> were saturated structures, namely hexadecanoic acid (25%), octadecanoic acid (23%) and tetradecanoic acid (20%). A few unsaturated and branched fatty acids were also described with minor abundances (Table S4).

The following MICs were obtained for strain Marseille-P4005<sup>T</sup>: 0.032 ug/ml imipenem, 0.047 ug/ml daptomycin, 0.1 ug/ml rifampicin, 0.25 ug/ml vancomycin, 0.75 ug/ml benzylpenicillin, 0.38 ug/ml ceftriaxone, 0.5 ug/ml amoxicillin, 2 ug/ml linezolid, 8 ug/ml oxacillin, 32 ug/ml trimetho-sulfamethoxazole, and 8 ug/ml doxycycline. MICs > 256 ug/ml were obtained for colistin, amikacin, tobramycin, clindamycin, ceftazidime and ciprofloxacin, suggesting a natural resistance towards these antibiotics.

### Genome characteristics and comparison

The genome was 3,218,823-bp- long with a G+C content of 56.4 mol% and includes two contigs. The Prokka annotation revealed 3,239 genes. Of them, 3,182 genes were protein-coding genes, and 67 genes were RNA (9 rRNA including 3 16S rRNA, 57 tRNA, 1 mRNA). 1,585 (48.93%) are hypothetical proteins, 163 (5.03%) are ORFans, and 215 (6.63%) present a signal peptide according to SignalP-5.0 (Almagro Armenteros et al. 2019). The distribution of all Prokka annotations, RNA genes (tRNAs, rRNAs), antibiotic resistance genes (CARD), GC content, and GC skew is displayed in a graphical circular map for each genome (Fig. 2).

Using OrthoANI analysis, strain Marseille-P4005<sup>T</sup> exhibited the highest similarity (78.48%) with *Agathobaculum desmolans* strain ATCC 43058<sup>T</sup> and the lowest one (67.58%) with *Flavonifractor plautii* strain ATCC 2986<sup>T</sup> (Fig. 3). Moreover, the analyses of amino acid sequences showed the maximum AAI between strain Marseille-P4005<sup>T</sup> with *Agathobaculum butyriciproducens* NB2B-10-MRS<sup>T</sup> (61.62%) while the minimum AAI was shared with *Flavonifractor plautii* strain ATCC 2986<sup>T</sup> (19.27%). The comparison of the genomic characteristics of strain Marseille-P4005<sup>T</sup> with those of closely related strains is reported in (Table 2).

The dDDH analysis showed the maximum value between strain Marseille-P4005<sup>T</sup> with *Butyricoccus pullicaecorum* strain DSM 23266<sup>T</sup> (29.1%) while

the minimum value was shared with *Intestinibacillus massiliensis* strain Marseille-P3216<sup>T</sup> (20.8%).

(Table 3).

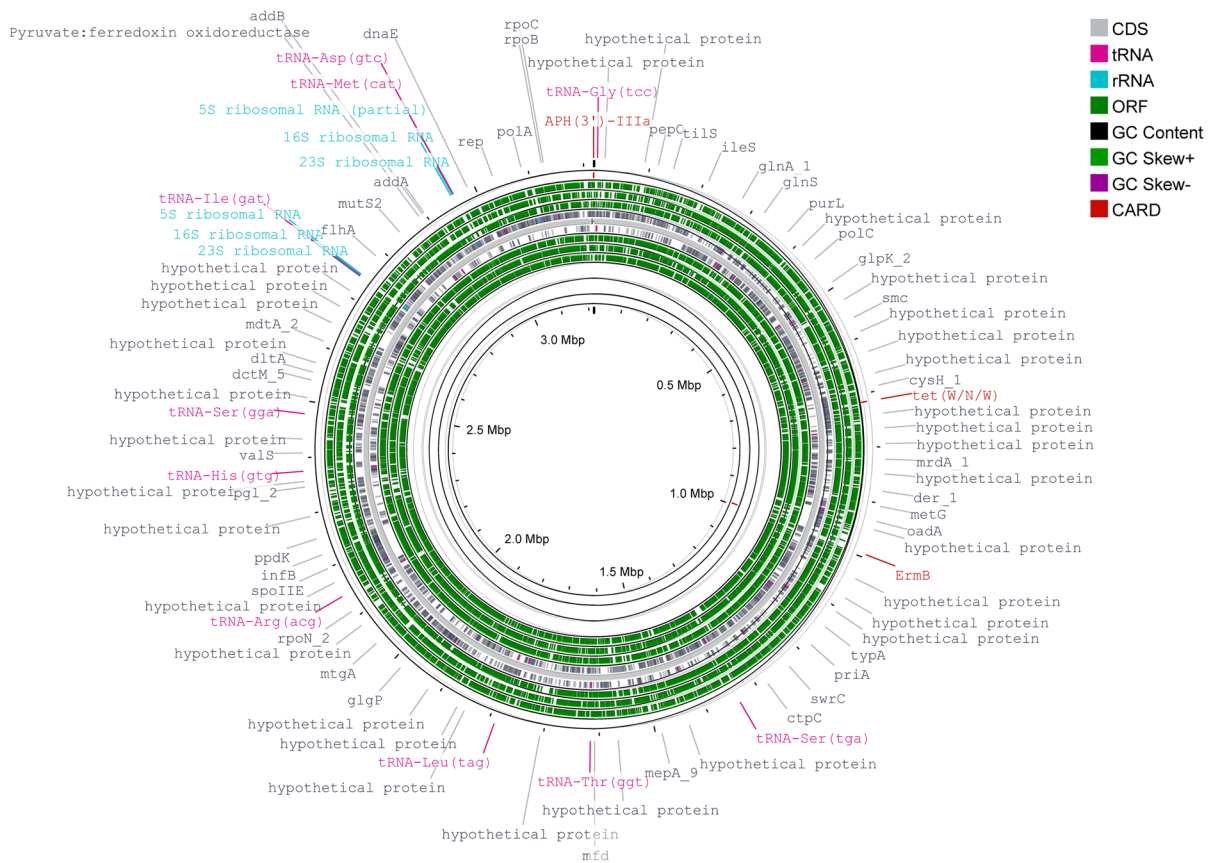
Values of OrthoANI, AAI and dDDH were lower than the critical values 95%, 95–96% and 70%, respectively, recommended to delineate a novel bacterial species (Ahn et al. 2016; Thompson et al. 2013; Ludwig et al. 2021). The genome-based tree confirmed the close relationship of strain Marseille-P4005<sup>T</sup> with *Agathobaculum* species (Fig. S2).

The pangenome analysis demonstrated that strain Marseille-P4005<sup>T</sup> was clearly distinct from other compared *Agathobaculum* species (Fig. 4).

The comparison of the number of genes associated with a COG functional cluster for the strain with the closest species is represented in Fig. 5. 2148 protein-coding genes were assigned to COG categories. No proteins belonging to the chromatin structure and dynamics, nuclear structure, cell motility cytoskeleton, extracellular structures and RNA processing and modification within the genome. A high proportion of genes assigned to transcription (10.19%) and carbohydrate transport and metabolism (10.33%) was observed in strain Marseille-P4005<sup>T</sup>.

For metabolic pathways based on KEGG Blast-KOALA analyses; 50.43% of protein coding genes (n=1605) of strain Marseille-P4005<sup>T</sup> are linked to known bacterial metabolic pathways (Table S5) including genetic information processing (14.70%, n=236), carbohydrate metabolism (13.64%, n=219), environmental information processing (12.02%, n=193), signalling and cellular processing (8.16%, n=131), and amino acid metabolism (5.73%, n=92). Those annotation results showed that strain Marseille-P4005<sup>T</sup> could use carbohydrates and amino acids resources available in their living environment. Strain Marseille-P4005<sup>T</sup>, for example, was able to use glucose via the glycolysis pathway to produce L-lactate and pyruvate but not acetate or ethanol (Fig. S3). Once pyruvate is produced, cross-feeders may convert it to short-chain fatty acids (SCFAs) as butyrate (Koh et al. 2016). Butyrate consumption is known to improve the integrity of the gut's tight junctions and may have anti-inflammatory properties (Den Besten et al. 2013; Lee and Hase 2014). Additionally, our strain showed that 12% of protein-coding genes are involved in environmental information processing, suggesting adaptability to the surrounding environment.





**Fig. 2** Circular genome map of strains Marseille-P4005<sup>T</sup> generated by the CGView server. From outside to the center: CDS (Complete coding potential sequence), Prokka annotation, RNA genes, ORFs (Open Reading Frames) on both forward

and reverse strands, positive and negative GC skew, GC content. Antibiotic resistance genes marked in red based on CARD (Comprehensive Antibiotic Resistance Database)

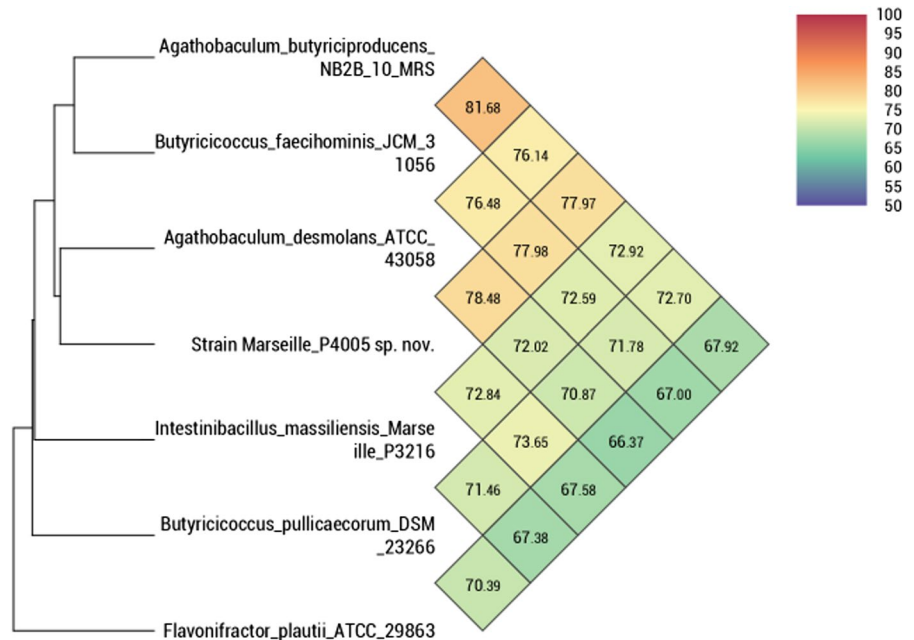
Interestingly, our strain was predicted to use phenylalanine to produce indole, which is a major inter-cellular signal molecule within the gut microbial ecosystem, regulating various aspects of bacterial physiology, including spore formation, resistance to drugs, virulence and biofilm formation (Lee and Lee 2010). Through having a gene coding for tryptophan synthase beta chain (*trpB*) enzyme, strain Marseille-P4005<sup>T</sup> could produce L-tryptophan from indole (Fig. S4). Tryptophan is an essential amino acid (not produced by the human host), metabolized into serotonin (5-hydroxytryptamine) that acts as an important neurotransmitter in the modulation of emotional control, food intake, sleep, and pain processing (Israelyan and Margolis 2018).

Based on the literature, we found that similar species such as *A. butyriciproducens* could produce

butyrate, a key metabolite for human health (Ahn et al. 2016). In contrast, we did not find coding genes for the enzymes necessary to produce butyrate when performing KEGG analysis for strain Marseille-P4005<sup>T</sup>. Thus, it could be expected that strain Marseille-P4005<sup>T</sup> may have health-associated properties through indole and tryptophane endogenous production.

Using the ResFinder bioinformatic tool and CARD database, antibiotic resistance genes *APH(3')-IIIa*, *erm(B)*, and *tet(W)* were detected within the genome of strain Marseille-P4005<sup>T</sup>, with high identity percentages of 100%, 98.78% and 97.18% for each gene, respectively. The *APH(3')-IIIa* gene confers resistance to amikacin, *erm(B)* gene confers resistance to erythromycin, lincomycin, clindamycin, quinupristin,pristinamycin-IA,virginiamycin-S while

**Fig. 3** Heatmap generated with OrthoANI values using OAT software version 0.93.1 between Marseille-P4005<sup>T</sup> and the closely related species



*tet(W)* gene confers resistance to doxycycline, tetracycline and minocycline. Other resistance genes were predicted with lower identity percentages (Table 4).

Using AntiSMASH bioinformatic tool, two BGCs or regions had been predicted in strain Marseille-P4005<sup>T</sup> genome that encode potential secondary metabolites (Fig. S5). The first BGC region is related to ranthipeptides, a newly emerging class of natural products belonging to the ribosomally synthesized and post-translationally modified peptide (RiPP) superfamily. Ranthipeptides were investigated to have a role in quorum sensing and bacterial cellular metabolism that could help in engineering industrially interesting organisms (Chen et al. 2020). The second BGC region was assigned to a NRPS that was described as a defense mechanism that can have a role in the biosynthesis of pharmaceutical natural products (Akey et al. 2012). Among all putative BGCs for compounds with antibiotic properties, we found short sequences in the first BGC region with low similarity related to: isoindolinomycin and persiamycin A (5% similarity), and formicamycin, xantholipin, polyketomycin and dutomycin (similarity 4%) (Fig. 6). Such findings suggest that strain Marseille-P4005<sup>T</sup> has the capacity to produce hitherto unknown molecules of pharmaceutical interest including ranthipeptide and NRPS.

In addition, strain Marseille-P4005<sup>T</sup> showed no toxin genes based on ToxFinder-1.0 bioinformatic tool for toxins detection (ResFinder, 2022).

#### Prevalence and relative abundance in human microbiomes

The ecophysiological role of our strain Marseille-P4005<sup>T</sup> was investigated using the IMNGS platform through the query of the public metagenomes of the sequence read archive. Among 500,048 metagenomes of the sequence read archive, we found 84,361 human metagenomes, including 62,549 with a designated anatomical niche of origin with at least 100 samples per niche (Table S6). We observed that strain Marseille-P4005<sup>T</sup> is much more prevalent in the human gastrointestinal tract and reproductive system than in other anatomical niches. This seems consistent with its anaerobic metabolism and it could be of particular interest because it is part of the healthy anaerobic human microbiota (Million et al. 2017). We measured the relative abundance in the positive samples (Fig. 7). Interestingly, the relative abundance was highest in the gastrointestinal tract (34.19%) followed by the reproductive system (17.98%), and was much lower in the skin (1.33%) and oral cavity (0.41%). Taken all together, these results suggest that strain Marseille-P4005<sup>T</sup> might be a human commensal

**Table 2** Comparison of genomic characteristics

Scaffolds/ contigs	Strain Marseille- P4005 <sup>T</sup>		<i>Agathobaculum desmolans</i> ATCC 43058 <sup>T</sup>		<i>Agathobaculum butyrificiproducens</i> NB2B-10-MRS		<i>Butyrivicoccus pul- licaeorum</i> DSM 23266 <sup>T</sup>		<i>Butyrivicoccus pul- licaeorum</i> DSM 31056 <sup>T</sup>		<i>Intexinibacillus massiliensis</i> Marseille- P3216 <sup>T</sup>		<i>Flavonifractor plau- titii</i> ATCC 29863 <sup>T</sup>	
	Value	% of total	Value	% of total	Value	% of total	Value	% of total	Value	% of total	Value	% of total	Value	% of total
Size (bp)	<b>3,218,823</b>	<b>100</b>	<b>2,643,795</b>	<b>100</b>	<b>3,002,197</b>	<b>100</b>	<b>1,033,537</b>	<b>100</b>	<b>2,995,907</b>	<b>100</b>	<b>2,813,233</b>	<b>100</b>	<b>3,813,599</b>	<b>100</b>
G+C con- tent (%)	1,811,955	56.4	1,282,240	48.5	1,588,162	52.9	1,782,608	54.5	1,665,724	55.6	1,673,592	59.49%	2,322,481	60.9
Total genes	<b>3,239</b>	<b>100</b>	<b>2,678</b>	<b>100</b>	<b>2,942</b>	<b>100</b>	<b>1,059</b>	<b>100</b>	<b>2,890</b>	<b>100</b>	<b>2,759</b>	<b>100</b>	<b>3,732</b>	<b>100</b>
Protein-cod- ing genes	3,182	98.24	2,624	97.98	2,843	96.63	1,042	98.39	2,820	97.57	2,701	97.89	3,675	98.47
Total RNA genes	67	2.06	54	2.02	99	3.36	60	1.60	70	2.42	58	2.10	57	1.52
Hypothetical proteins	1585	48.93	1369	51.12	1558	52.95	597	56.37	1481	51.24	1346	48.78	1943	52.06
Proteins assigned to COGs	2148	66.31	1732	64.67	1752	59.55	634	59.86	1857	64.25	1822	66.03	2279	61.06
Proteins with peptide signals	215	6.63	167	6.23	207	7.03	76	7.17	224	7.75	245	8.88	256	6.85
Contigs	2	-	97	-	5	-	2	-	39	-	108	-	344	-

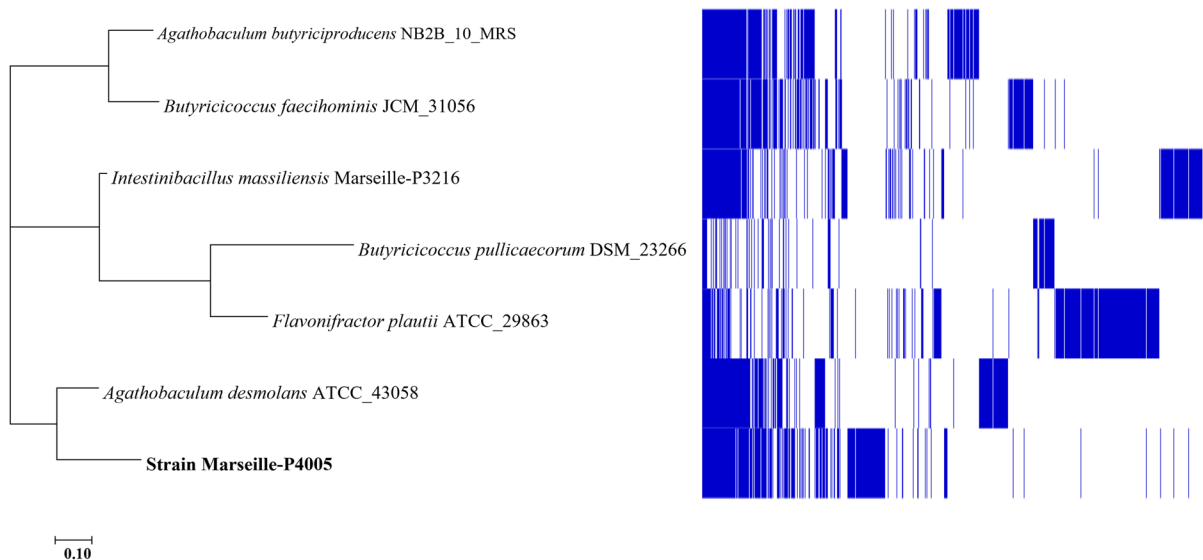
Values in bold = the total (100%) value; NA not available

P4005: Strain Marseille-P4005<sup>T</sup> (OMP000000000.1), AD *Agathobaculum desmolans* ATCC 43058<sup>T</sup> (JNIN01000001.1), AB *Agathobaculum butyrificiproducens* NB2B-10-MRS (JAMXVA010000001.1), BP *Butyrivicoccus pullicaeorum* DSM 23266<sup>T</sup> (FUXXW01000001.1), BF *Butyrivicoccus faecihominis* JCM 31056<sup>T</sup> (BMQH01000001.1), IM *Intexinibacillus massiliensis* Marseille-P3216<sup>T</sup> (FTLU00000000.1), FP *Flavonifractor plautii* ATCC 29863<sup>T</sup> (AGGK000000000.1)

**Table 3** Digital DNA-DNA hybridization values (%) between strain Marseille-P4005<sup>T</sup> and the related species using formula 2 of the GGDC web server (Estimation was based on identities /HSP length)

Strains	AB	AD	BF	BP	FP	IM	P4005
AB	100	22	28.1	24.3	25.5	20.9	24.8
AD		100	23	23.8	19.4	20.1	22.7
BF			100	21.8	19.8	20.7	25
BP				100	32.5	20.9	29.1
FP					100	18.1	23.5
IM						100	20.8
P4005							100

P4005 Strain Marseille-P4005<sup>T</sup> (OMPN00000000.1), AD: *Agathobaculum desmolans* ATCC 43058<sup>T</sup> (JNPN01000001.1), AB *Agathobaculum butyriciproducens* NB2B-10-MRS (JAMXVA010000001.1), BP *Butyricoccus pullicaecorum* DSM 23266<sup>T</sup> (FUXW01000001.1), BF *Butyricoccus faecihominis* JCM 31056<sup>T</sup> (BMQH01000001.1), IM *Intestinibacillus massiliensis* Marseille-P3216<sup>T</sup> (FTLU00000000.1), FP *Flavonifractor plautii* ATCC 29863<sup>T</sup> (AGCK00000000.1)

**Fig. 4** Pangenome-based tree showing the position of Marseille-P4005<sup>T</sup> among related closed species. Pangenomic analysis was carried out using the Roary software. The scale represents a 10% nucleotide sequence divergence

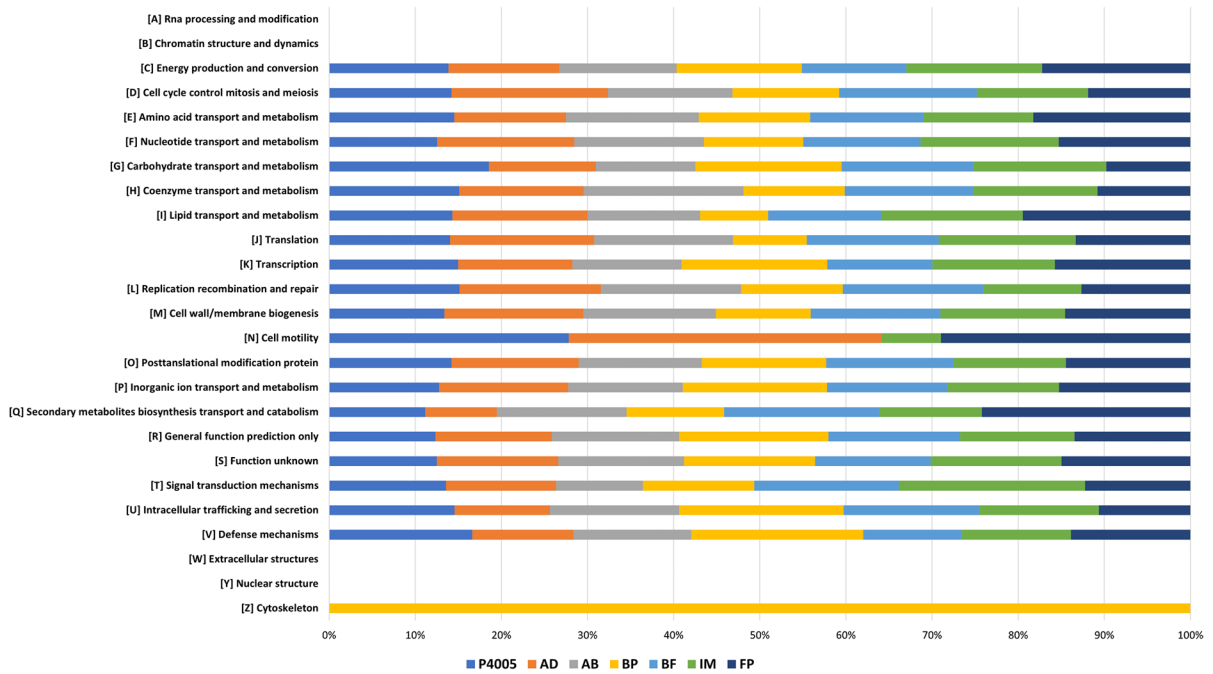
associated with the gastrointestinal tract and reproductive system. Future studies are needed to clarify its precise role in these ecosystems.

## Conclusion

According to our findings, based on whole genome sequences analyses combined with the phenotypic data, we propose *Agathobaculum massiliense* sp. nov. as a novel species within the *Agathobaculum* genus, for which Marseille-P4005<sup>T</sup> is the type strain.

## Description of *Agathobaculum massiliense* sp. nov.

*Agathobaculum massiliense* (mas.si.li.en'se. L. neut. adj., massiliense, of Massilia, the latin name of Marseille, France, where the type strain was isolated). The type strain is Marseille-P4005<sup>T</sup> (=CSUR P4005=CECT 9669). It was isolated from a stool of a healthy control. Optimal growth of strain Marseille-P4005<sup>T</sup> occurs at 37 °C after 48 h of incubation under strict anaerobic conditions on 5% sheep blood-enriched Columbia agar. Growth pH is 7. No salt tolerance is observed. Colonies are punctiform,

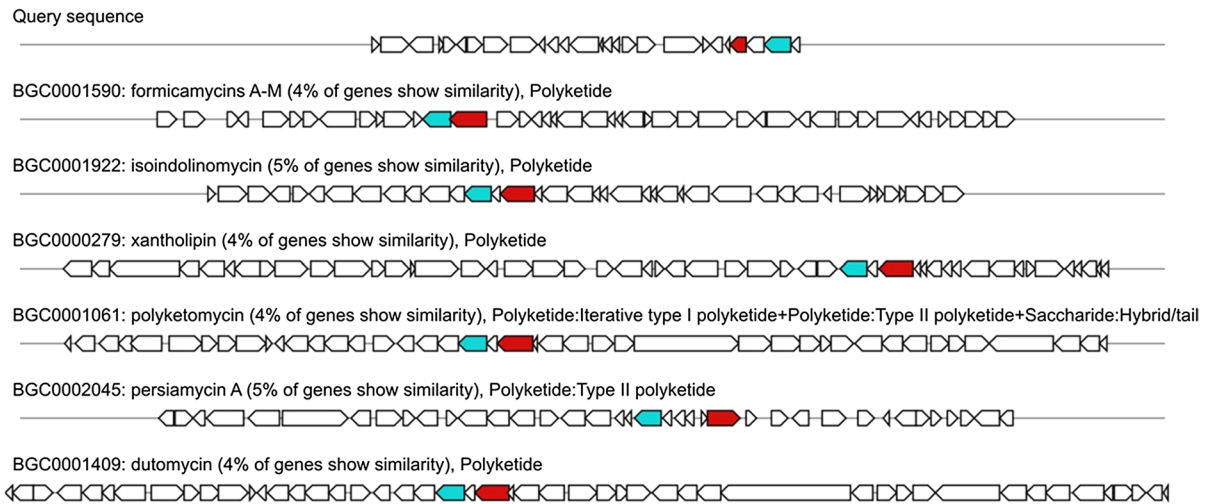


**Fig. 5** Comparison of the 25 general cluster of orthologous groups (COGs) functional clusters of strain Marseille-P4005<sup>T</sup> and closely related species. P4005: Strain Marseille-P4005<sup>T</sup>, AD *Agathobaculum desmolans* ATCC 43058<sup>T</sup>, AB *Agathob-*

*aculum butyriciproducens* NB2B-10-MRS, BP *Butyricicoccus pullicaecorum* DSM 23266<sup>T</sup>, BF *Butyricicoccus faecihominis* JCM 31056<sup>T</sup>, IM *Intestinibacillus massiliensis* Marseille-P3216<sup>T</sup>, FP *Flavonifractor plautii* ATCC 29863<sup>T</sup>

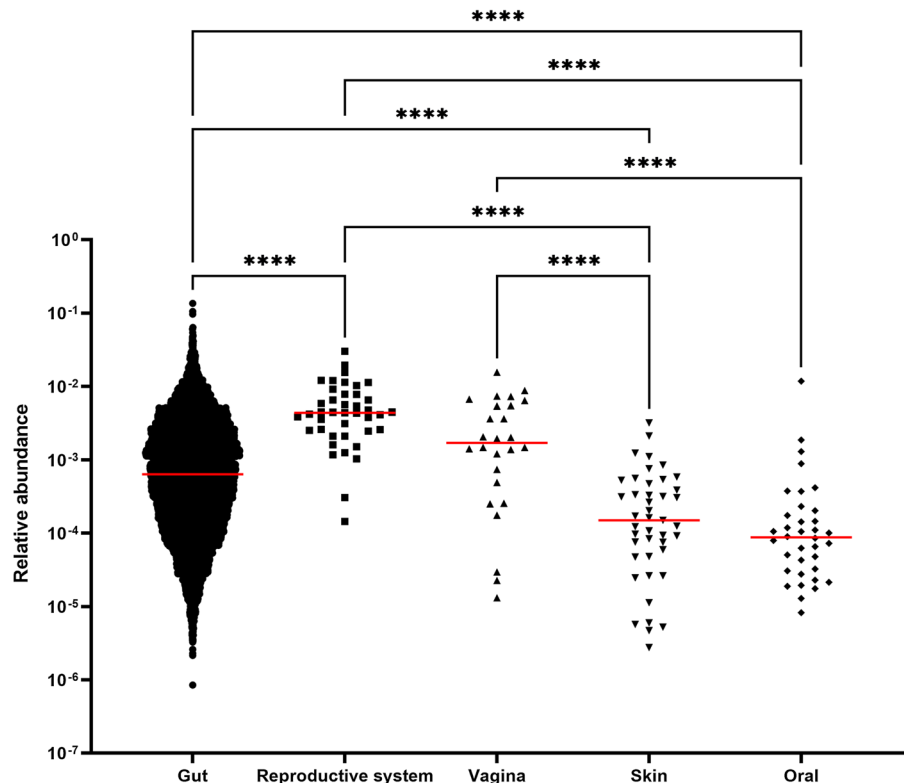
**Table 4** Antibiotic resistance genes detected in the genome of strains Marseille-P4005<sup>T</sup> using ResFinder and CARD databases

	Resistance gene	% Identity of matching region	% Length of reference sequence	AMR gene family	Drug class
Strain Marseille-P4005 <sup>T</sup>	APH(3')-IIIa	100.0	100.0	APH(3')	Aminoglycoside antibiotic (amikacin)
	<i>erm(B)</i>	98.78	98.79	Erm 23S ribosomal RNA methyltransferase	Macrolide, lincosamide, streptogramin A, streptogramin B, erythromycin, lincomycin, clindamycin, quinupristin, pristinamycin-IA, virginiamycin-S
	<i>tet(W)</i>	97.18	100.0	Tetracycline-resistant ribosomal protection protein	Doxycycline, tetracycline, minocycline
	<i>van(G)</i>	42.07	102.01	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide antibiotic
	<i>van(W)</i>	41.26	155.76	<i>vanW</i> , glycopeptide resistance gene cluster	Glycopeptide antibiotic
	<i>van(T)</i>	35.31	52.39	glycopeptide resistance gene cluster, <i>vanT</i>	Glycopeptide antibiotic



**Fig. 6** Biosynthetic gene clusters (BGCs) for antibacterial compounds related to the first BGC detected in the genome of strain Marseille-P4005<sup>T</sup> with genes encoding ranthipeptide compounds using AntiSMASH v.6.0.1

**Fig. 7** Relative abundance of strain Marseille-P4005<sup>T</sup> in human metagenomes based on its presence in different anatomical niche using IMNGS platform database (imngs.org). The figure created by GraphPad Prism v.9 using non-parametric Mann–Whitney U test. \*\*\*\*  $p < 0.0001$  statistically highly significant



slightly convex, white and nonhaemolytic. It measures  $1.03 \pm 0.21 \mu\text{m}$  in length and  $0.4 \pm 0.1 \mu\text{m}$  in width. Using API<sup>®</sup> 50CH strip, positive reactions were obtained with glycerol, erythritol, dulcitol,

D-mannitol, D-tagatose and potassium 5-cetogluconate. Using an API<sup>®</sup> ZYM strip, positive activities were observed with alkaline phosphatase, lipase (C14), leucine arylamidase, trypsin, acid phosphatase,

naphtol phosphohydrolase and  $\beta$ -galactosidase. Strain Marseille-P4005<sup>T</sup> exhibited positive reaction only to gelatin when using API<sup>®</sup> 20A strip. The major fatty acids are the saturated structures as: hexadecanoic acid (25%), octadecanoic acid (23%) and tetradecanoic acid (20%). The genome was 3,212,687-bp- long with G+C content of 56.4 mol%. The pathogenic potential of strain Marseille-P4005<sup>T</sup> is unknown. Strain Marseille-P4005<sup>T</sup> was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR), under reference CSUR P4005 and the Spanish Type Culture Collection under reference CECT 9669.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectrum

The MALDI-TOF MS spectrum of 'Agathobaculum massiliense' strain Marseille-P4005<sup>T</sup> is available online (<https://www.mediterranee-infection.com/urms-data-base>).

#### Collection deposit

Strain was deposited in the Collection de Souches de l'Unité des Rickettsies under deposition number CSUR P4005 and the Spanish Type Culture Collection under deposition CECT 9669.

#### Accession numbers

The 16S rRNA and the genome of Marseille-P4005<sup>T</sup> were deposited in Genbank under accession numbers LT985392.1 and OMPN00000000.1, respectively.

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**Author contributions** R.M.W carried out the technical characterization of strain Marseille-P4005<sup>T</sup>, drafted the manuscript and analyzed the data; M.Z. analysed and verified the genomic characterization; M.B. isolated the strain; M.T.A performed a critical review of the manuscript, C.I.L. review the manuscript,

S.B. described the morphology features by using SEM; S.A. deposited the strain; C.A. performed the genome sequencing, A.C. analyzed the genome, P.E.F review the manuscript; M.M. supervised the project; D.R. conducted project conceptualization, resources and funding acquisition. All authors reviewed the manuscript.

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#### Declarations

**Conflict of interest** The authors would like to declare that Didier Raoult is a consultant in microbiology for the Hitachi High-Tech Corporation from March 2018 until March 2020. Otherwise, the authors declare no other conflict of interest.

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## **Article. 4**

### ***Thomasclavelia ramosa* is a signature biomarker in Patients with Alcoholic Liver Disease and Associated Hepatocellular Carcinoma: Culturomics and Metagenomics Case-Control Study**

**Reham MAGDY WASFY, Patrick BORENTAIN, Babacar MBAYE, Maryam TIDJANI ALOU, Aurelia CAPUTO, Claudia ANDRIEU, Giovanna MOTTOLA, Didier RAOULT, Matthieu MILLION and Rene GEROLAMI**

**« Draft Article.»**

1 ***Thomasclavelia ramosa* is a signature biomarker in Patients with Alcoholic**  
2 **Liver Disease and Associated Hepatocellular Carcinoma: Culturomics and**  
3 **Metagenomics Case-Control Study**

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## 20 **Abstract**

21 **Background:** alcohol abuse has deleterious effects on human health by disrupting the  
22 functions of many organs and systems, especially the liver, leading to fatal complications  
23 such as hepatocellular carcinoma (HCC). Gut microbiota has been implicated in the  
24 pathogenesis of alcoholic liver diseases (ALD) and a variety of organ malignancies such as  
25 HCC. Current studies revealed that gut microbiota can be used as a biomarker to predict  
26 cancers. **Methods:** a case-control study was conducted on patients with ALD without HCC  
27 (ALD-NoHCC) (n=16) and ALD with HCC (ALD-HCC) (n=19) and controls (n=24) groups.  
28 The culturomics approach was used for the first time, in addition to 16S rRNA sequencing, to  
29 analyze the patients' fecal samples. The taxonomic composition of the gut microbiota and  
30 correlation analysis to the fecal and clinical indicators were studied. **Results:** Compared to  
31 the control group, the gut microbiota of the patients in the ALD-NoHCC and ALD-HCC  
32 groups showed significant changes using 16S rRNA sequencing. In ALD-NoHCC,  
33 *Mediterraneibacter gnavus* was significantly increased, while *Thomasclavelia ramosa* and  
34 *Gemmiger formicilis* were significantly increased in the ALD-HCC group. Alpha diversity  
35 was significantly lower in both disease groups compared to the control. Using culturomics, a  
36 total of 292 species were isolated from ALD samples, whereas 254 species were isolated  
37 from the control samples ( $p= 0.0342$ ). *T. ramosa* was confirmed to be significantly enriched  
38 in all ALD samples by culturomics. Patients in both disease groups showed a significant  
39 decrease in fecal total cholesterol and triglycerides ( $p <0.0001$ ). **Conclusions:** our study  
40 reported *T. ramosa* in HCC for the first time. This species can be used as potential  
41 noninvasive biomarkers for diagnosing the progression of ALD to HCC, especially since it  
42 has been documented in different types of cancers. Therefore, experimental studies are  
43 needed to investigate their potential carcinogenic role in addition to disease prediction models  
44 for ALD and HCC risk prediction.

45

46 **Keywords:** *Thomasclavelia ramosa*, Alcohol-related liver disease, Hepatocellular carcinoma,  
47 Culturomics, 16S rRNA sequencing.

## 48 **Introduction**

49 Alcoholic liver disease (ALD) is among the leading causes of morbidity and mortality,  
50 accounting for 0.9% of all global deaths and 47.9% of all liver cirrhosis-related deaths [1].  
51 Severe alcoholic hepatitis, the most aggressive type of the disease, has a fatality rate of 20-  
52 50%, and its pathophysiology is still mostly unknown [2]. Furthermore, there is no approved  
53 treatment for alcoholic hepatitis, and existing medications, such as steroids, are marginally  
54 helpful and limited by the risk of sepsis [3,4], and liver transplantation is the only curative  
55 choice for these patients [5]. As a result, there is an urgent need to understand the  
56 pathogenesis of alcoholic hepatitis better and find innovative treatments for these patients.

57 Gut microbiota plays a vital role in the maturation of the host immune response [6]  
58 and the maintenance of intestinal barrier integrity, which restricts pathogen proliferation in  
59 the gut [7]. Gut dysbiosis has been reported in both rodent models [8] and patients with ALD  
60 [9], and bacterial products such as endotoxins have been linked to the development of ALD  
61 and progression to cirrhosis in experimental and human diseases [10,11]. Interestingly, recent  
62 studies have investigated the role of fecal microbiota transplantation (FMT) in ALD [12].

63 In ALD, the gut microbiome showed an increase in *Proteobacteria* and *Fusobacteria*  
64 and a reduction in *Bacteroidetes* [13]. *Lachnospiraceae* and *Ruminococcaceae* families are  
65 decreased, whereas *Enterobacteriaceae* and *Fusobacteriaceae* are significantly increased in  
66 ALD-related cirrhosis [14]. ALD-related dysbiosis was associated with increased  
67 *Bifidobacteria* and *Streptococci* and decreased anti-inflammatory *Clostridium septum* and  
68 *Faecalibacterium prausnitzii*. The density of these protective strains is inversely correlated  
69 with specific clinical parameters such as bilirubin levels [15]. However, there is insufficient  
70 data to evaluate whether there are unique alterations in the fecal microbiota in well-defined  
71 cases of ALD and associated complications, such as hepatocellular carcinoma (HCC).

72 Generally, HCC is induced by chronic liver disease, and its major risk factors include  
73 viral hepatitis infection, diabetes, nonalcoholic fatty liver disease (NAFLD), and ALD [16].  
74 HCC is the most common form of liver cancer, forming almost 90% of cases [17]. Moreover,  
75 gut dysbiosis has been reported to contribute to HCC by inducing inflammation [18]. In terms  
76 of morbidity and mortality, liver cancer ranks third among other cancers, resulting in a  
77 significant worldwide cancer burden [19,20]. However, the etiology and exact molecular  
78 mechanism of HCC have yet to be fully understood.

79           Moreover, gut dysbiosis has been reported to contribute to HCC by inducing  
80 inflammation [18]. Recent research suggests that gut microbiota can possibly promote liver  
81 cancer by inhibiting tumor immunosurveillance. Treatment with broad-spectrum antibiotics  
82 decreased spontaneous HCC growth and inhibited liver metastasis of tumors of different  
83 origins in mice, indicating that gut microbiota could play a pro-tumorigenic role [21].  
84 Numerous studies have examined gut microbiota characteristics linked to Immune  
85 Checkpoint Inhibitor (ICI) therapy outcomes in patients with HCC [22–24], suggesting that  
86 modifying gut microbiota could be used to enhance immunotherapy against HCC.  
87 Furthermore, some experimental data from human studies [25–32] and animal models  
88 [21,33–39] indicate that gut microbiota is related to HCC occurrence.

89           In addition, several metagenomic studies have investigated gut dysbiosis in patients  
90 with ALD based on 16S rRNA amplicon sequencing [9,40,41] or shotgun (whole-genome)  
91 [42]. However, no study described gut dysbiosis in patients with ALD or HCC based on the  
92 culturomics approach. Culturomics is a high-throughput culture method based on diversified  
93 culture conditions, which was found to be complementary to metagenomics [43], for  
94 exploring gut microbiota composition by discovering novel bacteria [44]. Previous case-  
95 control studies have reported the value of culturomics in defining the missing repertoire of  
96 probiotics and beneficial species that could be used in FMT, which requires viable isolated  
97 strains [45,46].

98           Across the diverse species, different species have been described to be associated with  
99 HCC, such as *Enterobacter ludwigii* [28] and *Escherichia coli* [26]. However,  
100 *Thomasclavelia ramosa*, which was found to be associated with some types of cancers such  
101 as colorectal [47–49] and nasopharyngeal [50] cancers, hasn't been reported before in  
102 patients with HCC.

103           The recently revised genome-based bacterial taxonomy classified *Thomasclavelia*  
104 *ramosa* (new genus *Thomasclavelia*) into the family *Coprobacillaceae* within the order  
105 *Erysipelotrichales* [51]. Previously, this Gram-positive, non-mobile, spore-forming anaerobic  
106 bacillus was known for a long time as *Clostridium ramosum* and *Erysipelatoclostridium*  
107 *ramosum* [52]. Being a commensal of the human intestinal flora, *T. ramosa* plays only a  
108 pathogenic role in adults with any degree of compromised immune system such as  
109 immunosuppressive conditions [53]. *T. ramosa* was identified as a genotoxic species, but the

110 mechanisms by which this species damages DNA in a study investigating colorectal cancer is  
111 not well established [48].

112 Accordingly, this study aims to characterize the microbial signature in patients with  
113 ALD and ALD-associated HCC using both culturomics and large-scale sequencing (v3v4  
114 region 16S rRNA amplicon sequencing).

## 115 **Material and Methods**

### 116 **Study Design and ethics statement**

117 A case–control study was carried out in the Hepatology Department of Marseille University  
118 Hospital (south-eastern France), Marseille, France, according to STROBE statement  
119 guidelines [54] from January to June 2022. The HEPATGUT study was approved by the  
120 local ethics committee of l’Institut Hospitalo-Universitaire Méditerranée Infection, Marseille,  
121 France (IHUMI, 2020-004), approved by the Protection of Persons Committee (Approval No.  
122 CPP: 21.04391.000046—21075), and carried out according to the 2013 Declaration of  
123 Helsinki (1975; World Medical Association, 2013) [55]. Informed consent was obtained from  
124 the study subjects before their enrolment.

### 125 **Study Population**

126 The HEPATGUT study included a total of 35 patients with ALD and 24 controls. All 59 fecal  
127 samples were intended to be analyzed using 16S rRNA sequencing. Eleven ALD and ten  
128 controls have been studied by culturomics and are reported in the present article. Healthy  
129 controls without chronic diseases or on regular medications were recruited using a snowball  
130 approach [56]. Age, gender, weight, height, alcohol consumption, and dietary habits were  
131 collected. Alcoholism was defined, according to the National Institute of Alcohol Abuse and  
132 Alcoholism, as the consumption of five or more drinks (male) or four or more drinks (female)  
133 in the space of about 24 hours [57]. The diagnosis of patients with ALD was made according  
134 to the European Association for the Study of the Liver (EASL) guidelines [58].

135 The exclusion criteria were: probiotics/antibiotics intake in the previous month prior  
136 to the collection of stools, vegetarians (those who eat only vegetables, fruits, grains, nuts, and  
137 occasionally eggs or dairy products), as this dietary habit has been associated with distinct  
138 gut microbiota [59], participants under 18 years old, non-alcoholic steatohepatitis (NASH),  
139 concurrent systemic diseases, patients infected with viral hepatitis, autoimmune liver disease,  
140 a history of acute gastroenteritis eight days before enrolment and participants who drank

141 alcohol only once a month. To clarify gut microbiota signature according to the degree of  
142 liver damage, we divided the patients into two groups based on the presence or absence of  
143 associated HCC. The groups were matched with age and body mass index (BMI) (**Fig. 1**).

#### 144 **Clinical measurements**

145 Liver stiffness measurements in cirrhotic patients were conducted using a FibroScan®  
146 instrument (Echosens, Paris, France). Measurements with more than ten successful  
147 acquisitions were obtained (with a rate of > 60% and an interquartile range of < 30%). In  
148 addition, routine biochemistry, including prothrombin index (PT), platelets count, total  
149 bilirubin (TBIL), serum albumin (ALB), alanine aminotransferase (ALT), aspartate  
150 aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP)  
151 and serum creatinine, were measured. The fecal samples from all participants were collected  
152 as described before [45] and stored at - 80° C until used.

#### 153 **High-throughput culturomics approach**

154 The stool samples (11 samples from patients with ALD and ten samples from controls) were  
155 cultured according to the culturomics approach previously established in our laboratory [43].  
156 The number of samples was chosen because it is a time-consuming approach in which one  
157 sample takes approximately four weeks to be analyzed. In addition, the culturomics study  
158 here is intended to be an exploratory study to gain insights about cultured microbiota in  
159 patients with ALD and HCC. Fast culturomics was applied using four culture conditions, as  
160 comprehensively detailed by Naud et al. [60]. We applied the same culturomics methodology  
161 described in our previous study [45]. A subculture of the isolated colonies was performed for  
162 purification. The colonies were then identified using Matrix Assisted Laser Desorption/  
163 Ionization Mass Spectrometry (MALDI-TOF/MS) (Bruker Daltonics, Bremen, Germany)  
164 according to the manufacturer's instructions [61,62].

#### 165 **Measurement of fecal parameters (ethanol, glucose, total protein (TP), triglycerides 166 (TGC), and total cholesterol**

167 One gram of stool was suspended in 5 mL of high-performance liquid chromatography  
168 (HPLC) water in HeadSpace glass vials. A calibration range was prepared from an ethanol  
169 stock solution in water with concentrations ranging from 0.5 to 100 mM. Standards and  
170 samples were spiked with isopropanol at 100 mM (internal standard). Measurements were  
171 performed using an HS-GC-MS system (Perkin Elmer, Villebon sur Yvette, France)



172 combining an HS40 headspace injector, a Clarus 500 gas chromatograph, and an SQ8 S mass  
173 spectrometer. All vials were positioned onto the headspace sample tray after homogenization  
174 by shaking. They were heated one by one at 60 °C for 10 min to vaporize the alcohols that  
175 were automatically transferred to the gas chromatography system by overpressure (1 min, 25  
176 psi) followed by a 1.8 s depressurization (needle/transfer line/GC inlet at 70/80/150 °C).  
177 Alcohols were introduced into a ZB-BAC2 chromatography column (30 m, 0.32 mm ID, 1.2  
178 µm; Phenomenex, Le Pecq, France) maintained at 40 °C and separated using Helium as a  
179 carrier gas at 10 psi. Compounds were individually monitored by mass spectrometry with a  
180 selected ion recording (SIR) method: ethanol m/z 31 and isopropanol m/z 45. The MS inlet  
181 line and electron ionization source were set at 150 °C. All data were collected and processed  
182 using Turbomass 6.1 (Perkin Elmer) software. Internal calibration was calculated using the  
183 peak areas from the associated SIR chromatograms.

#### 184 **16S rDNA gene sequencing**

185 PCR amplification of the bacterial 16S rRNA gene V3-V4 region was performed using  
186 primers(FwOvAd\_341F:5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACG  
187 GGNGGCWGCAG3' ;RevOvAd\_785R:5'GTCTCGTGGGCTCGGAGATGTGTATAAGA  
188 GACAGGACTACHVGGGTATCTAATCC3') as being described in details in our previous  
189 study [45].

#### 190 **Bioinformatics analysis**

191 The raw sequencing data for all samples were deposited into the NCBI Sequence Read  
192 Archive database (accession number: PRJEB62828). Noisy sequencing data were excluded,  
193 and chimeric sequences were identified and removed by Chimera Slayer. The clean data were  
194 clustered into operational taxonomic units (OTUs) at the 97% similarity threshold using  
195 UCLUST algorithm after the removal of singletons.

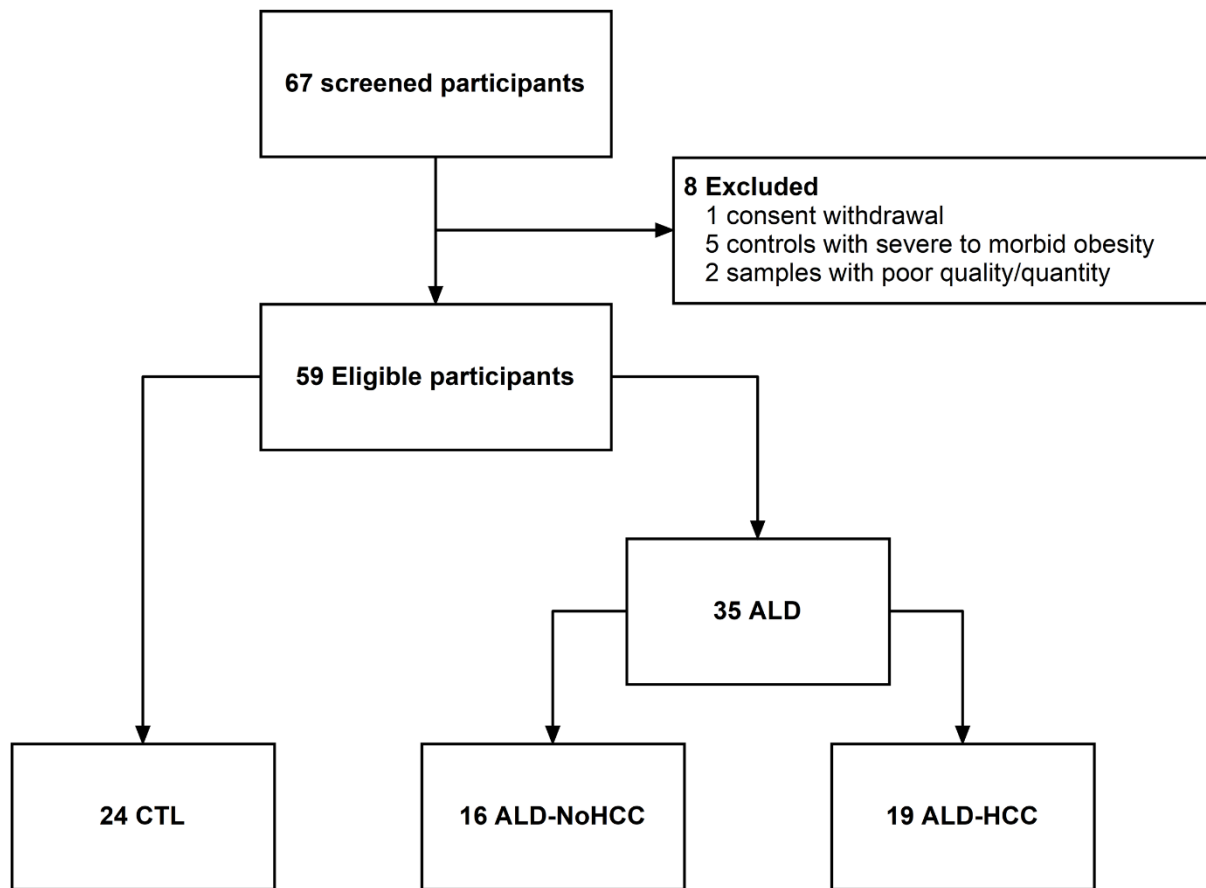
196 The alpha and beta diversity were calculated using MicrobiomeAnalyst database  
197 (<https://www.microbiomeanalyst.ca/>; Accessed on 30 September), based on the profile of the  
198 relative abundance of OTUs in a single sample. Alpha-diversity was evaluated through three  
199 indexes (Chao1, Simpson, and Shannon) to compare bacterial richness and diversity across  
200 samples. Principal Coordinates analysis (PCoA) was conducted using Bray-Curtis  
201 dissimilarity to visualize the microbial structure and distribution among the samples.  
202 Permutational multivariate analysis of variance (PERMANOVA) were used to assess beta  
203 diversity. Linear discriminant analysis (LDA) effect size (LEfSe) used the Kruskal-Wallis

204 rank sum test combined with LDA to detect features with significant different abundances of  
205 various taxa among groups via Microbiome Analyst Platform  
206 (<https://www.microbiomeanalyst.ca/>; Accessed 30 September 2023). A log LDA score >2  
207 was the threshold for discriminating between groups. STAMP software (version 2.1.3) was  
208 used analyzing metagenomic profiles via applying effect sizes and confidence intervals in  
209 assessing biological importance. Shared taxa present in all groups were defined as the core  
210 microbiota. Heatmaps showing correlations between the variables were created using the  
211 Spearman's rank-correlation analysis by Morpheus software  
212 (<https://software.broadinstitute.org/morpheus>)

### 213 **Statistical analysis**

214 It is essential to understand well that culturomics is a qualitative approach able to decipher  
215 the presence or absence of viable microbial species but is currently inadequate to evaluate the  
216 absolute or relative abundance of microbial species or strains. In contrast, amplicon  
217 sequencing is adequate for detecting the presence or absence of the DNA sequence (but not  
218 the viability) and its relative abundance (but not the absolute abundance) in the sample  
219 analyzed. Accordingly, only a comparison of frequency has been used for culturomics.  
220 Conversely, both frequency comparison and analyses based on relative abundance (LDA)  
221 have been used for 16S amplicon sequencing data. The microbial composition in each disease  
222 stage was analyzed at all taxonomic levels till the species level, and the average relative  
223 abundance of each microbiota was calculated. For the figures, OTUs not assigned to a  
224 cultured species have been removed to improve readability.

225 The results are expressed as the mean  $\pm$  standard deviation. The normal distribution  
226 was determined using either D'Agostino-Pearson or the Kolmogorov-Smirnov test. One-way  
227 analysis of variance was performed to compare continuous variables between two groups.  
228 Differences between groups were determined by using the non-parametric Kruskal-Wallis  
229 and Mann-Whitney U test. Chi-square test was used to compare categorical data. Spearman  
230 rank correlation was used to calculate the relationship between microbial makers and clinical  
231 indicators. To test for significant detection frequency difference, two-sided Fisher's exact test  
232 was used. All statistical analyses were calculated using GraphPad Prism Software for  
233 Windows (GraphPad Software, San Diego, CA, USA) (version 9.0). *P* values less than 0.05  
234 were considered as significant differences.



235

236 **Figure. 1** Flowchart of the subjects included in the study. ALD: Alcoholic liver disease;  
 237 HCC: Hepatocellular carcinoma; CTL: Controls. The chart was created by EDGE  
 238 Diagrammer software®.

## 239 Results

### 240 Characteristics of the study participants

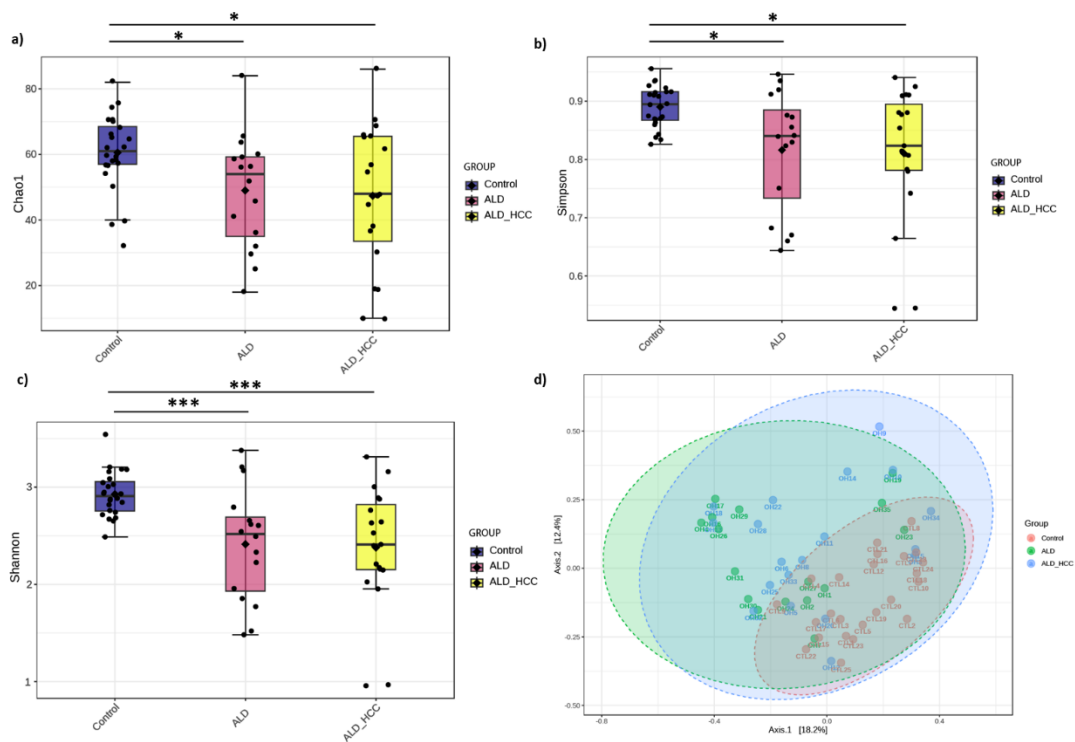
241 After a strict inclusion and exclusion process, a total of 59 participants were enrolled in our  
 242 study, including 35 patients with ALD and 24 controls. The ALD group was subdivided into  
 243 ALD without HCC (ALD-NoHCC) (n=16) and with HCC (ALD-HCC) (n=19). The clinical  
 244 characteristics of the groups are shown in **Table 1**.

### 245 Overview of gut microbiota shift in patients by 16S rDNA sequencing

246 The 16S rDNA gene sequencing was performed on the stool samples from all the  
 247 participants. The sequencing run expressed good-quality monitoring parameters, yielding a  
 248 total of 4,329,380 reads (1,246,349 for ALD-NoHCC, 1,544,568 for ALD-HCC, and  
 249 1,538,463 for control groups) after excluding low-quality reads. On average, each sample had  
 250 73,379 ±54,786 reads. The number of reads per sample was higher in the ALD-HCC group

251 (81,293 ±86,694) than in ALD-NoHCC (77,897 ±35,166) or control groups (64,103 ±26,923)  
252 but no significant difference was noticed between the groups (ALD-NoHCC vs ALD-HCC ( $p$   
253 = 0.5669); among the three groups ( $p= 0.5587$ ). A total of 2,840,773 clean tags were  
254 obtained, of which 1980 OTUs were matched. Of these OTUs, 1565 (79.04 %) were  
255 successfully assigned at the phylum level, 886 (44.75 %) at the genus level, and 493 (24.90  
256 %) at the species level.

257 The overall intestinal microbiota structure significantly differed among the ALD-  
258 NoHCC, ALD-HCC, and control groups. The Chao1 index ( $p= 0.0367$ ), Simpson index ( $p=$   
259 0.0168), and Shannon index ( $p = 0.0005$ ), which reflect the alpha diversity, were significantly  
260 lower in the ALD-NoHCC and ALD-HCC groups than in the control group (**Table S1; Fig.**  
261 **2**). However, no significant difference was noticed in alpha diversity indices between ALD-  
262 NoHCC and ALD-HCC groups. PCoA was performed using Bray–Curtis distance, applying  
263 pairwise comparison. The ALD-NoHCC and ALD-HCC groups showed a significant  
264 separation pattern compared to the control group (**Fig. 2**) ( $p = 0.001$ ; F-value= 2.9824;  
265  $R^2=0.0963$ ).



266

267 **Figure. 2** Comparison of alpha diversity (a, b, c) and beta diversity (d) in patients with  
 268 Alcoholic liver disease (ALD; n=16), ALD-associated hepatocellular carcinoma (HCC; n=  
 269 19), and control (n=24) groups. Alpha diversity in box plots. Each boxplot represents the data  
 270 range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the  
 271 mean (black diamond). Principal coordinate analysis (PCoA) plot of beta diversity. \* p-value  
 272 < 0.05, \*\*\* p-value < 0.001.

273 **Table 1** Demographic and clinical characteristics of the study participants.

Characteristics	Control (n=24)	ALD-NoHCC (n=16)	ALD-HCC (n=19)	P1 value	P2 value	P3 value	P4 value
<b>Demographic</b>							
Age (years)	63.82 ± 7.65	60.13 ± 10.14	65 ± 5.80	0.146 <sup>M</sup>	0.857 <sup>t</sup>	0.110 <sup>M</sup>	0.131 <sup>A</sup>
Gender (M/F)	10/14	11/5	19/0	0.093 <sup>C</sup>	<0.0001 <sup>C****</sup>	0.0085 <sup>C**</sup>	0.0003 <sup>C***</sup>
Female sex (%)	14 (58.33 %)	5 (31.25 %)	0 (0 %)	--	--	--	--
Male sex (%)	10 (41.66 %)	11 (68.75 %)	19 (100 %)	--	--	--	--
BMI (kg/cm <sup>2</sup> )	25.2 ± 2.5	27.5 ± 5.98	26.89 ± 4	0.137 <sup>t</sup>	0.151 <sup>t</sup>	0.721 <sup>t</sup>	0.261 <sup>A</sup>
<b>Co-morbidities</b>							
Hypertension (%)	4 (17 %)	6 (37.5 %)	12 (63.16 %)	0.159 <sup>F</sup>	0.004 <sup>F**</sup>	0.181 <sup>F</sup>	0.0074 <sup>C**</sup>
Dyslipidemia (%)	0 (0%)	1 (6.25 %)	3 (15.79 %)	0.4 <sup>F</sup>	0.079 <sup>F</sup>	0.608 <sup>F</sup>	0.123 <sup>C</sup>
Smoking (%)	0 (0%)	13 (81.25 %)	6 (31.58 %)	<0.0001 <sup>F****</sup>	0.005 <sup>F**</sup>	0.006 <sup>F**</sup>	<0.0001 <sup>C****</sup>
Digestive symptoms	4 (17 %)	4 (25 %)	4 (21.05 %)	0.691 <sup>F</sup>	>0.9999 <sup>F</sup>	>0.9999 <sup>F</sup>	0.811 <sup>C</sup>
<b>Liver functions</b>							
AST (U/L)	NA	54.94 ± 28.10	58.95 ± 38.23	--	--	0.980 <sup>M</sup>	--
ALT (U/L)	NA	90.63 ± 223.14	34.79 ± 17.01	--	--	0.838 <sup>M</sup>	--
GGT (U/L)	NA	192.88 ± 305.55	267.21 ± 377.53	--	--	0.282 <sup>M</sup>	--
TBIL (umol/L)	NA	36.51 ± 56.06	25.28 ± 15.04	--	--	0.898 <sup>M</sup>	--
ALP (U/L)	NA	154.13 ± 60.59	177 ± 96.64	--	--	0.574 <sup>M</sup>	--
Serum albumin (g/L)	NA	33.48 ± 6.36	34.91 ± 4.96	--	--	0.482 <sup>t</sup>	--
<b>Routine tests</b>							
Prothrombin time (%)	NA	67.93 ± 22.49	74.35 ± 18.13	--	--	0.379 <sup>t</sup>	--
PLT count (g/L)	NA	149.75 ± 82.22	163.72 ± 87.21	--	--	0.664 <sup>M</sup>	--
Serum creatinine (umol/L)	NA	81.13 ± 28.55	68.71 ± 15.00	--	--	0.208 <sup>M</sup>	--
<b>Complications</b>							
Ascites	0 (0%)	6 (37.5 %)	6 (31.58 %)	--	--	0.736 <sup>F</sup>	--
Encephalopathy	0 (0%)	7 (43.75 %)	4 (21.05 %)	--	--	0.273 <sup>F</sup>	--
Cirrhosis	0 (0%)	14 (87.5 %)	19 (100%)	--	--	0.202 <sup>F</sup>	--
CTP-A	0 (0%)	8 (50 %)	11 (57.89 %)	--	--	0.740 <sup>F</sup>	--
CTP-B	0 (0%)	7 (43.75 %)	8 (42.11 %)	--	--	>0.9999 <sup>F</sup>	--
CTP-C	0 (0%)	1 (6.25 %)	0 (0%)	--	--	0.457 <sup>F</sup>	--

275 Continuous variables were expressed as means  $\pm$  standard deviation. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate  
276 aminotransferase; GGT: Gamma-glutamyltransferase; TBIL: Total bilirubin; PLT: Platelets; ALP: Alkaline phosphatase; CTP: Child-Turcotte-  
277 Pugh score; ALD: Alcoholic liver disease; HCC: hepatocellular carcinoma; *P1*: Comparisons between ALD-NoHCC and controls groups; *P2*:  
278 Comparison between ALD-HCC and controls groups; *P3*: Comparison between ALD-NoHCC and ALD-HCC groups; *P4*: Comparisons among  
279 groups; <sup>M</sup>: Mann-Whitney U test; <sup>t</sup>: Unpaired *t*-test; <sup>A</sup>: ANOVA statistical test; <sup>C</sup>: Chi-square test and <sup>F</sup>: Fisher's exact test. \*\*  $p < 0.05$ , \*\*\*  $p <$   
280  $0.001$ , \*\*\*\*  $p < 0.0001$ . NA: not available.

## 281 **Abundance profile of gut microbiota in patients with ALD**

282 To show the abundance of bacteria in all fecal samples, ten phyla, 17 classes, 23 orders, 37  
283 families, and 64 genera were identified, and the top ten dominant gut microbiota are shown in  
284 (Fig. S1). Linear discriminant analysis (LDA) effect size modeling was applied to identify  
285 specific bacterial taxa associated with ALD-NoHCC and ALD-HCC groups (Fig. S2; Table  
286 S2). There were markedly significant differences in the community compositions in ALD-  
287 NoHCC and ALD-HCC patients compared with the control. At the phylum level, *Bacillota*  
288 and *Bacteroidota* were significantly abundant phyla in the control group. At the class level,  
289 *Clostridia* and *Bacteroidia* were abundant in controls, while *Alphaproteobacteria*, *Bacilli*,  
290 and *Erysipelotrichia* were abundant in the ALD-HCC group. At the family level,  
291 *Enterobacteriaceae* and *Carnobacteriaceae* were abundant in the ALD-NoHCC group, while  
292 *Streptococcaceae*, *Erysipelotrichaceae*, and *Odoribacteraceae* were abundant in the ALD-  
293 HCC group (Fig. S2).

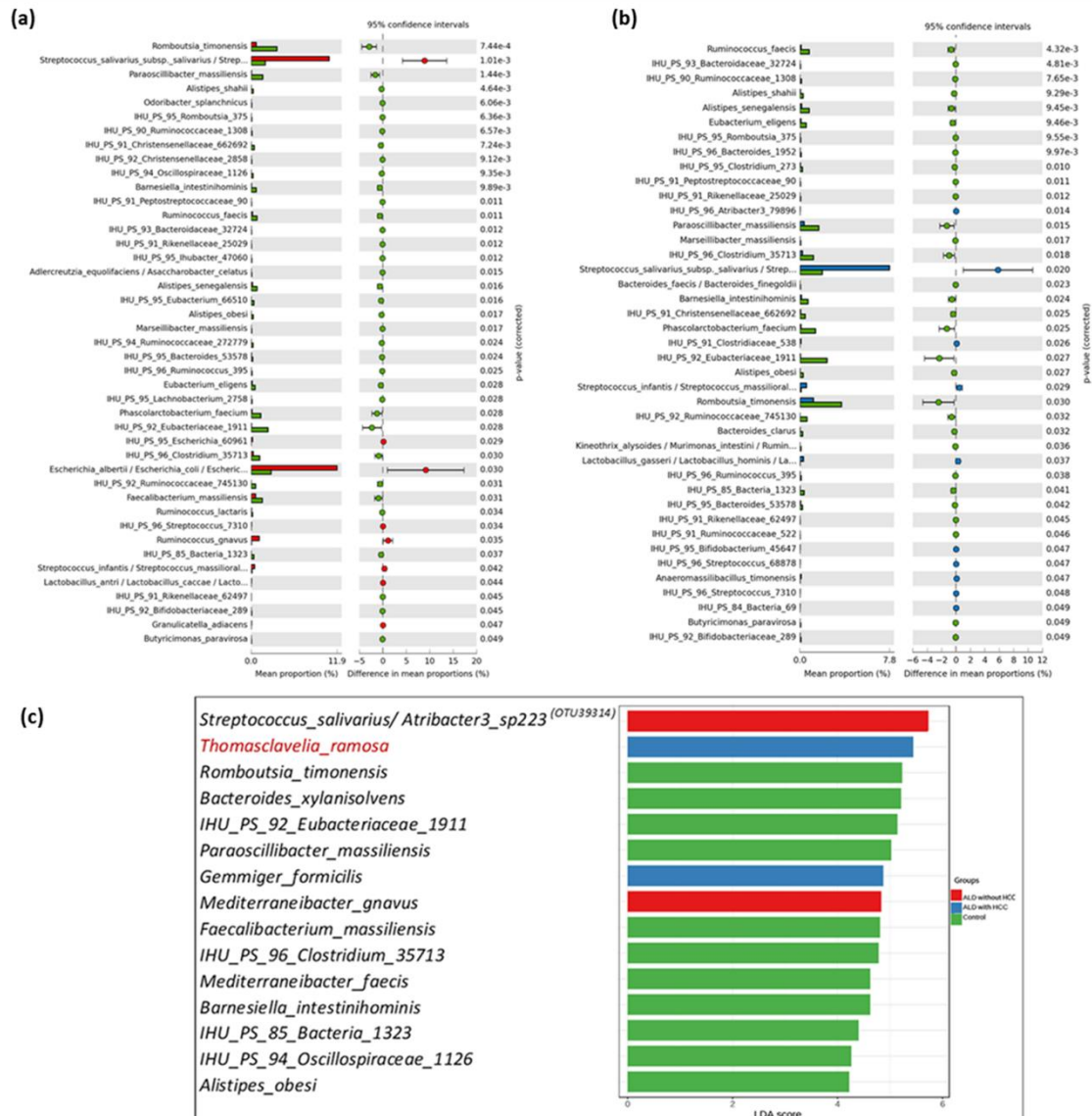
## 294 **Development of HCC is associated with distinct changes in the fecal microbiome**

295 There were 21 significantly different genera in all the groups. Significantly abundant genera  
296 in the ALD-NoHCC group were IHU\_PG\_91\_ *Bifidobacteriaceae*\_1548, *Escherichia*, and  
297 *Granulicatella*. The four most abundant genera in the ALD-HCC group included  
298 *Streptococcus*, *Gemmiger*, *Turicibacter*, and *Odoribacter* (Fig. S2; Table S2). Interestingly,  
299 *Streptococcus* and *Turicibacter* were also highly abundant in patients with ALD-NoHCC  
300 expressing elevating liver enzymes, denoting their role in the progression of the disease.  
301 These significantly different gut microbial taxa can be used as potential noninvasive  
302 biomarkers for the diagnosis of the progression of ALD to HCC.

## 303 **A microbiome signature for HCC distinct from ALD-NoHCC at the species level**

304 Compared to the control group, the gut microbiota of all ALD patients showed a significant  
305 abundance of *Streptococcus\_salivarius/Atribacter\_sp223* (OTU39314), *Escherichia\_albertii/*  
306 *Escherichia\_coli* (OTU2689), *Mediterraneibacter\_gnavus* (formerly, *Ruminococcus\_gnavus*),  
307 and *Thomasclavelia\_ramosa*. To identify potential microbiota biomarkers for HCC, we  
308 performed different comparisons between each disease group vs. the control group and  
309 comparisons of the three groups altogether. Interestingly, the ALD\_NoHCC and ALD\_HCC  
310 groups showed significant changes. In ALD\_NoHCC, *Mediterraneibacter\_gnavus* was  
311 significantly increased, while *Thomasclavelia\_ramosa* and *Gemmiger\_formicilis* were  
312 significantly increased in the ALD\_HCC group (Fig. 3).





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**Figure. 3** (a) Extended error bar plot of species differentially abundant between alcoholic liver disease with no carcinoma (ALD-NoHCC) and control groups. (b) Extended error bar plot of species differentially abundant between ALD with carcinoma (ALD-HCC) and control groups; (>1% difference in abundance,  $p < 0.05$ , Welch's t-test) created by STAMP software. (c) Histogram of the linear discriminant analysis (LDA) scores for differentially abundant species in the three groups. A logarithmic LDA score  $> 2$  indicated a higher relative abundance in the corresponding group than in other groups. The color of the circles, dendrogram, and histogram bars denote sample grouping (red (ALD-NoHCC group), blue (ALD-HCC), and green (control group)).

### 323 **Measures of fecal parameters**

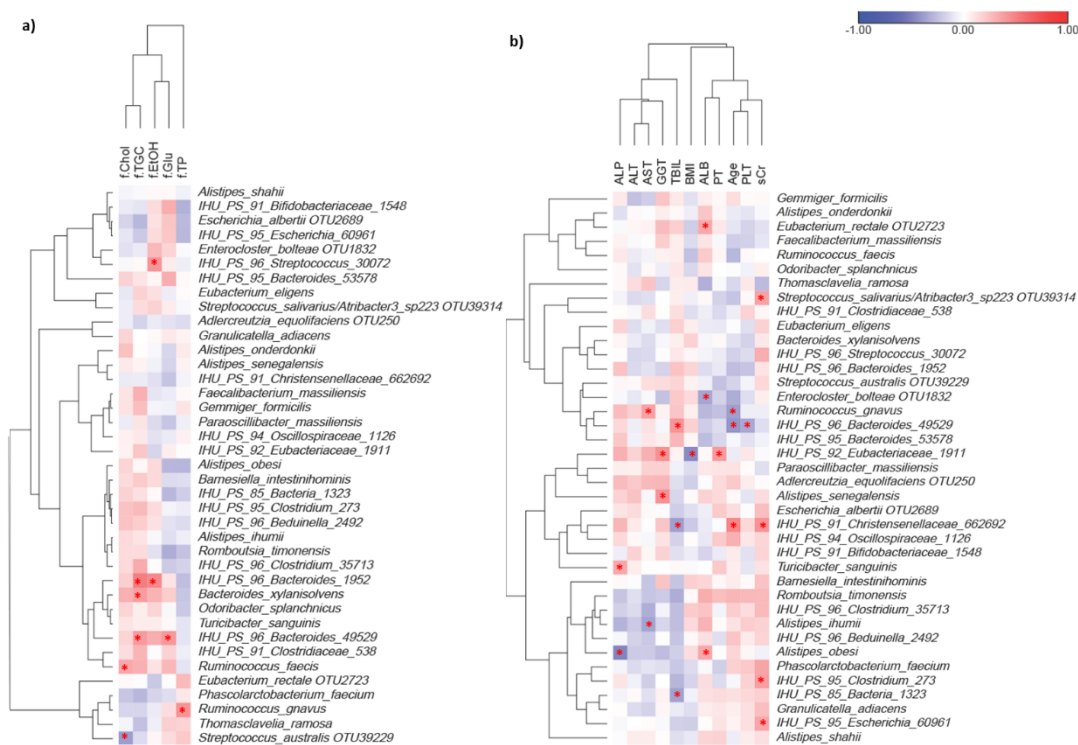
324 Patients with ALD-NoHCC and ALD\_HCC showed a significant decrease in fecal total  
325 cholesterol and TGC ( $p < 0.0001$ ) compared to the control group with no significant  
326 difference between ALD-NoHCC and ALD-HCC. However, fecal glucose, TP, and ethanol  
327 showed no significant difference among all the groups.

### 328 **Correlation analysis of species abundance with fecal biochemical and serum biomedical** 329 **indicators**

330 To identify the correlations between clinical variables and microbiota, associations between  
331 significant abundant species and continuous clinical variables were calculated by the  
332 Spearman correlation coefficient in all participants. Species heatmap revealed the correlation  
333 between species and clinical variables (**Fig. 4**). Five fecal and nine serum biomedical  
334 indicators and age and BMI were measured. 39 OTUs assigned to species level were  
335 associated with the fecal biochemical indicators (**Fig. 4a**). The abundance of *Bacteroides*  
336 species such as *IHU\_PS\_96\_Bacteroides\_49529*, *Bacteroides xylanisolvens*, and  
337 *IHU\_PS\_96\_Bacteroides\_1952* were significantly positively correlated with fecal TGC.  
338 *Mediterraneibacter\_gnavus* correlated positively with TP, while *Streptococcus\_australis*  
339 OTU39229 and *Ruminococcus faecis* were significantly negatively and positively correlated  
340 with fecal cholesterol, respectively.

341 The serum biomedical indicators were also found to be associated with the abundance  
342 of 39 OTUs assigned to species. The increased AST levels were significantly positively  
343 correlated with the abundance of *Mediterraneibacter gnavus*, but negatively with the  
344 abundance of *Alistipes ihumii*. The ALP levels were positively correlated with the abundance  
345 of *Turicibacter sanguinis*, but negatively with *Alistipes obesi*. The serum ALB levels were  
346 positively correlated with the abundance of *Alistipes obesi* and *Eubacteriu\_rectale* OTU2723,  
347 while negatively correlated with *Enterocloster\_bolteae* OTU1832. The serum creatinine  
348 levels were positively correlated with the abundance of  
349 *Streptococcus\_salivarius/Atribacter3\_sp223* (OTU39314) (**Fig. 4b**).

350 *T. ramosa* showed a negative correlation with serum creatinine and albumin and a  
351 positive correlation with AST, although statistically insignificant. *Gemmiger formicilis* was  
352 negatively non-significantly correlated with ALT and AST but positively correlated with  
353 GGT. Among all the species in the heatmap, *Alistipes senegalensis* showed a significant  
354 positive correlation with GGT. *M. gnavus* was negatively correlated with age.



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357 **Figure. 4** Heatmap of the correlation analysis between significant abundant species and both  
 358 fecal (a) and clinical (b) parameters in patients with alcoholic liver disease (ALD; with and  
 359 without carcinoma). Red color indicates a positive correlation while blue color indicates a  
 360 negative correlation; Spearman`s correlations were calculated using GraphPad Prism v.9; \*  
 361  $p < 0.05$ . The figure was created using the MORPHEUS tool available at  
 362 <https://software.broadinstitute.org/morpheu>.

363 BMI: Body mass index; PT: Prothrombin; PLT: Platelets; TBIL: Total bilirubin; ALB: serum  
 364 albumin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; sCr: Serum  
 365 creatinine; GGT: Gamma-glutamyltransferase; ALP: Alkaline phosphatase; f.Chol: fecal total  
 366 cholesterol; f.TGC: fecal triglyceride; f.Glu: fecal glucose; f.TP: fecal total protein and  
 367 f.EtOH: fecal ethanol.

## 368 Diversity assessment by culturomics approach

369 Overall, twenty-one samples were analyzed using three culture conditions. 11 samples were  
370 analyzed for patients with ALD, and 17,308 colonies were isolated and tested using MALDI  
371 TOF MS with an average of  $1573 \pm 333$  colonies per sample. For control samples, ten  
372 samples were analyzed, and 14,780 colonies were isolated, with an average of  $1478 \pm 265$   
373 colonies per sample ( $p= 0.4793$ ). A detailed list of the isolated bacterial species is listed  
374 in **File Table S3**. A total of 292 species (with an average of  $66 \pm 19$  per sample) were  
375 isolated in ALD samples, whereas 254 species (with an average of  $50 \pm 13$  per sample) were  
376 isolated in control samples ( $p= 0.0342$ ). In ALD samples, 177 bacterial isolates (60.41 %) were Gram-positive and 204 species (69.62 %) were anaerotolerant. In addition, among the  
377 292 species isolated in the ALD group, 175 species were known in the human gut, 70 species  
378 were known in humans but not in the gut, 30 species were known but not previously found in  
379 humans, and 17 species were identified as new species (**File Table S3**). In the control group,  
380 197 species were known in the human gut, 12 unknown species were isolated from the human  
381 gut, among which four new genera were identified (**File Table S3**), 13 were known but not  
382 previously found in humans ( $p= 0.0399$ ), and 44 were already known in humans but not  
383 previously found in the gut. 159 species were shared between ALD and control groups, while  
384 133 species were uniquely detected in the ALD group (**File Table S3**). By comparing the  
385 species isolated in the ALD group to a recently published cultured human bacteria repertoire  
386 describing 3242 bacterial species, 116 shared species, and 17 species were uniquely defined  
387 in the ALD group. Moreover, 22 species were shared between ALD and control groups (**File**  
388 **Table S3**).

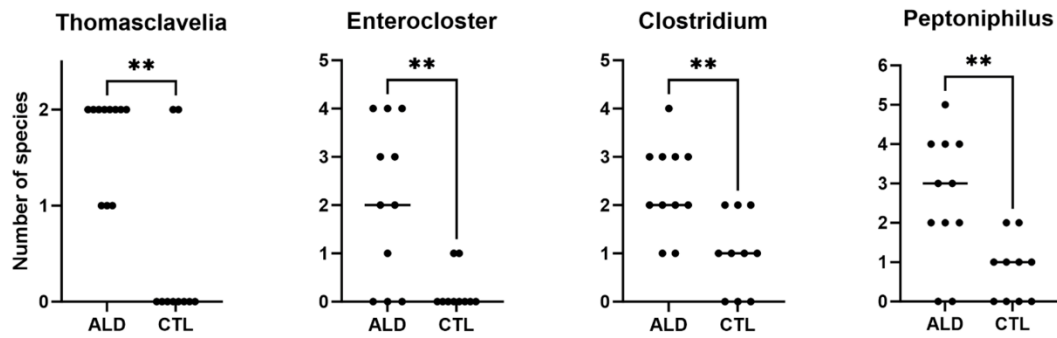
390 In ALD samples, six bacterial phyla were isolated, with mostly *Bacillota* (172  
391 species), followed by 50 *Bacteroidota*, 43 *Actinomycetota*, 15 *Pseudomonadota*, two  
392 *Fusobacteria* and one *Synergistetes*. In control samples, six phyla were isolated, with a  
393 majority of *Bacillota* (143 species; 56.30%), followed by 50 (19.69%) *Bacteroidota*,  
394 40 (15.27%) *Actinomycetota*, 12 (4.72%) *Pseudomonadota*, 1 (0.4%) *Synergistetes*, and  
395 1 (0.4%) *Verrucomicrobia* (**File Table S3**). No significant difference was found between  
396 both groups except for the *Bacillota* phylum, which was significantly enriched in the ALD  
397 group ( $p= 0.004$ ).

398 **Enrichment of *Thomasclavelia ramosa* species in patients with ALD**

399 The 292 species isolated from ALD samples belonged to 44 different families and 131  
400 different genera. Among them, 14 were the best-represented genera in terms of the diversity  
401 in the species, including *Bacteroides* (15 species), *Enterococcus* (14)  
402 *Clostridium* (11), *Streptococcus* (11), *Alistipes* (ten), *Peptoniphilus* (eight), *Prevotella* (eight),  
403 *Bifidobacterium* (seven), *Parabacteroides* (six), *Staphylococcus* (six), *Blautia* (five),  
404 *Enterocloster* (five), *Lachnoclostridium* (five), *Limosilactobacillus* (five). In control samples,  
405 a total of 111 genera were identified, with the seven most-represented genera  
406 including *Bacteroides* (15species), *Enterococcus* (11), *Alistipes* (12), *Peptoniphilus* (9), *Bifid*  
407 *obacterium* (7), *Limosilactobacillus* (7), and *Clostridium* (6) (File Table S3). 50 genera were  
408 only represented in the ALD group and not the control group. Four bacterial genera from  
409 the *Bacillota* phylum were found to be significantly enriched in the ALD group, including  
410 genera *Enterocloster* ( $p = 0.0058$ ), *Clostridium* ( $p = 0.0021$ ), *Peptoniphilus* ( $p= 0.0044$ ) and  
411 *Thomasclavelia* ( $p= 0.0016$ ) (**Fig. 5a, b**).

412 At the cultured species level, *Clostridium* and *Enterocloster* species such as *E. bolteae*  
413 and *T. ramosa* were significantly enriched in ALD patients. Surprisingly, *T. ramosa* was  
414 detected in all ALD patients ( $n=11$ ) including those with ALD-HCC ( $n= 5$ ) and ALD-  
415 NoHCC ( $n= 6$ ). Moreover, *Facklamia hominis* and *Peptoniphilus harei* were only enriched in  
416 ALD patients without HCC.

a.



b.

Species isolated by culturomics	Frequency Difference	ALD (n=11)	Control (n=10)	p-value	Genera with a significant diversity difference between ALD and CTL
<i>Thomasclavelia ramosa</i>	9	11	2	***	<i>Thomasclavelia</i>
<i>Enterocloster bolteae</i>	7	7	0	**	<i>Enterocloster</i>
<i>Enterocloster clostridioformis</i>	6	6	0	**	
<i>Oscillibacter massiliensis</i>	7	7	1	*	<i>Thomasclavelia</i>
<i>Thomasclavelia [Clostridium] innocuum</i>	6	8	2	*	<i>Enterocloster</i>
<i>Hungatella hathewayi</i>	6	8	2	*	<i>Clostridium</i>
<i>Peptoniphilus harei</i>	5	5	0	*	<i>Peptoniphilus</i>
<i>Mediterraneibacter gnavus</i>	5	5	0	*	
<i>Clostridium perfringens</i>	6	6	1	*	
<i>Finegoldia magna</i>	6	6	1	*	
<i>Limosilactobacillus fermentum</i>	7	7	2		
<i>Anaerococcus vaginalis</i>	4	4	0	*	
<i>Anaerostipes caccae</i>	4	4	0	*	
<i>Clostridium sporogenes</i>	4	4	0	*	
<i>Enterocloster aldenensis</i>	4	4	0	*	
<i>Facklamia hominis</i>	4	4	0	*	
<i>Peptoniphilus grossensis</i>	4	4	0	*	
<i>Peptoniphilus meridionalis</i>	4	4	0	*	
<i>Streptococcus constellatus</i>	4	4	0	*	
<i>Phascolarctobacterium faecium</i>	4	0	4	*	
<i>Alistipes communis</i>	4	3	8	*	

c.

Genus diversity per sample	ALD-NoHCC						ALD-HCC					Control									
	OH1	OH2	OH4	OH6	OH7	OH21	OH3	OH5	OH8	OH9	OH10	CTL13	CTL14	CTL7	CTL8	CTL15	CTL17	CTL4	CTL6	CTL12	CTL21
<i>Thomasclavelia</i>	2	2	2	2	2	1	2	2	2	1	1	0	0	0	0	0	0	2	0	2	0
<i>Enterocloster</i>	4	4	2	1	3	0	4	3	0	0	2	0	1	0	0	0	0	0	0	1	0
<i>Clostridium</i>	4	3	3	2	3	2	1	3	2	2	1	1	1	1	2	0	1	0	2	2	0
<i>Peptoniphilus</i>	5	3	3	4	2	4	2	4	0	0	2	1	0	0	2	0	1	0	1	2	1

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419 **Figure. 5 (a)** The number of species per sample identified by culturomics in the genera with  
 420 a significant enrichment between patients with alcoholic liver disease (ALD) and the control  
 421 (CTL) group; **(b)** Species with a significant frequency difference between ALD and control  
 422 groups by the culturomics approach. Multiple non-parametric *t*-tests and Barnard's bilateral  
 423 exact test were used to test the *p*-value. \* *p*-value < 0.05, \*\* *p* value < 0.001. **(c)** Enriched  
 424 genera in each sample, represented by the number of species in each genus per sample.

## 425 **Missing Repertoire in Patients with ALD**

426 For identification of potential probiotic species, all of the bacterial species that were  
427 identified both by culturomics and metagenomics were considered in the control samples but  
428 not in ALD samples (**File Table. S3**). The common species between both approaches in the  
429 control group were three, including *Akkermansia muciniphila*, *Dialister succinatiphilus* and  
430 *Metaprevotella massiliensis*. However, the ALD group showed no shared species between  
431 both approaches. The core microbiome was represented by 59 species forming the species  
432 shared between both groups by the two approaches including (**File Table. S3**).

433 Comparing the species identified by metagenomics and culturomics in ALD and  
434 control groups, 121 species were found exclusively in the control samples or in other words  
435 forming the missing repertoire in ALD patients (**File Table. S3**). These species belonged  
436 overwhelmingly to the *Bacillota* phylum (63; 52.06%), the *Bacteroidetes* (22; 18.18 %) and  
437 the *Actinobacteria* (16; 13.22 %), followed by a low number of species from the  
438 *Proteobacteria* (8; 6.61 %) phylum. Among the missing repertoire, strikingly, 78 species  
439 (64.46%) were strictly anaerobic.

440 The literature was searched for each species to find a possible probiotic use for  
441 humans. Seven species, *Akkermansia muciniphila*, *Lactobacillus iners*, *Lactobacillus ruminis*,  
442 *Levilactobacillus brevis*, *Limosilactobacillus mucosae*, *Limosilactobacillus reuteri* and  
443 *Propionibacterium freudenreichii*, were found to have possible probiotic features (**File Table**  
444 **S3**). All of them were isolated by culturomics in the control samples and are readily available  
445 in our laboratory's Collection de Souches de l'Unité des Rickettsies (CSUR) collection.

446 Overall, the hitherto unknown diversity was assessed and defined as the number of  
447 new species added to the species not previously known from the human gut by sample for  
448 culturomics analysis and as the number of unidentified OTU for metagenomics analysis [46].  
449 The difference was significant by culturomics for new species plus previously known species  
450 which had not been previously found in humans ( $7 \pm 4.38$  in ALD vs.  $2.5 \pm 0.97$  in  
451 controls,  $p = 0.0006$ ) suggesting a different  $\alpha$ -diversity (**File Table S3**).

## 452 **Discussion**

453 Our study is the first to characterize gut microbiota in ALD-NoHCC, ALD-HCC and controls  
454 using a culturomics approach, then compared to 16S rRNA sequencing. The main findings of  
455 this study include (i) the increased frequency and diversity of four genera in ALD, namely  
456 *Thomasclavelia*, *Enterocloster*, *Clostridium*, and *Peptoniphilus*, by culturomics, and (ii) the  
457 association between *Thomasclavelia ramosa* and ALD-HCC. Several metagenomic studies  
458 have outlined gut microbiota composition of patients with ALD based on 16S rRNA  
459 amplicon sequencing [9,40,41] or shotgun (whole-genome) [42].

460 It is generally believed that the intestinal microbiome plays a vital role in the genesis  
461 of ALD [63]. The current study supports this paradigm at the species level and demonstrates  
462 that ALD and HCC are associated with changes in the microbiome. The development of HCC  
463 is further associated with changes in the intestinal microbial composition that are distinct  
464 from those associated with ALD-related cirrhosis per se.

465 It has been reported that increased gut permeability permits the translocation of  
466 bacterial antigens, including endotoxins, contributing to ALD and liver damage [64]. Among  
467 the highly abundant species detected in our HCC groups were *T. ramosa*, and *Gemmiger*  
468 *formicilis*, in addition to *Streptococcus salivarius* and *Mediterraneibacter gnnavus*.

469 Being a normal resident of the human intestinal flora, *T. ramosa* plays only in rare  
470 occasions a pathogenic role, leading to invasive infections most commonly in older adults  
471 with any type or degree of compromised immune system such as septicemia in patients with  
472 hematological cancers [65], severely infected pressure ulcers [66], osteomyelitis [67],  
473 infected thoracic aortic aneurysm [68], septic arthritis and septicemia in chronic renal failure  
474 [69], peritonitis [70], Fournier's gangrene [71], and intracranial abscess [72]. Interestingly,  
475 similar to the best known and more important human opportunistic pathogen from the  
476 *Clostridia* class such as *Clostridium difficile* [73], *T. ramosa* was also found to be a causative  
477 agent of pseudomembranous colitis [74]. Moreover, various types of cancers (hematological  
478 cancers [65], colon carcinoma [66], and Ewing`s sarcoma [75] were the most commonly  
479 reported in comorbidities or immunocompromising conditions, followed by different causes  
480 of invasive infections [53].

481 A high risk for *T. ramosa* infection is likely to be associated with underdevelopment  
482 and dysfunction of the intestinal mucosal barrier in young children and immunodeficient  
483 adults/elderly patients, which enable the unique virulence factors such as IgA proteases of



484 this commensal anaerobic bacterium to help to invade the host and cause infection at various  
485 locations, scaping the host defense [76]. However, rare reports described *T. ramosa* as the  
486 causal agent of invasive infections in apparently immunocompetent individuals with  
487 complicated benign disorders or with indwelling medical devices due to sepsis after bowel  
488 perforation in a child [77], postpartum pyomyoma [78], spondylodiscitis in an older patient  
489 with benign prostatic hyperplasia [79], secondary infection in an intracranial hydatid cyst of a  
490 child with pleural empyema [80], and endocarditis in an elderly French patient with  
491 bioprosthetic aortic valve replacement [81]. *T. ramosa* was also reported to cause  
492 osteomyelitis after open fracture [82] indicating the potential ubiquitous nature of its spores,  
493 which have probably originated from the soil and entered the body through an open traumatic  
494 wound [82].

495 The antibiotic resistance in *T. ramosa* strains is not widespread. This anaerobic  
496 bacillus showed a high level of resistance only to rifampin, aminoglycosides,  
497 fluoroquinolones, and tetracyclines [76,79]. Metronidazole, a broad-spectrum beta-lactam  
498 with a beta-lactamase inhibitor, and meropenem appear to be the antibiotics of choice that  
499 can be used in combination or as a monotherapy to treat them [83]. Furthermore, *T.*  
500 *ramosa* was enriched in patients with colon cancer and exhibited genotoxicity in an  
501 experimental model producing small-molecule metabolites (indolimines) that induce cell  
502 cycle arrest and DNA damage in human colorectal cancer [48]. However, the mechanisms by  
503 which this species damages DNA and the relevance for human disease require further  
504 investigation.

505 Despite reporting *Gemmiger formicilis* to be enriched in patients with ALD-HCC in  
506 our study, this species was found to be significantly reduced in nasopharyngeal carcinoma  
507 [50], and was reported to be significantly negatively correlated with 5-HT (5-  
508 hydroxytryptamine) [50]. Meanwhile, *T. ramosa* was significantly positively correlated with  
509 5-HT in patients with nasopharyngeal carcinoma [50]. 5-HT is an important neurotransmitter  
510 that is mainly secreted by the central nervous system and enterochromaffin cells [84]. A  
511 variety of research has found that 5-HT can stimulate the development and progression of  
512 multifarious cancers, such as prostate carcinoma [85], HCC [86,87], colon cancer [88], small-  
513 cell lung cancer [89], and breast cancer [85]. A previous study has shown that the metabolites  
514 of *T. ramosa* could stimulate the secretion of 5-HT from enterochromaffin cells in the gut,  
515 which can promote the level of 5-HT in plasma [86]. These series of discoveries mentioned  
516 above have induced us to get a scenario that these species can promote the secretion of 5-HT

517 to facilitate the progression of HCC. However, they should be further investigated in  
518 extensive experimental and clinical studies.

519 *Mediterraneibacter gnavus* was reported as a potential pathogen in infectious disease  
520 and exhibited an increased abundance in inflammatory bowel disease [87,88]. In 2019, a  
521 study reported that *M. gnavus* was the producer of inflammatory polysaccharides, a promotor  
522 in TNF- $\alpha$  secreted by dendritic cells [89]. Therefore, alcohol enhances the sensitivity of  
523 Kupffer cells to product TNF- $\alpha$  [90], which can be increased by *M. gnavus*. In addition, our  
524 results showed that *M. gnavus* expressed a positive correlation with AST, which is in  
525 agreement with another study [91]. Liver function indexes ALT and AST are the easiest  
526 markers to assess hepatocellular damage and usually are the screen tests for liver disease  
527 [92].

528 The presence of oral species such as *Streptococcus salivarius* in our patients is  
529 confirmed by another study showing its abundance in ALD patients with cirrhosis compared  
530 to healthy controls [42]. One cannot completely rule out the possibility that this species could  
531 be transient food-associated microbes.

532 Our results showed also significant abundance of *E. coli* in ALD-NoHCC group that  
533 most of their patients were cirrhotic. This correlates with one study discovered that gut  
534 microbial profiles related to HCC among patients with cirrhosis were featured by the elevated  
535 *E. coli* abundances in the feces [26]. Meanwhile, predicting HCC presence based on fecal *E.*  
536 *coli* counts may be very potential. Consequently, *E. coli* overgrowth in the intestine possibly  
537 facilitates HCC formation. Moreover, the dysbiosis degree related to HCC elevates as cancer  
538 development stage increases. By introducing the more comprehensive integrated index called  
539 the degree of dysbiosis ( $D_{dys}$ ), a previous study found that HCC cases had higher fecal  
540 microbial levels of pro-inflammatory bacteria compared with healthy individuals [27].

541 *Akkermansia* abundance nonsignificantly decreased in our ALD-NoHCC group but  
542 increased in ALD-HCC group compared to the control. This was quite similar to another  
543 study which reported its relative decrease in cirrhotic and HCC patients [29]. Those results  
544 were also supported by reporting *Akkermansia muciniphila* in the control group but not in  
545 ALD group using culturomics approach. *A. muciniphila* is a Gram-negative anaerobic  
546 commensal that uses host-derived mucins as carbon and nitrogen source and promotes barrier  
547 function partly by enhancing mucus production [93]. Recovery of ethanol-induced  
548 *Akkermansia muciniphila* depletion ameliorates ALD [94].

549 Patients with ALD were found to have a decreased alpha microbiome diversity in the  
550 former studies [91,95]. The reduction of microbial diversity was one of the significant types  
551 of gut disease-associated dysbiosis [96]. The prior research proved that individuals with  
552 decreased microbial richness have more tendency to develop low-grade inflammation [97]. In  
553 our study, as noted by others for cirrhosis in general [98–100], there is a decrease in bacterial  
554 diversity in those with ALD-NoHCC and ALD-HCC groups. Likewise, our results, Zheng et  
555 al. discovered a remarkable reduction of fecal microbial alpha-diversity among cirrhotic  
556 patients and those who developed HCC [31]. Besides, beta-diversity was largely different in  
557 patients with cirrhosis, and the HCC group had markedly higher gut microbial diversity  
558 compared with the cirrhotic group [31].

559 Our study reported a high abundance of the *Bacillota* and *Bacteroidota* phyla in  
560 controls, which was in line with the results of another study, which found that the diversity of  
561 *Bacillota* decreased from controls to cirrhosis and HCC patients [28]. However, former  
562 studies reported lower *Bacteroidetes* in individuals with ALD-associated cirrhosis [101,102],  
563 which was inconsistent with our findings. In fact, ethanol treatment in mice was found to  
564 induce a decrease in *Bacillota* and *Bacteroidota* [8]. The different findings of the phyla may  
565 be the result of the difference in observed objects and events.

566 Meanwhile, *Alphaproteobacteria* class was abundant in our HCC group while Zhang  
567 et al. reported an increase in *Gammaproteobacteria* in patients with HCC [28]. At the family  
568 level, *Streptococcaceae*, *Erysipelotrichaceae* and *Odoribacteraceae* were the most abundant  
569 in our ALD-HCC group, while *Enterobacteriaceae* and *Carnobacteriaceae* were most  
570 abundant in ALD-NoHCC. Ponziani et al. showed [29] the *Streptococcus* and  
571 *Enterobacteriaceae* were significantly enriched in fecal microbiota of all patients with  
572 cirrhosis (NAFLD-related) and HCC. However, one study reported an increase in  
573 *Enterococcaceae* in HCC patients [28]. Despite our results reporting a significant abundance  
574 of Ruminococcaceae and Bacteroides in the control group, a previous study showed they  
575 were enriched in HCC patients compared with cirrhotic patients [29].

576 The abundance of butyrate-producing genera and species (*Clostridium*,  
577 *Ruminococcus*) increased our ALD group by culturomics. However, they showed a decrease  
578 in early HCC patients compared with controls by another study [103]. Moreover, one study  
579 identified the optimal 30 microbial markers between non-HCC and early HCC cas; among  
580 them were Bacteroides, Alstipes, Feacalibacterium,acterium and *Ruminococcaceae*.

581 However, those taxa were identified as highly abundant in our control group. This  
582 discrepancy could be attributed to different populations, sample sizes, and methodologies.

583 Interestingly, *Streptococcus* and *Turicibacter* were highly abundant in both the ALD  
584 groups of patients with elevated liver enzymes and HCC groups, denoting their role in the  
585 progression of the disease. These significantly different gut microbial taxa can be used as  
586 potential noninvasive biomarkers for the diagnosis of the progression of ALD.

587 Our results showed that patients with ALD-NoHCC and ALD\_HCC showed a  
588 significant decrease in fecal total cholesterol and TGC compared to the control group.  
589 However, one study reported that dietary cholesterol drives fatty liver-associated liver cancer  
590 by modulating gut microbiota and metabolites in a mouse model [37]. High dietary  
591 cholesterol led to the sequential progression of steatosis, steatohepatitis, fibrosis and  
592 eventually HCC in mice [37]. This finding should be widely investigated in future studies  
593 especially our patients didn't report significant hypercholesteremia. Moreover, fecal ethanol  
594 showed no significant difference among all the groups despite reporting significant  
595 enrichment in ethanol producing bacteria in ALD group by culturomics such as *E. boltea*.  
596 Interestingly, *E. boltea* has been described to produce ethanol known for its damaging effect  
597 on the liver, in our previous study on chronic HBV patients [45].

598 At present, no available treatment can be applied to prevent HCC in addition to the  
599 treatment of underlying disease. However, targeting the gut microbiota for HCC prevention is  
600 a promising tool where several small-scale clinical trials revealed that antibiotic treatments  
601 like rifaximin and norfloxacin can extend the survival time of cirrhotic cases [104,105]. In  
602 addition, some approaches with a high safety profile and low risk of severe adverse effects,  
603 like probiotics, prebiotics, synbiotics, and FMT can be used effectively. Probiotics can  
604 improve gut microbial balance and stimulate bacterial products and metabolites [106]. At  
605 present, probiotics are just studied based on the murine HCC models, and relevant data in  
606 human beings are unavailable [21,36,107,108].

607 Our results suggested that *A. muciniphila*, *Lactobacillus iners*, *Lactobacillus ruminis*,  
608 *Levilactobacillus brevis*, *Limosilactobacillus mucosae*, *Limosilactobacillus reuteri* and  
609 *Propionibacterium freudenreichii* could be used as probiotics in patients with cirrhosis since  
610 they have been isolated from the control group and lost in ALD-NoHCC or ALD-HCC  
611 group. A detailed description of their possible probiotic roles in research or clinical trials are  
612 summarized in **(File Table.S3)**. Surprisingly, among our isolated probiotic species *P.*

613 *freudenreichii* was tested as a probiotic supplement in a clinical trial and was found to reduce  
614 the biologically effective dose of aflatoxin exposure and may thereby offer an effective  
615 dietary approach to decrease the risk of liver cancer [109]. *A. muciniphila* administration  
616 prevented hepatic injury, steatosis, and neutrophil infiltration in a mouse model with ALD  
617 [94]. Interestingly, one study found that probiotics containing *Bifidobacterium* and  
618 *Lactobacillus* species administration alleviated HCC occurrence in mice by restoring gut  
619 homeostasis and mitigating hepatic and intestinal inflammation, thereby preventing cirrhosis  
620 from progressing into HCC [36].

621 Fecal microbiota transplantation is a process that administers fecal matter solution  
622 obtained from a healthy donor to the recipient intestinal tract, to induce the health benefits or  
623 treat a certain disorder directly [110]. Recently, FMT has been shown to have a role in some  
624 types of cancer, such as non-small cell lung cancer, colorectal cancer, and melanoma  
625 [111,112]. In particular, FMT can enhance the efficacy of ICI therapy against these types of  
626 cancer. In addition to enhancing the antitumor effect of ICI, it is a potential strategy for HCC  
627 treatment [112]. Likewise, FMT may treat or prevent HCC. Hence, our results provide  
628 insights into the potential use of those probiotic species in future well-structured large-scale  
629 human trials.

630 In short, the discordance between culturomics and metagenomics results has been a  
631 surprising finding since the very first development of this approach in 2009 with less than  
632 20% of common species [113]. Both approaches have several biases that could explain the  
633 discrepancies and complementarity [114,115]. It is a central point for the culturomics  
634 approach that was developed in our lab ten years ago to consider that, theoretically, all  
635 bacteria could grow [113]. Indeed, culturomics aims to reproduce the natural micro-  
636 environment and is very successful in discovering several hundreds of new bacterial species.  
637 However, several species and strains remain uncultured to date. Moreover, the sample's  
638 preparation could have a considerable impact on culturability, including the mode of  
639 sampling, time of exposure to oxygen, and mode of freezing. For instance, the use of  
640 cryoprotectants could have dramatically improved culturability [116]. In addition, it was  
641 detected that culturability depends on the abundance of the bacterial species in the sample  
642 [117].

643 Regardless of the discrepancy between metagenomics and culturomics [118,119],  
644 culturomics allowed us to extend gut microbiota's known diversity in our patients.

645 Furthermore, culturomics is a validated technique with consistent species identification that  
646 avoids one of the previously mentioned disadvantages of the metagenomic approach,  
647 particularly poor taxonomic resolution [115]. Eventually, no method is ideal, and culturomics  
648 and metagenomics are necessary and complementary for explaining the dysbiosis associated  
649 with ALD and HCC.

## 650 **Conclusions**

651 Although we have gained some insight into the interactions between gut microbial  
652 communities and the host, a comprehensive understanding of the gut microbiota's function  
653 during ALD and HCC is lacking.

654         Characteristics of the ALD and ALD-HCC associated dysbiosis in our study showed  
655 enrichment in potentially pathogenic *T. ramosa*, *M. gnavus* and *G. formicilis*. Unlike earlier  
656 research, our study reported the abundance of *T. ramosa* for the first time in patients with  
657 ALD-HCC by two complementary techniques, such as the use of various diagnostic  
658 techniques (culturomics with different culture conditions), while previous studies utilized the  
659 metagenomic approach solely. Additionally, microbial culturomics allows us to obtain live  
660 bacterial species that could contribute to the treatment and the pathophysiology of HCC.

661         Overall, it would be interesting to compare the shifts in microbiota composition with  
662 detailed data on liver function assessed using additional methods like liver biopsy. Our  
663 findings should be further verified through large-scale studies and animal experiments  
664 especially to investigate the mechanisms by which *T. ramosa* and *G. formicilis* contribute to  
665 carcinogenesis. Microbiome and metabolome analysis in patients with ALD and HCC are  
666 recommended for further studies to understand the correlations with specific biochemical  
667 parameters better. In addition, our study provides insights to develop a specific PCR with a  
668 very high predictive value to detect *T. ramosa* and *G. formicilis* in stool samples for better  
669 and quick diagnosis. Our results should be confirmed in more extensive studies in the future.

## 670 **Study limitations**

671 Our study had some limitations that should be noted. Firstly, the number of patients involved  
672 in culturomics analysis was small; thus, our findings need to be confirmed in a more  
673 extensive cohort. However, the sample size determines the statistical power to identify a  
674 statistically significant difference. A small sample size may lack the ability to detect a slight  
675 difference (small effect size). On the contrary, if a difference is statistically significant with a  
676 small sample size, it means that the difference (size effect) and the strength of the association

677 are essential, and power of association is the first of the Bradford Hill criteria for causality  
678 [120].

679 However, we identified associations between ALD-HCC and the gut microbiome,  
680 providing direct causal evidence by cultivation and isolation of strains such as *T. ramosa*  
681 whose enrichment was confirmed by two different complementary techniques. Those strains  
682 could be further explored in experimental studies; moreover, this evidence could be  
683 supported by animal models that will be beneficial to elucidate the mechanism of gut  
684 microbiota in ALD and HCC. Secondly, we only used 16S rRNA gene sequencing data, and  
685 hence, metagenomics needs to be involved for further investigation and functional analysis.  
686 In our study, the data on alpha-fetoprotein (AFP) was not available for all the patients with  
687 HCC. AFP is the most widely used tumor marker in detecting patients with HCC and has  
688 been proven to have the capability of prefiguring the prognosis [121]. However, to  
689 investigate the relationship between gut microbial diversity and HCC, a correlation analysis  
690 was performed with other available clinical markers of cirrhosis.

#### 691 **Data and materials availability**

692 The raw sequencing data of ALD patients and healthy participants are available in the NCBI  
693 Sequence Read Archive with accession number PRJEB6282. All the cultivated species are  
694 deposited and available in our Collection de Souches de l'Unité des Rickettsies and (CSUR).  
695 The clinical metadata was also available in supporting materials.

#### 696 **Author Contributions**

697 R.M.W. contributed to the investigation, formal analysis, original draft writing, and  
698 visualization. P.B. contributed to the study design, clinical assessment, review, and editing.  
699 B.M. performed culturomics of the control samples. M.T.A. contributed to the review and  
700 editing. A.C. contributed to bioinformatic analysis. C.A. contributed to 16S RNA sequencing.  
701 G.M. contributed to the formal biochemical analysis. D.R. contributed to conceptualization  
702 and project administration. R.G. contributed to the study design, review, and editing. M.M.  
703 contributed to supervision, formal analysis, writing, review and editing, project  
704 administration, and visualization. All authors have read and agreed to the published version  
705 of the manuscript.

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710 **Data Availability Statement**

711 The metagenomic files are available online under bio project number PRJEB62828. All the  
712 cultured species in this study are preserved and available for further investigation. They are  
713 deposited in the Collection de Souches de l’Unité des Rickettsiesand (CSUR) collection  
714 numbers as *Thomasclavelia ramosa* CSUR Q5641. Probiotic species are available and  
715 deposited in our CSUR collection *Propionibacterium freudenreichii* CSUR Q7880 / CSUR  
716 Q7178.

717 **Acknowledgments**

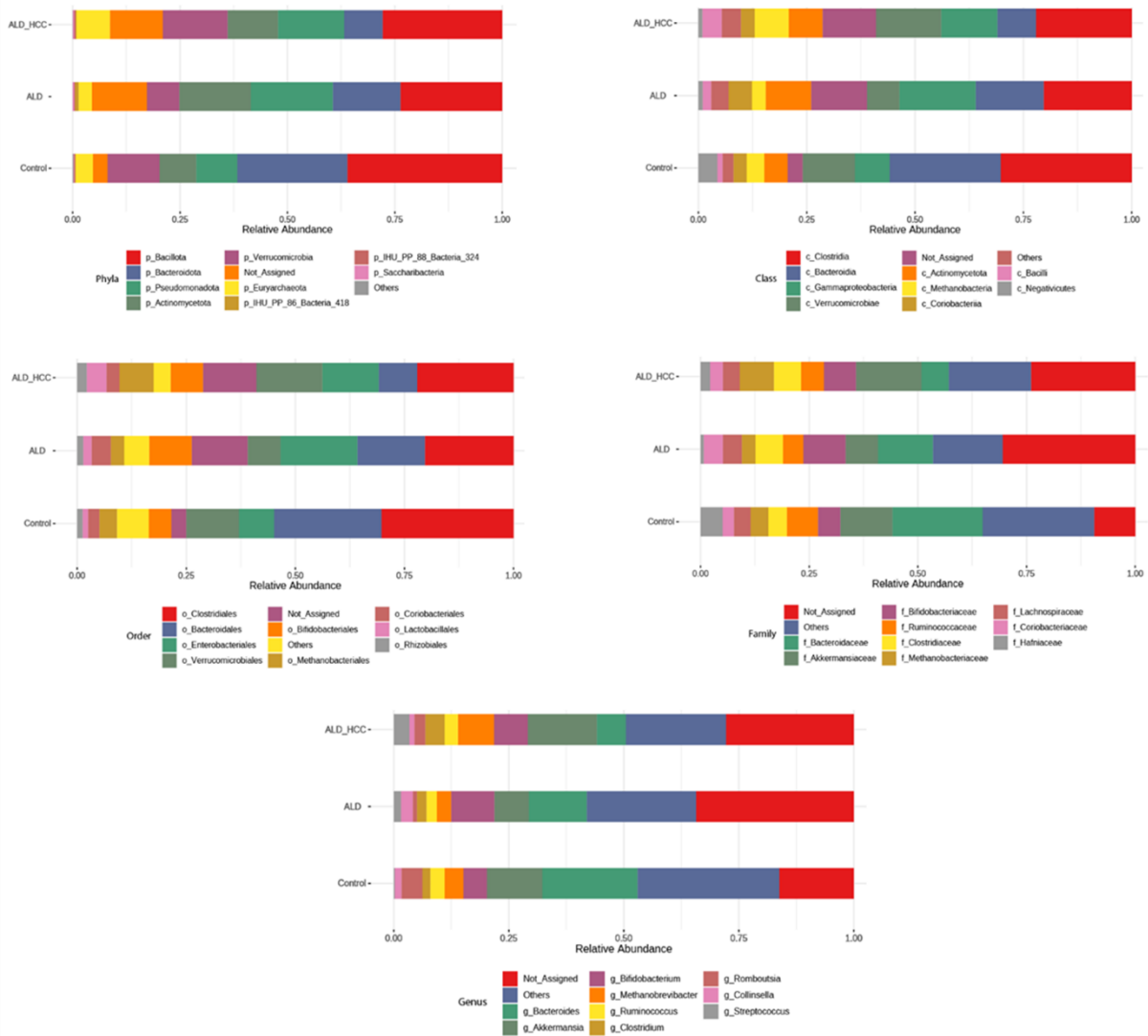
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720 **Conflicts of Interest**

721 The authors declare no conflict of interest.

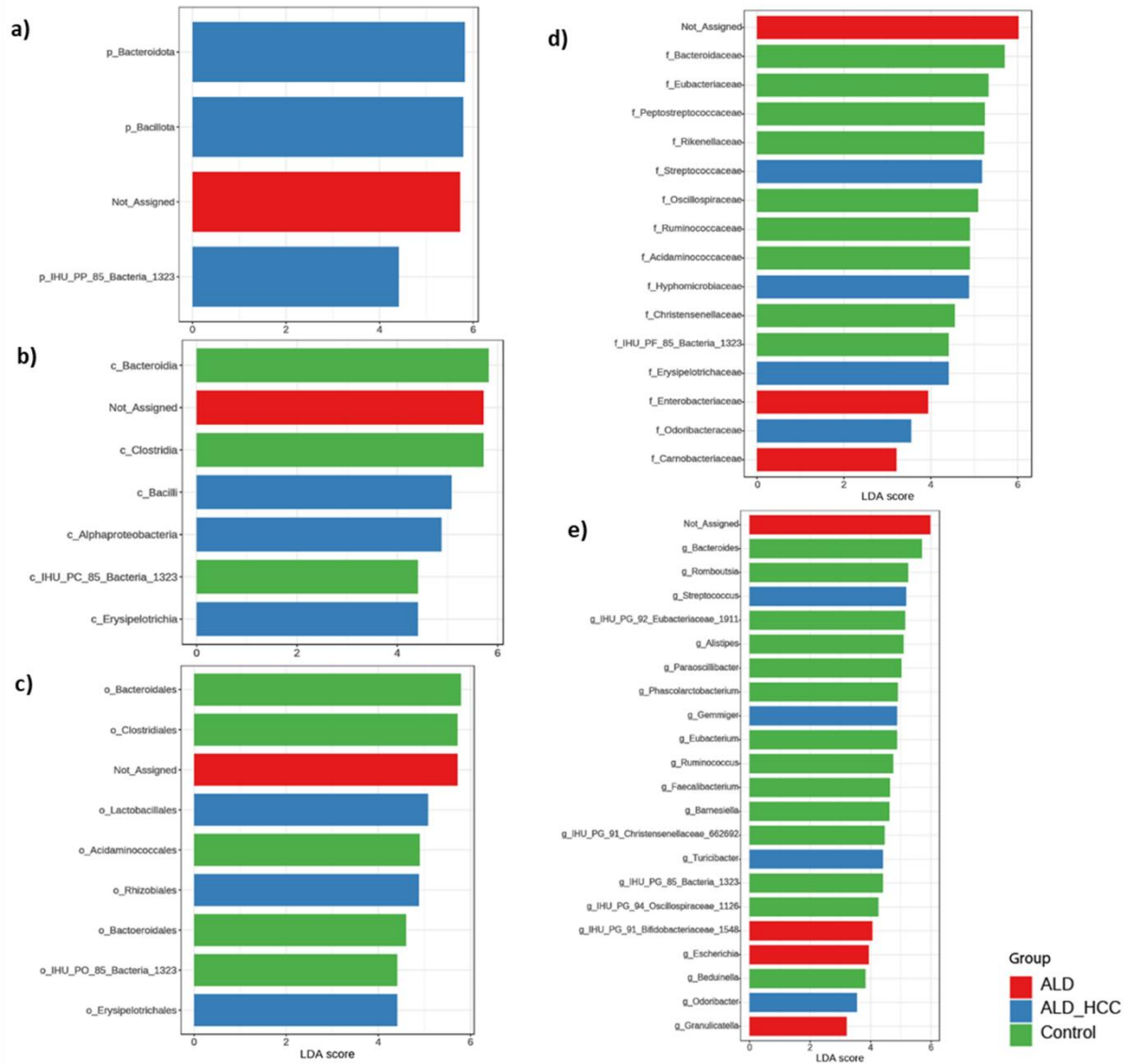


722 **Supplementary figures**



723

724 **Figure. S1** Bar charts with the cumulative relative abundance (%) the legend only contains  
 725 the top ten abundant different bacterial taxa in disease and control groups. ALD: Alcoholic  
 726 liver disease (with no carcinoma); ALD-HCC: ALD with hepatocellular carcinoma.



727

728 **Figure. S2** Histogram of the linear discriminant analysis (LDA) scores for differentially  
729 abundant taxa **(a)** p\_phylum; **(b)** c\_class; **(c)** o\_order; **(d)** f\_family; **(e)** g\_genus. A  
730 logarithmic LDA score > 2 indicated a higher relative abundance in the corresponding group  
731 than in other groups. ALD: Alcoholic liver disease (with no carcinoma); ALD-HCC: ALD  
732 with hepatocellular carcinoma.

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## **CHAPTER III**

### **Collaborative Studies and Annexes**

## **Context 1**

**Systematic review: *Akkermansia muciniphila***

## **Article 5**

### ***Akkermansia muciniphila*: The state of the art, 18 years after its first discovery**

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# *Akkermansia muciniphila*: The state of the art, 18 years after its first discovery

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*Akkermansia muciniphila* (*A. muciniphila*) is an anaerobic, Gram negative and mucin-degrading bacterium of the phylum Verrucomicrobia isolated in 2004 from human feces. Although it is a common resident in the human intestinal tract, it has also been detected in other anatomical sites. Genomic studies have revealed that *A. muciniphila* can be divided into different phylogroups with distinct metabolic properties. There is growing evidence regarding its beneficial impact on human health. Indeed, *A. muciniphila* is considered as a promising next-generation probiotic for treating cancer and metabolic disorders. The large-scale production of *A. muciniphila* is, therefore, a challenge. Beside mucin-based medium, other culture strategies have enabled its isolation. The administration of both live and pasteurized forms of *A. muciniphila* has shown to be promising in animal models. Alternatively, the administration of various prebiotics has also been assessed for enhancing its abundance in the human gut. Future prospects include human clinical trials, some of which are currently ongoing. This paper provides an overview of what is currently known about *A. muciniphila*'s phenotypical and genotypic traits, as well as its culture techniques and its connections to a number of human diseases and its potential application as an effective next generation probiotic.

## KEYWORDS

human health, metabolic diseases, cancer, culture, probiotic, microbiota, *Akkermansia muciniphila*

## Introduction

Within the human microbiome, the gut microbiota has, to date, been the most characterized, and its function and importance in maintaining the balance between human health and pathology has been widely investigated. Alteration of the composition of the gut microbiota has been linked to several diseases, including inflammatory bowel syndrome (1),

type 2 diabetes (2), and cancer (3) as well as eating disorders (4) and psychological disorders (5). Different phyla are reported in the gut, the two phyla Firmicutes and Bacteroidetes represent 90% of gut microbiota. Other reported phyla include Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia (6). *A. muciniphila* is the only species in the Verrucomicrobia phylum that has been reported in the gastro-intestinal tract. Discovered and isolated from the stool of a healthy individual in 2004 by Derrien et al. (7), *A. muciniphila* relies on mucin for carbon, nitrogen and energy. Since then, it has been reported that it constitutes between 1% and 3% of the fecal microbiota and is present in more than 90% of healthy adults tested, but decreases in the elderly (8, 9). The majority of the research studies reporting the presence of *A. muciniphila* presence in the human digestive tract are based on metagenomic analysis, but only few studies have reported its isolation. The capacity of *A. muciniphila* to degrade and use mucin as a unique source of carbon and nitrogen gives it significant importance in the human gastro-intestinal tract, giving the opportunity to other bacteria to survive and grow by using the metabolites resulting from mucin degradation. These metabolites also play a role in the inflammatory status of the host (10). *A. muciniphila* was found to regulate the immune system, improve the gut barrier function and ameliorate metabolism in the case of obesity and diabetes, especially *in vitro* or in mice models (11–13). Furthermore, an association was found between high relative abundance of *A. muciniphila* and a lower incidence of obesity (14). Its abundance is found to decrease in different kind of diseases such as cancer (15–17), type 2 diabetes (18), inflammatory diseases (19) and liver diseases (20). These findings allowed the association between the presence of *A. muciniphila* and the healthy status of human beings, given that its abundance significantly decreases in many diseases. For this reason, it could be used as a marker of certain diseases with differing severity. Due to its beneficial effects on the human body, recent studies have also promoted its use as a probiotic (21, 22). To date, there are three validly published studies that reported the safety of use and the beneficial role of *A. muciniphila* in obese humans as a probiotic (12, 23, 24), while two clinical trials are in progress to evaluate the effects of the use of *A. muciniphila* in obese patients with type 2 diabetes and in hyperglycemic adults (NCT04797442/NCT05114018). The purpose of this review is to summarize what is currently known about *A. muciniphila* in terms of both the phenotypical and the genotypical characteristics, as well as its culture methods. We will also discuss its relationships with many human diseases. And most importantly, we will discuss the already established human trials and those that are still in progress focusing on and its possible use as a promising probiotic.

## The *Akkermansia* genus

Since its discovery by Derrien et al. (7) in 2004, the *Akkermansia* genus, which is a part of the division Verrucomicrobia contains only

two known species: *A. muciniphila* and *A. glycaniphila* (25). However, a recent study analyzing metagenome-assembled genomes of *Akkermansia* suggested the presence of two more putative species (26), while another study cited the presence of eight different species in the genus *Akkermansia* (27). *Akkermansia* spp. are Gram-negative, non-motile, non-spore forming and anaerobic bacteria. Cells are oval shaped with a mean diameter of 0.6–1.0  $\mu\text{m}$  (7).

## Taxonomy

A study analyzed 23 whole genome sequences of the *Akkermansia* genus and revealed that these strains formed four clades, divided into four species based on dDDH values (28), while a more recent study has divided *Akkermansia* strains into five distinct group (29). Moreover, it has been shown that single nucleotide polymorphisms (SNPs) were not evenly distributed throughout the *A. muciniphila* genomes, while genes in regions with high SNPs are found to be related to metabolism and cell wall/membrane envelope biogenesis (28).

When it comes to *A. muciniphila*, many genomic studies have been conducted in order to study its genomic diversity. *A. muciniphila* can be subdivided into three phylogroups, with high nucleotide diversity and distinct metabolic and functional profiles (30). However, a recent study has reported the presence of four different *Akkermansia* phylogroups, based on pangenome analysis (31). Another study analyzed different *A. muciniphila* strains from different phylogroups and revealed that each phylogroup has some specific phenotypes such as oxygen tolerance or sulfur assimilation. These phenotypes can influence the colonization of the gastrointestinal tract (32).

## Metabolic characteristics

This genus uses mucin as its only carbon and nitrogen source, but it has been proven that it can grow in a medium containing glucose, N-acetylglucosamine and N-acetylgalactosamine, when provided with other protein sources (7, 25). The uptake of these sugars can also be enhanced by adding mucin, revealing the role of other mucin-derived components in its growth (33).

*A. muciniphila* has numerous candidate mucinase-encoding genes but surprisingly lacks genes coding for canonical mucus-binding domains (27). This capacity to degrade mucin might be essential to the survival of other bacteria in the human gut, as mucin degradation by *A. muciniphila* provides metabolites that supports the growth of other bacteria such as *Anaerostipes caccae* by changing the transcriptional profile to induce an increase in the expression of mucin degradation genes and a reduction in the expression of ribosomal genes (34). Among the various studies aiming to identify the enzymes involved in mucin

degradation, one has succeeded in identifying a novel phospholipid-regulated  $\beta$ -galactosidase involved in mucin degradation (35). Further work revealed other beta galactosidases involved in the complex mucin degradation machinery (36). *A. muciniphila* can survive without the addition of vitamins to the medium. It was even proven in a recent study that some *A. muciniphila* strains were able to synthesize vitamin B12 (31).

## Resistance to antimicrobial agents

*A. muciniphila* and *A. glycaniphila* strains have been shown to be resistant to ampicillin and vancomycin (7, 25, 37, 38). Specifically, *A. muciniphila* Muc<sup>T</sup> was also found to be resistant to other antibiotics, including metronidazole and penicillin G, but susceptible to doxycycline, imipenem, and piperacillin/tazobactam (38). This antibiotic profile can change from one strain to another. For example, another *A. muciniphila* strain isolated in 2017 was sensitive to penicillin, imipenem, ceftriaxone and amoxicillin but resistant to ofloxacin (37). In 2015, a study aimed at assembling the genome of a strain sequenced directly from a human stool sample detected its presence, by performing an in-silico prediction of eight beta lactamase genes. Moreover, three macrolide resistance genes were detected with only one sharing 65% similarity with a known macrolide gene. Finally, resistance to vancomycin, chloramphenicol, sulfonamide, tetracycline and trimethoprim was associated with only one gene (39).

## *A. muciniphila* distribution within the human body

### Digestive tract

*A. muciniphila* is a common bacterial component of the human intestinal tract (9). A study by Collado et al. found that the presence of *A. muciniphila* presence increases from 16% of the samples of one-month-old infants to 90% at 12 months, while it is present in all the adult samples. Similarly, levels also increase in early life to reach levels similar to that observed in adults within a year. On the other hand, this level decreases significantly in the elderly (8).

Aiming to characterize the whole gut microbiota, Mailhe et al. collected samples from various parts of the digestive tract: the stomach, duodenum, ileum, and the left and right colon and analyzed those samples using culturomics and metagenomics. They succeeded in cultivating *Akkermansia muciniphila* in the left colon. In terms of metagenomic analysis, the Verrucomicrobia phylum, represented by the *Akkermansia*

genus, was detected in the duodenum, ileum, and the right and left colon (40). Moreover, Ye et al., detected *Akkermansia*-like sequences in three out of six duodenal fluid samples (41). Another study exploring the duodenal and rectal microbiota in luminal contents and biopsy tissues in healthy volunteers found Verrucomicrobia sequences in duodenal biopsies, mucus and rectal biopsies (42). The presence of *Akkermansia* sequences was reported in the jejunal fluid, the pancreas and the bile with mean relative abundance of 0.01%, 0.05% and 0.01%, respectively, in a study exploring disturbances in the microbiome in patients undergoing pancreaticoduodenectomy (43). Analysis based on 16S rRNA genes uncovered the presence of *Akkermansia* sequences in ileocecal biopsies of patients with primary sclerosing cholangitis (PSC), ulcerative colitis and in non-inflammatory controls, with no significant differences between the different groups (44). The presence of Verrucomicrobia or *Akkermansia*-like sequences were detected much more frequently in the large intestine (45, 46) (Figure 1).

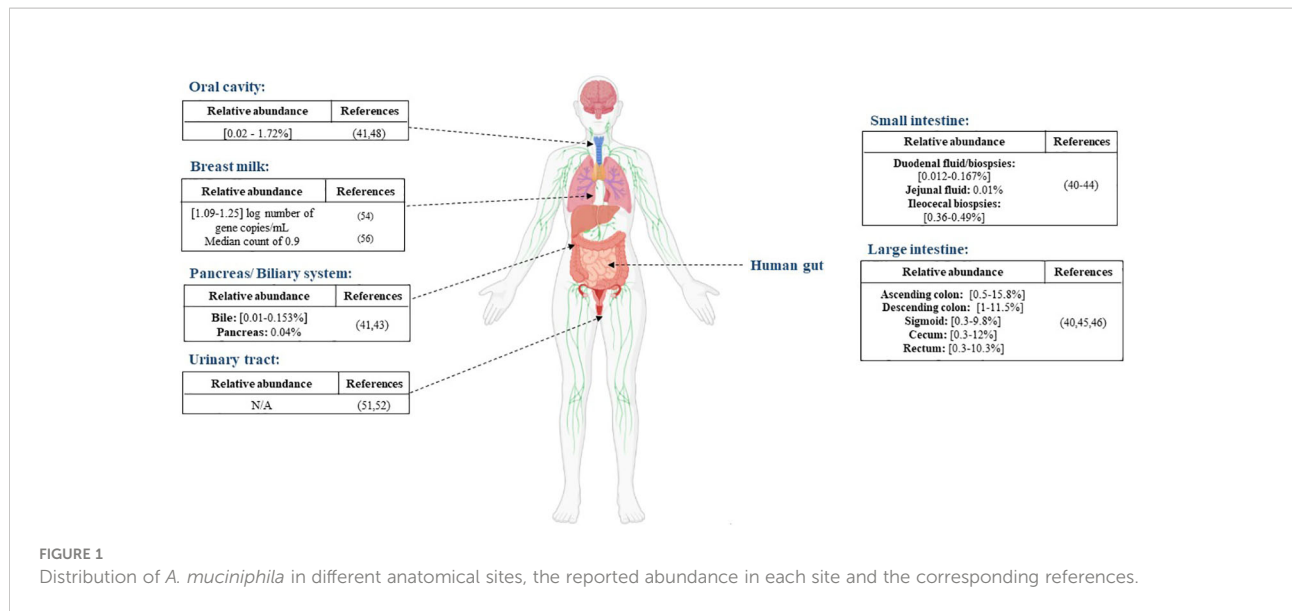
### Oral cavity

There is no significant evidence of the presence of *A. muciniphila* in the oral cavity. Le et al. highlighted an absence of *A. muciniphila* in the oral cavities of 47 pediatric patients after PCR screening (47). A study performed in 2017 by Coretti et al. assessed the subgingival microbiota of smokers and non-smokers with chronic periodontitis compared to a control group. They found that the Verrucomicrobia phylum was significantly lower in people with chronic periodontitis (48). *A. muciniphila* was also detected in the saliva sample of a choledocholithiasis patient. He was the only positive patient out of six and the relative abundance was very low (41) (Figure 1). While its presence is not abundant in the oral cavity, *A. muciniphila* can be a potential therapeutic agent for periodontitis. In an experimental periodontitis mouse model, *A. muciniphila* showed protective effects by decreasing inflammatory cell infiltration and reducing alveolar bone loss (49).

### Urinary tract

Although urine was long considered sterile, some studies have proved the presence of resident microorganisms by using culture and molecular based techniques (50). Few studies have detected the presence of *A. muciniphila* in the urine. Mansour et al. analyzed tissue and urine samples from patients with bladder carcinoma in order to compare the microbiota in both type of samples. Sequencing results showed that the *Akkermansia* genus was present in both type of samples but





was over-represented in the tissue samples compared to the urine samples (51). Another sequencing-based study reported a decrease in the levels of the phylum Verrucomicrobia in urine samples from an elderly Type 2-diabetes mellitus group compared with a control group (52).

## Human breast milk

Human milk contains nutrients providing immunological and other health benefits to new-born babies. Studies on human milk show that it provides a source of commensal microorganisms for the new-born gut (53). As for *A. muciniphila*, its presence in human breast milk was reported for the first time in a study conducted by Collado et al. (54) The study showed that *A. muciniphila* was found in milk samples taken from women shortly after giving birth (colostrum), as well as at one and six months, with mean concentrations of 1.25, 1.09, and 1.20 log number of gene copies/mL, respectively. Moreover, they demonstrated that *A. muciniphila* was more abundant in overweight mothers than in normal weight mothers. Another study in 2014 discovered the presence of *Akkermansia*-like species using 16S rRNA sequencing in human breast tissue samples of 43 women (aged 18 to 90 years) (55). In addition, *A. muciniphila* was also found by qPCR analysis in milk colostrum samples collected from 11 women after an elective caesarean section, with a median count of 0.9 (56). Furthermore, metagenomic analysis of breast milk samples from healthy Korean mothers detected the presence of the *Akkermansia* genus (57). Finally, in a study aiming to evaluate the impact of maternal breast milk composition on children who develop coeliac disease (CD), milk samples were collected from

mothers with a genetic predisposition to CD and a control group. The genus *Akkermansia* was found in milk from mothers in the CD group and the control group but was more abundant in the CD group (58). The presence of *A. muciniphila* in the human breast milk might be due to its ability to use human milk oligosaccharides (59). Although it is important to note that this ability is strain dependent (60).

## *A. muciniphila* culture methods

The *Akkermansia* genus was isolated for the first time from a human stool sample, with *A. muciniphila* being the type species using a basal medium supplemented with 0.25% gastric mucin and 0.7% rumen fluid. The human stool was serially diluted into sterile anaerobic Ringer's solution containing 0.5 g cysteine. Each dilution was inoculated in the medium as described previously. Pure colonies were isolated using the same medium containing 0.75% agar. Since then, other studies have used the same medium in order to isolate other *Akkermansia* strains (61). Twelve years later, the same medium enabled the cultivation, from reticulated 193 python faeces (25) of *A. glycaniphila*, which depends on mucin as its only energy source for carbon and nitrogen. In a recent study focusing on distinguishing the fast and slow growing bacteria of the faecal microbiota by changing the dilution rates in mucin-supplemented media, *A. muciniphila* was isolated in low dilution rates (62). It was also suggested that the growth of *A. muciniphila* is promoted in a media rich in sugar and mucin (63). To understand how *A. muciniphila* adapts to mucin, transcriptomic and metabolomic analysis showed an upregulation of genes related to energy metabolism and cell

growth in the presence of 0.5% of mucin, correlated with smaller diameter of the cells, a sign of bacterial division, and proliferation. Moreover, enzymes such as fucosidase, beta-galactosidase and hexosaminidase were also overexpressed to degrade mucins into oligosaccharides and eventually monosaccharides to use them as a source of energy (64). Another study comparing the growth of *A. muciniphila* in static and dynamic culture simulating the physiological conditions in the colon showed that the biomass of *A. muciniphila* in dynamic culture was significantly higher after 48 hours compared to under static conditions. The same study tested the growth of *A. muciniphila* in five different culture conditions: human mucin, porcine mucin, brain heart infusion (BHI) medium only, or BHI supplemented with porcine mucin or human mucin. *A. muciniphila* can grow in all the media tested, but the lowest biomass was found in BHI only, and human mucin is the most ideal for the cell growth (65).

However, some studies have proved that *A. muciniphila* can be isolated without mucin-based media culture, such as from a blood culture sample after 72 hours of subculture on Columbia agar with 5% sheep blood (37). Similarly, another strain was isolated from a stool sample after diluting it in pre-reduced phosphate-buffered saline (PBS), plating on Columbia blood agar supplemented with 5% horse blood, and subjected to two to four days of incubation at 37°C under an H<sub>2</sub>-CO<sub>2</sub>-N<sub>2</sub> (1:1:8 [vol/vol/vol]) gas mixture (66). Finally, culturomics techniques enabled the isolation of *A. muciniphila* from fresh stool samples using the following anaerobic culture conditions at 37 °C: culture bottle containing 5% sheep blood and 5% rumen fluid, YCFA medium, YCFA solid medium, reinforced clostridiales solid medium, brain heart infusion (BHI) solid medium, Columbia solid medium and, finally, De Man, Rogosa and Sharpe (MRS) solid medium (67, 68) (Table 1).

The growth of *A. muciniphila* has been proven to be pH dependent. The optimum pH was 6.5. Low pH strongly inhibits its growth, explaining its abundance in the distal colon in comparison to the proximal colon (69, 70). *A. muciniphila* also showed high tolerance to oxygen (up to 72 hours) (71). When oxygen is present at nanomolar concentrations, its growth rate and yield were increased compared to those observed in strict anaerobic conditions. This is due to the presence of cytochrome bd complex that can function as a terminal oxidase (72). *A. muciniphila* showed high tolerance to different temperatures (4°C, 22°C, and 37°C). In contrast, cell viability showed significant decrease at 44°C. In this study, its stability and tolerance to the different gastrointestinal conditions were evaluated. Interestingly, *A. muciniphila* showed stability after exposure to simulated gastrointestinal conditions. Other evaluations might be needed in order to understand the effect of stress on the metabolism and the adhesion properties of the bacterium (71).

Other studies have tested different growth conditions for *A. muciniphila*. For example, as mentioned before, a study proved that *A. muciniphila* is also able to grow on human milk *in vitro* and degrade its oligosaccharides, which is explained by proteomic analysis showing an increase in the expression of glycan degrading enzymes such as  $\alpha$ -L-fucosidases,  $\beta$ -galactosidases, exo- $\alpha$ -sialidases and  $\beta$ -acetylhexosaminidases (59). *A. muciniphila* does not code for the enzyme that mediates the conversion of fructose-6-phosphate (Fru6P) to glucosamine-6-phosphate (GlcN6P), which is essential in peptidoglycan formation. This finding suggests that N-acetylglucosamine found in mucin is crucial for the growth of *A. muciniphila*, thus explaining its importance and the adaptation of *A. muciniphila* to its components (73). In contrast, bile salts were found to impede the growth of

TABLE 1 *A. muciniphila* culture methods.

Strain	Sample	Medium used/Culture conditions	Authors	Year
<i>A. muciniphila</i>	Stool sample	0.4 g KH <sub>2</sub> PO <sub>4</sub> ; 0.53 g Na <sub>2</sub> HPO <sub>4</sub> ; 0.3 g NaCl; 4 g NaHCO <sub>3</sub> ; 0.3 g NH <sub>4</sub> Cl; 0.25 g Na <sub>2</sub> S <sub>7</sub> -9H <sub>2</sub> O; 0.1 g MgCl <sub>2</sub> .6H <sub>2</sub> O; 0.11 g CaCl <sub>2</sub> ; 1 ml alkaline trace element solution; 1 ml acid trace element solution; 0.5 mg resazurin and 1 ml vitamin solution 0.25% gastric mucin and 0.7% rumen fluid.	Derrien et al. (7)	2004
	Blood culture sample	Incubation of blood culture sample for 96 hours Colonies isolated after 72 hours of subculture on Columbia agar with 5% sheep blood	Dubourg et al. (37)	2017
	Fecal sample	Mucin-supplemented media, low dilution rates	Adamberg et al. (62)	2018
	Intestinal microbiota samples	Bacterial growth media (containing sugars, nitrogen, vitamins, minerals, hematin, amino acids and mucin)	Yousi et al. (63)	2019
	Stool samples	Culturomics in anaerobic conditions with the following media: Culture bottle with 5% sheep blood and 5% rumen fluid/YCFA liquid medium and solid/Reinforced clostridiales solid/Brain heart infusion solid/Columbia solid/De Man, Rogosa and Sharpe solid at 37°C	Lagier et al. (67) Diakite et al. (68)	2016/ 2020
	Stool sample	Columbia blood agar supplemented with 5% horse blood and two to four days of incubation at 37 °C under a H <sub>2</sub> -CO <sub>2</sub> -N <sub>2</sub> (1:1:8 [vol/vol/vol]) gas mixture	Ogata et al. (66)	2020

Table resuming the different studies that have succeeded in cultivating *A. muciniphila*, the year of publication, the origin of the sample and the media and culture conditions used in each study.

*A. muciniphila*, except for sodium deoxycholate which increased its growth (74).

## A. muciniphila and health

### Cancer

The association between cancer and changes in the gut microbiota in humans has been widely investigated. More specifically, the role of *A. muciniphila* in different types of cancer has been assessed (75). One study highlighted the decrease in the abundance of faecal *A. muciniphila* among non-small-cell lung cancer patients compared to controls (15), through metagenomic and metabolomic profiling, while an increase was detected using real time PCR on gut mucosal tissues samples of colorectal cancer patients compared to controls (16). Similarly, an abundance of *A. muciniphila* along with other bacteria was significantly increased in patients with different gastrointestinal cancer such as esophageal, gastric and colorectal cancer, compared to the control group (17) (Table 2).

A study performed on pancreatic cancer xenograft mice model showed an increase in *A. muciniphila* in the guts of mice receiving Gemcitabine treatment, as well as a decrease in tumour volume (92). In a prostate cancer mice model, the relative abundance of *A. muciniphila* in the gut was decreased. However, this decrease was reversed after receiving androgen deprivation therapy (93).

In other studies concentrating on the role of gut microbiota in the response to anti-PD1 (Programmed cell Death protein 1) immunotherapy, the presence of species such as *Bifidobacterium breve*, *Bifidobacterium longum*, *Faecalibacterium prausnitzii* and, most importantly, *A. muciniphila* in the gastro-intestinal tract of cancer patients was associated with a stronger immune response to the therapy and subsequently an extended survival of these patients (94). In another study based on anti-PD1 therapy for non-small cell lung cancer (NSCLC), two genera, *Akkermansia* and *Olsenella*, were significantly higher in the stable disease group than in the progressive disease group (95). Similarly, gastric cancer patients showed an enrichment for the genus *Akkermansia* before and after radical distal gastrectomy (96).

In epithelial tumours, metagenomic analysis of stool samples from patients receiving immune checkpoint inhibitors showed correlations between clinical responses to the treatment and the relative abundance of *A. muciniphila* (97). The same team also found an increase in *A. muciniphila* levels in patients responding favourably to immune checkpoint blockade treatment in a cohort of renal cell carcinoma patients (98).

In a study of anti-colon cancer therapy based on treatment with FOLFOX, it was demonstrated that the abundance of *A. muciniphila* significantly increased in patients receiving the

treatment, which was positively correlated with the therapeutic effect (99).

In terms of colorectal cancer (CRC), it has been demonstrated that CRC tissues increase the expression of mucin2 compared to normal mucosa (100).

Finally, in a randomized trial evaluating the impact of probiotic supplementation on the outcome of gut microbiome and metastatic renal cell carcinoma (mRCC), patients who had received a treatment and had been supplemented with probiotics present a higher abundance of *A. muciniphila* in the gut (101). Furthermore, there was a positive and significant association between the presence of *A. muciniphila* and the clinical benefit of the treatment (101).

### Metabolic diseases

The abundance of *A. muciniphila* is decreased in many metabolic disorders, such as inflammatory bowel diseases, appendicitis and obesity (76, 77, 79) suggesting its association with healthy intestine and normal mucosa. Eating disorders, such as binge eating disorder (78) (Table 2) have also been associated with a decrease in the levels of *A. muciniphila*.

These findings reveal the importance of *A. muciniphila* as a biomarker of health status (102). Many studies targeted treating metabolic diseases have focused on tracking the levels of *A. muciniphila* to assess the success of the therapy (103).

### Liver diseases

Liver diseases are associated with changes in the gut microbiota, specifically a decrease in the levels of *A. muciniphila*. Grander et al. suggested that the decrease in levels of *A. muciniphila* in alcoholic liver disease is indirectly correlated with disease severity (20) (Table 2). In contrast, other studies have highlighted an increase in *A. muciniphila* after treatment. For example, in non-alcoholic liver disease mice models, it was reported that treatment with Bilberry anthocyanins increases the levels of *A. muciniphila* in the digestive tract, associated with the efficacy of the treatment on NAFLD (104). Similarly, another study using an alcoholic liver disease mice model showed that treatment with berberine also cause an increase in the levels of *A. muciniphila* (105).

### Obesity

*A. muciniphila* levels are negatively correlated with obesity. Studies have shown that the abundance of *A. muciniphila* decreases significantly in overweight/obese preschool children (81), and in obese adult women (82) compared to the normal weight/lean group. Moreover, its abundance is even lower in severe obesity (80) (Table 2). The presence of *A. muciniphila* is also associated with the normal weight gain in pregnant women (106). The beneficial effects of *A. muciniphila* can also be

TABLE 2 Association between *A. muciniphila* and different clinical diseases.

Type of diseases	Pathology	Samples	Cohort	Technique	Abundance of <i>A. muciniphila</i>	Other findings	References
Metabolic disorders	Acute appendicitis	appendices, cecal biopsies and faecal samples	70 patients with appendicitis/400 controls	rRNA-based FISH	↓	<i>A. muciniphila</i> is inversely related to the severity of the disease.	(76)
	Inflammatory bowel disease (IBD)	Biopsies	46 IBD/20 controls	Real-time PCR	↓	x	(77)
	Binge eating disorder (BED)	Stool samples	101 obese patients with/without BED	Sequencing and subsequent bioinformatics	↓	x	(78)
	Ulcerative colitis (UC)	Colonic biopsies and mucus brushings	20 patients with active UC/14 with quiescent UC/20 healthy controls	Real-time PCR	↓	Inverse relationship between <i>A. muciniphila</i> and inflammation	(19)
	Alcoholic liver disease (ALD)	Fecal samples	21 patients with ALD/16 non-obese healthy controls	Quantitative PCR	↓	Decrease of faecal <i>A. muciniphila</i> indirectly correlated with hepatic disease severity	(20)
	Obesity	Fecal samples	164 participants with variable geographical origin, diet, age, and gender	Metagenomics	↓	Fecal salinity was associated with obesity and a depletion in anti-obesity <i>A. muciniphila</i>	(79)
			21 adult women with severe or moderate obesity	Metagenomics/ Quantitative PCR	↓	Significant lower <i>A. muciniphila</i> abundance in severe obesity than in moderate obesity	(80)
			20 overweight children/20 control children	Quantitative PCR	↓	x	(81)
			17 lean/15 obese females		↓	x	(82)
			134 Danish adults with prediabetes/134 controls	Sequencing	↓	x	(83)
Type 2 diabetes (T2D)	Fecal samples	182 lean/obese individuals with T2D	Metagenomic/ Metabolomics	↓	Significant decrease of <i>A. muciniphila</i> abundance in lean individuals with T2D than without T2D, but not in the comparison of obese individuals with and without T2D.	(18)	
		345 patients with T2D/nondiabetic controls	Sequencing	↑	x	(84)	
		70 female T2DM patients/70 healthy females		↓	Decreased Akkermansia muciniphila was associated with high Fasting blood glucose and urine glucose	(85)	
		50 CDI patients/50 healthy controls	Real-time Quantitative PCR	↑	x	(86)	
		11 NSCLC patients/8 controls	Metagenomics/ Metabolomics	↓	x	(15)	
Cancer	Non-small cell lung cancer (NSCLC)	Stool samples	18 CRC patients/18 non-CRC controls	Quantitative PCR	↑	x	(16)
	colorectal cancer (CRC)	gut mucosal tissues	130 gastrointestinal cancer patients/147 healthy controls	16S rRNA sequencing	↑	x	(17)
	Gastrointestinal cancer	Stool samples					

(Continued)

TABLE 2 Continued

Type of diseases	Pathology	Samples	Cohort	Technique	Abundance of <i>A. muciniphila</i>	Other findings	References
Other diseases	Allergic asthma	stool samples	92 children (between 3 and 8) with asthma/88 healthy children	Quantitative PCR	↓	x	(87)
	Atopic dermatitis (AD)/ Food allergy	Fecal samples	82 children with AD with absence and presence of food allergy	16S rRNA microbial analysis	↑	Fecal microbiome of children with AD and food allergy harbored relatively more <i>A. muciniphila</i> than children with AD without food allergy	(88)
	Psoriasis	Fecal samples	14 psoriasis patients/14 healthy controls	16S rDNA sequencing	↓	x	(89)
	CaOx dihydrate (COD) and monohydrate (COM) lithiasis	Fecal samples	24 patients diagnosed with CaOx lithiasis	Real-time PCR	↓	x	(90)
	Autism spectrum disorder (ASD)	Fecal samples	23 children with ASD/22 typically developing siblings/9 unrelated community controls	Real-time Quantitative PCR	↓	x	(91)

Table resuming the different studies that associated *A. muciniphila* with different diseases, the cohort, type of sample, and technique used in each study, as well as the change in the abundance of *A. muciniphila* and the references.

↑: Increase in abundance, ↓: Decrease in abundance.

observed in obese adults after a six-week calorie restriction period followed by a six-week weight stabilization diet. The adults included in this study had a healthier metabolic status when the abundance of *A. muciniphila* was high. Moreover, *A. muciniphila* was associated with other microbial species related to health (107).

However, another study on obese patients undergoing bariatric surgery, gastric banding or the Roux-en-Y gastric bypass procedure showed that the relative abundance of *A. muciniphila* was inversely correlated with the severity of obesity but was not associated with glucose homeostasis markers. Furthermore, a significant increase in the relative abundance of *A. muciniphila* was observed after the Roux-en-Y gastric bypass procedure but was not correlated with metabolic improvement (80).

When it comes to the mechanism of *A. muciniphila* in controlling obesity, evidence have shown that *A. muciniphila* stimulates glucagon-like peptide-1 (GLP-1) production by intestinal cells, leading overall to an improvement in insulin sensitivity, glucose tolerance and suppressing appetite (108).

## Diabetes

In relation to diabetes, some studies have provided evidence revealing the association between *A. muciniphila* and the metabolism of glucose and its dysregulation. Allin et al. showed that abundance of *A. muciniphila* is decreased in individuals with prediabetes (83). One study showed that in lean individuals with T2D, the levels of *A. muciniphila* are lower

compared to the control group, which is not the case with obese T2D patients (18). Another study also showed a decrease in *A. muciniphila* in T2D patients, associated with higher fasting blood glucose and urine glucose (85). However, one metagenomic study on a Chinese population found that some of the genes in *A. muciniphila* were enriched in type 2 diabetic subjects, perhaps due to differences in genes and lifestyle (84) (Table 2). In type 1 diabetes (T1D), NGS analysis of stool samples from T1D patients receiving probiotics showed an elevation of *Bifidobacterium animalis*, *A. muciniphila* and *Lactobacillus salivarius* associated with reduced fasting blood glucose levels and improvement of glycosylated hemoglobin levels (109). Plovier et al. recently highlighted the effect of pasteurized *A. muciniphila* to diminish fat mass development, insulin resistance, and dyslipidemia in mice. They also demonstrated that the outer membrane protein Amuc 1100 is involved in the bacterial-to-host contact through Toll-like receptor 2 signaling. Moreover, this protein partially mimics the effects of *A. muciniphila* on insulin resistance and gut barrier modification (12).

## Inflammatory bowel diseases

Earley et al. quantified *A. muciniphila* in colonic biopsies and mucous swabs from patients with active ulcerative colitis and quiescent ulcerative colitis. They demonstrated that patients with active ulcerative colitis had a reduced abundance of *A. muciniphila* compared to quiescent ulcerative colitis and controls (19). Studies focusing on inflammatory bowel disease have shown that mucolytic bacteria levels increase in IBD

patients. However levels of *A. muciniphila* reduce, mainly due to the potential anti-inflammatory role of *A. muciniphila* (77). Another observational study has suggested that the relative abundance of *A. muciniphila* is inversely correlated to pain reduction in a cohort of IBS patients (110).

## Other diseases

The depletion of *A. muciniphila* has also been associated with several allergic disorders, suggesting a potential educational role toward immunity. For example, decreased levels of *A. muciniphila* and *Faecalibacterium prausnitzii* in stool samples of patients with allergic asthma have been reported (87). In children with atopic dermatitis (AD), the presence of a microbial signature made it possible to differentiate between the presence and absence of food allergies. The fecal microbiome of children with AD and food allergies contains relatively less *B. breve*, *B. adolescentis*, *F. prausnitzii*, and *A. muciniphila* and more *E. coli* and *B. pseudocatenuatum* than children with AD without food allergies (88). Tan et al. also reported a decrease in the abundance of *A. muciniphila* in patients with psoriasis (89). In a study comparing the intestinal dysbiosis between CaOx dihydrate (COD) and monohydrate (COM) lithiasis, a large decrease in the mean values of the mucin-degrading *A. muciniphila* was observed, which is significantly more intense in COD than in COM lithiasis (90). Vakili et al. highlighted an increase in levels of *A. muciniphila* in patients with clostridium difficile infection (CDI) (86).

A decrease in *A. muciniphila* levels is also associated with many psychological disorders. For example, a study in children with autism showed a decrease in levels of *A. muciniphila* and *Bifidobacteria* species when compared with unaffected children (91). Another study showed that the abundance of *A. muciniphila* is reduced in ulcerative colitis patients suffering from depression, revealing a potential connection between psychological disorders and gut bacteria via the gut-brain axis (111) (Table 2). Finally, the protein Amuc\_1100 was shown to have an antidepressant role in a chronic unpredictable mild stress (CUMS) mice model by down-regulating the brain-derived neurotrophic factor (BDNF) and inflammation in the hippocampus (112).

## *A. muciniphila*: A new probiotic?

### The development of *A. muciniphila* for clinical use

The consumption of certain beneficial microbes, known as probiotics, has been known to affect the gut microbiota. This is because the consumption of these organisms can trigger a variety of health benefits for the host (113). It has been noted

that most of the probiotics sold on the market are microorganisms from the *Bifidobacterium* and *Lactobacillus* genera (114). They are safe to use and approved by the United States Food and Drug Administration (FDA) (115). Recently, however, new microbes identified by next generation sequencing methods are emerging and are also associated with health promotion. The safety of these microbes, called next generation probiotics (NGPs), as well as their formulation and administration are currently being processed (115). *A. muciniphila* has emerged as a potential NGPs due to its various benefits on health (116). For this purpose, an efficient and scalable workflow has been developed for the cultivation and preservation of *A. muciniphila* cells. This study resulted in viable *Akkermansia* colonies with high yields and stability, with a survival up to  $97.9 \pm 4.5\%$  for one year if stored in glycerol-amended medium at  $-80^{\circ}\text{C}$  (117) (Figure 2).

In recent years, there has been a lot of focus on the use of nonviable bacterial supplements (pasteurized forms) known as paraprobiotics (118) (Figure 2) as an alternative to live bacteria to lower the risk of infection. For example Druart et al. demonstrated that pasteurized *A. muciniphila* is safe to use as a food ingredient based on rat models (119). The safety of *A. muciniphila* products has also been recently reported in humans (12, 107). The pasteurized form is achieved when the bacteria suspension was heated at  $70^{\circ}\text{C}$  for 30 minutes, as described by Plovier et al. (12) By comparing the effects of live and pasteurized *A. muciniphila* on normal diet-fed mice, Ashrafiyan et al. showed that both forms of *A. muciniphila* could modulate lipid and immune homeostasis and improved health by modulating gut microbiota, while all these effects were dominantly observed in the pasteurized form (120). Another study conducted by Grajeda-Iglesias et al. demonstrated that pasteurized *A. muciniphila* was more efficient than the live version in elevating the intestinal concentrations of polyamines, short-chain fatty acids, 2-hydroxybutyrate, as well multiple bile acids. All these metabolites have been described to be associated with human health (121). Recent studies also started focusing on postbiotics, which refers to using inactivated cell components to promote health (122). In the case of *A. muciniphila*, many studies started focusing on the potential use of its extracellular vesicles (EVs) as postbiotics (Figure 2). For example, a study by Ghaderi et al. showed that live and pasteurized forms of *A. muciniphila* and its EVs can affect the expression of the endocannabinoid system and peroxisome proliferator-activated receptors (PPARs) genes involved in metabolic pathways, suggesting the potential possibility to use them as probiotic, paraprobiotic and postbiotic respectively in order to prevent metabolic diseases (123). Furthermore, *in vitro* study showed that treatment with *A. muciniphila* or its EVs could influence the expression of genes involved in the serotonin system and thus can be used as a serotonin modulation therapy (124).

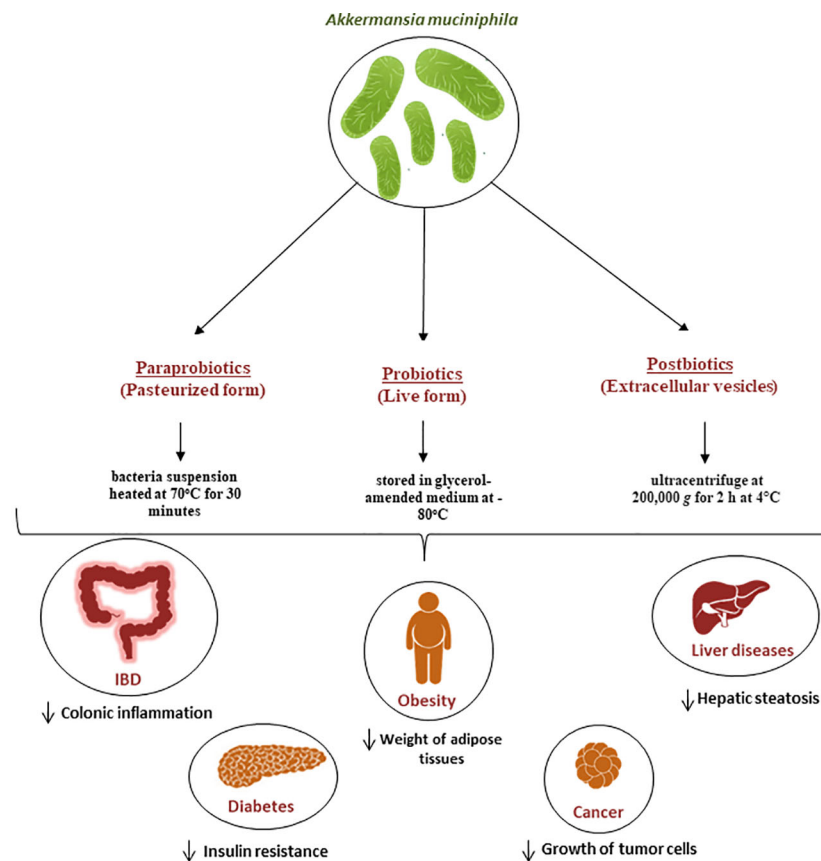


FIGURE 2

Role of *A. muciniphila* as a next generation probiotic for various metabolic diseases. Since its isolation in 2004, It has been demonstrated that *A. muciniphila* is crucial for the immune and metabolic systems regulation. It is now regarded as a "next-generation probiotic" for cancer and metabolic diseases including diabetes, liver diseases and obesity. Additionally, the pasteurized form known as paraprobiotic and the extracellular vesicles known as postbiotics are currently being used for the same diseases and have proven to be efficient in the treatment of these diseases.

## Mice models

Many studies have focused on the causal link between *A. muciniphila* and improvements in metabolism (Figure 2). It has been shown that daily oral supplementation with live *A. muciniphila* at the onset of obesity, diabetes and gut barrier dysfunction in mice at the dose of  $2.10^8$  bacterial cells per day improves glucose tolerance, reduces adiposity and inflammation, therefore partly protecting against diet-induced obesity in mice (108, 125). In addition, animals receiving live *A. muciniphila* no longer exhibited insulin resistance, nor infiltration of inflammatory cells (CD11c) in the adipose tissue, which is a key characteristic of obesity and associated low-grade inflammation (108). In addition, it was noted that live *A. muciniphila* prevented the development of metabolic endotoxemia as an effect associated with the restoration of a normal mucus layer thickness (108). It is worth mentioning that all these findings have subsequently been confirmed by different groups and extended to other specific

disorders such as atherosclerosis, hepatic inflammation and hypercholesterolemia (20, 126, 127). Furthermore, the administration of pasteurized *A. muciniphila* was correlated with an increase in energy expenditure in diet-induced obese mice, possibly explaining the mechanism by which administration of *A. muciniphila* can reduce body weight and fat mass gain (128).

The role and administration of *A. muciniphila* have been notably investigated in cancer. For instance, a study using prostate cancer mice model showed that the extracellular vesicles of *A. muciniphila* can be used as an immunotherapeutic agent for prostate cancer treatment, demonstrated by the decrease in tumors and the upregulation of immune cells such as tumor-killing M1 macrophages after injection of these vesicles in cancer bearing mice (129). Furthermore, the effect of the mucin degrading enzyme of *A. muciniphila* Amuc\_1434\* (130) was investigated in the inhibition of the proliferation of CRC tissues. The study has showed that the mucin degrading enzyme Amuc\_1434\* was able to inhibit the proliferation of CRC cells lines by mediating apoptosis *via* the

TRAIL pathway (131). However, Wang et al. suggested that treatment of CRC-mice with *A. muciniphila* increases the early level of inflammation and proliferation of the intestinal cells and therefore promotes the formation of tumors (132).

In liver diseases, recent studies have investigated the potential anti-fibrotic effects of heat-killed *A. muciniphila* Muc<sup>T</sup> on the activation of hepatic stellate cell (HSC), where they demonstrated that heat-killed *A. muciniphila* Muc<sup>T</sup> was safe and capable of improving LPS-induced HSC activation by modulating fibrosis markers (133). Moreover, oral supplementation in alcoholic steatohepatitis mice model induced a reduction in hepatic injury and steatosis, while enhancing mucus thickness and tight-junction expression (20). In an induced liver fibrosis mice model, it was revealed that treatment with live or pasteurized *A. muciniphila* or with its extracellular vesicles (EVs) can improve gut permeability, attenuating the expression of inflammatory biomarkers and subsequently preventing liver injury in treated mice (134). Another study showed that oral administration of *A. muciniphila* or its EVs could improve the anti-inflammatory responses eventually leading to a prevention from liver injury in mice (135). A recent study conducted by Rao et al. in mice explored the therapeutic effect of *A. muciniphila* in metabolic dysfunction-associated fatty liver disease (MAFLD). The study results indicated that *A. muciniphila* exhibited anti-MAFLD activity correlated with lipid oxidation and an improvement in gut-liver interactions by regulating the metabolism of L-aspartate (136).

The importance of *A. muciniphila* in maintaining good health and the negative correlation between its presence and obesity (81, 107) have initiated many studies focusing on using the mucin-degrading bacteria as a treatment. For example, studies performed on high-fat diet-fed mice models treated with *A. muciniphila* showed that this treatment prevents body weight gain, calorie intake and reduces the weight of adipose tissues, thus improving the induced metabolic disorders. In addition, it had many other beneficial effects such as improving glucose homeostasis and insulin sensitivity, inhibition of intestinal inflammation and restoration of damaged gut integrity (108, 137, 138). Administration of the pasteurized form had similar effects. A study by Ashrafiyan et al. showed that the pasteurized *A. muciniphila* and its EVs totally reduced the High-fat diet (HFD) induced intestinal inflammation and preserved intestinal permeability (139). Pasteurized *A. muciniphila* was shown to attenuate inflammatory response and improve intestinal barrier integrity. This is probably due to stimulating AMP-activated protein kinase (AMPK) and inhibiting Nuclear Factor-Kappa B (NF- $\kappa$ B) activation through the stimulation of TLR2 on intestinal epithelial cells (140). Aiming to understand the mechanisms of *A. muciniphila* involved in modulating the host metabolism, Yoon et al. identified a protein named P9 which induces glucagon-like peptide-1 (GLP-1) secretion and brown adipose tissue thermogenesis (141). Ashrafiyan et al.

demonstrated that *A. muciniphila* or its EVs significantly reduced the body and fat weight of HFD mice and improved intestinal barrier integrity and energy balance (142).

Another study has been conducted to prove that deficiency in *A. muciniphila* is correlated with a high incidence of diabetes in a NOD mouse model. This study showed that the oral transfer of *A. muciniphila* could delay the onset of diabetes through promoting mucus production, increasing the expression of antimicrobial peptide Reg3 $\gamma$ , lowering serum endotoxin levels and the expression of islet toll-like receptor (143). Chellakot et al. found that the administration of *A. muciniphila* EVs improved intestinal tight junction function, glucose tolerance in high-fat diet-induced diabetic mice and reduced weight gain, indicating a potential role for EVs in diabetes and thus indicating its use as a therapy (144).

The therapeutic role of *A. muciniphila* has also been studied in inflammatory bowel diseases. It has been found that treatment of ulcerative colitis dextran sulfate sodium (DSS)-induced mice with metformin alleviates the phenotype associated with an increase in the expression of mucin2 and in the abundance of *A. muciniphila* compared to the control group. Moreover, the administration of *A. muciniphila* decreases disruption of the mucus barrier and colonic inflammation (145). Similarly, another study conducted on a colitis DSS-induced mice model showed that the oral application of EVs protects against colitis phenotypes, such as body weight loss and inflammatory cell infiltration of the colon wall (146). Similar effects were also observed after treatment of the mice model with Amuc\_2109, a  $\beta$ -acetylaminohexosidase secreted by *A. muciniphila*. Treatment with Amuc-2109 also had anti-inflammatory effects by inhibiting the expression of inflammatory cytokines (147).

It was found that the outer membrane protein Amuc\_1100 of *A. muciniphila* promotes the biosynthesis of 5-HT, which is a neurotransmitter and a key signal molecule regulating the gastrointestinal tract functions and other organs (112). Wang et al. found that *A. muciniphila* or Amuc\_1100 improved gastrointestinal motility function and restored gut microbiota abundance and species diversity in antibiotic-treated mice. This finding represented an important approach through which *A. muciniphila* interacts with the host and further influences 5-HT-related physiological functions (148).

The anti-inflammatory and immunoregulatory roles of *A. muciniphila* has been assessed in other diseases in mice models. It was shown that the administration of pasteurized *A. muciniphila* in a mouse model of H7N9 influenza viral infection reduced mortality, given its anti-inflammatory and immunoregulatory roles (149). Likewise, the administration of *A. muciniphila* resulted in a decrease in inflammatory cell infiltration and bone destruction in a mouse model of calvarial infection (49). Treatment with *A. muciniphila* also resulted in decreased alveolar bone and systemic inflammation loss in an experimental *Porphyromonas gingivalis* induced periodontitis model (49, 150). These findings highlight the protective effects



of *A. muciniphila* and its use as a potential therapeutic agent to various diseases. However, Lawenius et al. showed that treatment with pasteurized *A. muciniphila* in mice reduces the accumulation of fat mass but does not protect against bone loss in a model of ovariectomized mice (151).

Moreover, another study performed in mice has reported that the presence of *A. muciniphila* and its EVs in the gut promote serotonin concentration, and also has an impact on serotonin signaling/metabolism through the gut-brain axis. These results suggest that *A. muciniphila* and its EVs can be considered as a new therapy for serotonin-related disorders (152). Ding et al. demonstrated that treatment of mice with depression induced by chronic restraint stress with *A. muciniphila* can reduce the depressive-like behavior of the mice, which was correlated with the increase in  $\beta$ -alanyl-3-methyl-l-histidine and edaravone (153).

## Human trials

Few studies on the use of *A. muciniphila* as a probiotic in humans have been conducted. The study by Plovier et al. was the first to demonstrate that the administration of live or pasteurized *A. muciniphila* is safe in humans in a cohort of 20 subjects with excess body weight. An exploratory study conducted by Depommier et al. on 32 overweight and obese insulin-resistant human volunteers also demonstrated that daily oral supplementation with either live or pasteurized *A. muciniphila* bacteria was safe and well-tolerated up for three months. Furthermore, they showed that pasteurized *A. muciniphila* improves insulin sensitivity and reduces insulinemia and plasma total cholesterol, while slightly decreasing body weight and fat mass compared to a placebo group (23). Moreover, the same team suggested that peroxisome proliferator-activated receptor alpha activation by mono-palmitoyl-glycerol might underlie some of the beneficial metabolic effects induced by *A. muciniphila* in human metabolic syndrome (24). Metabolome analysis illustrates that administration of *A. muciniphila* in prediabetic individuals leads to a decrease in some amino acids (tyrosine and phenylalanine), potentially explaining its hepato-protective role (154). Two clinical studies are ongoing to prove the efficacy of pasteurized *A. muciniphila* in improving insulin sensitivity, and to assess the weight-loss and glucose-lowering effects of *A. muciniphila* WST01 strain in overweight or obese patients with type 2 diabetes (Table 3).

## Enhancing the abundance of *A. muciniphila* with prebiotics/ other probiotics

One method of favorably modulating the gut microbiota is to administer growth-promoting substrates that can be used

preferentially by health-promoting bacteria to promote their growth and the production of associated desirable metabolites. The rationale of selectively enhancing beneficial microbes in the gut led to the concept of prebiotics, initially described in 1995 by Roberfroid and Gibson (158).

While some technological and regulatory hurdles may limit the use of certain strains of probiotics, it should be possible to use prebiotics and other dietary components to selectively enhance their growth in situ. The prebiotic paradigm has shifted in recent years, following the discovery of newly identified putatively beneficial gut microbiota members to target for enrichment. Through the development of new cultivation techniques and high-throughput sequencing, these studies have been able to explore the various impacts of specific fibers and products which represent untapped source of food bioactive on gut microbiota (159). For example, Anhe et al. showed that cranberry extract, rich in polyphenols, has been shown to improve diet-induced obesity and several features of metabolic syndrome (MetS) in mice, while increasing the abundance of *A. muciniphila* (160). Moreover, studies demonstrated that supplementation with grape polyphenols can promote increased intestinal abundance of *A. muciniphila* in mice fed either high-fat or low-fat diet, thus resulting in lower intestinal and systemic inflammation (161, 162). The administration of polymeric procyanidins in mice fed a high-fat/high-sucrose diet increases the proportion of *A. muciniphila* by eight times, producing beneficial effects on metabolic homeostasis (163). Another interesting fruit extract rich in polyphenols is camu-camu extract. This prebiotic can also improve the homeostasis of glucose and lipids while also increasing the abundance of *A. muciniphila* after five weeks of supplementation in HFD fed mice (164). Dietary supplementation with polysaccharides such as fucoidan decreased body weight in HFD-fed mice and also improved glucose intolerance and insulin resistance. Both fucoidans separately improved intestinal dysbiosis caused by a HFD and significantly increased the abundance of *A. muciniphila* (165). Inulin-type fructan prebiotics were found to significantly enhance the presence of *A. muciniphila*, linked to a decrease in obesity and fat mass and an improvement in insulin resistance in genetic obese and diet-induced leptin-resistant mice (166). An increase in the cecal content of *A. muciniphila* was detected by targeted qPCR following four weeks' supplementation with berberine in genetically obese mice, associated with an improvement in gut barrier function and hepatic inflammatory and oxidative stress (167). (167) Jiang et al. showed that total flavone (TFA) extracted from the flowers *Abelmoschus manihot* (TFA) can also enhance *A. muciniphila* in DSS-induced experimental colitis (168). Finally, dry extract of rhubarb root has also been shown to cause an increase in levels of *A. muciniphila* associated with the increased expression of *Reg3 $\gamma$*  in the colon, an anti-microbial peptide with an important role in the host defense system, thus protecting against metabolic disorders (169).

TABLE 3 Human studies or clinical trials on the use of *A. muciniphila* as a probiotic or enhancing its abundance through prebiotic administration.

	Prebiotic/Probiotic	Intervention	Cohort	Clinical case	Outcomes	Author/References	
Validly published studies	Prebiotics	Xylo-oligosaccharides (XOS)	1.4 g XOS, 2.8 g XOS or placebo taken daily	32 healthy subjects	x	↑ in <i>Akkermansia</i> sp. in those supplemented with the higher dose	(155)
		Resistant starch (RS)	Participants consumed a high (HC) or low carbohydrate (LC) diet followed by a baseline diet. *HC subjects consumed either a high RS (HRS – 66 g/d) or low RS (LRS – 4 g/d). *LC Subjects consumed either 48 g for HRS or 3 g for LRS.	39 subjects with reduced insulin sensitivity	x	↑ in the ratio of Firmicutes to Bacteroidetes. ↑ levels of <i>A. muciniphila</i>	(156)
	Probiotics/ Postbiotics	<i>A. muciniphila</i>	Oral administration of either live or pasteurized <i>A. muciniphila</i> or the membrane protein Amuc_1100* ( $1.5 \times 10^8$ CFU)	20 subjects with excess body weight	Obesity and type 2 diabetes	Administration of live or pasteurized <i>A. muciniphila</i> is safe in humans.	(12)
		<i>A. muciniphila</i>	daily oral supplementation of $10^{10}$ <i>A. muciniphila</i> bacteria either live or pasteurized (3 months) ( $10^{10}$ bacteria)	32 overweight/obese insulin-resistant volunteers	Obesity	1- <i>A. muciniphila</i> is safe and well tolerated. 2- Pasteurized <i>A. muciniphila</i> improved insulin sensitivity, reduced insulinemia and total plasma cholesterol. 3- Pasteurized <i>A. muciniphila</i> slightly decreased body weight and fat mass 4- <i>A. muciniphila</i> reduced the levels of markers for liver dysfunction and inflammation.	(23) (24)
Clinical trials in progress	Prebiotics	<i>Lactobacillus plantarum</i> , <i>Streptococcus thermophilus</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus rhamnosus</i> , <i>Bifidobacterium lactis</i> , <i>Bifidobacterium longum</i> , and <i>Bifidobacterium breve</i> .	Oral supplementation (6 weeks)	13 individuals	Obesity	Increase in the abundance of <i>A. muciniphila</i> after the intervention	(157)
		oat β-glucans	5 gr of oat β-glucan (12 weeks)	40 participants with type 2 diabetes mellitus	Type 2 diabetes	Follow up on <i>A. muciniphila</i> levels in fecal microbiota using (qPCR)	NCT04299763
		dietary fiber formulation	supplementation with 15g/day fiber powder (1 month)	20 healthy participants	x	Explore the change in <i>A. muciniphila</i> gut abundance.	NCT03785860
	Acetate	Supplementation with Acetate (Apple Cider Vinegar) (5 months).	10 patients on stable dose of antipsychotic medication for treatment of depression or anxiety.	Depression/ anxiety	Encourage the growth of <i>A. muciniphila</i>	NCT05022524	

(Continued)

TABLE 3 Continued

	Prebiotic/Probiotic	Intervention	Cohort	Clinical case	Outcomes	Author/References
	Camu Camu Capsules (CC)	2 capsules of Camu Camu daily in addition to antiretroviral therapy (12 weeks)	22 participant with HIV	HIV	1- Monitor <i>A. muciniphila</i> levels in stools. 2- Monitor gut damage and inflammation.	NCT04058392
		CC supplementation of 500 mg (3 months)	45 participants with Non-Small Cell Lung Cancer and melanoma receiving Immune Checkpoint Inhibitors	Non-Small Cell Lung Cancer and melanoma	1- Assess the safety and tolerability of CC prebiotic. 2- Discover if CC has the potential to enrich <i>A. muciniphila</i> and improve Immune Checkpoint Inhibitors efficacy.	NCT05303493
<b>Probiotics</b>	<i>Lactobacillus rhamnosus</i> Probio-M9	Daily oral dose (6 months)	46 patients receiving immunotherapy for liver cancer	liver cancer	Increase the abundance of <i>A. muciniphila</i> to improve effect of immunotherapy	NCT05032014
	<i>Lactobacillus Bifidobacterium</i> V9		46 Non-small Cell Lung Cancer Patients receiving immunotherapy	Cell Lung Cancer		NCT05094167
	fecal microbiota capsules	x	20 participants with Advanced Lung Cancer Treated With Immunotherapy	Advanced lung cancer	1- Selection of donor of fecal microbiota based on their fecal abundance in <i>F. prausnitzii</i> , <i>B. longum</i> , <i>A. muciniphila</i> and <i>Fusobacterium</i> spp. 2- Manipulating the microbial populations to enhance the efficacy of immunotherapy.	NCT04924374
	DS-01 (microbial consortia consisting of 24 strains across 12 species)	2 capsules daily (12 weeks)	100 men or women with IBS with constipation	Irritable bowel syndrome	Evaluate changes of <i>A. muciniphila</i> and other species	NCT04598295
	<i>A. muciniphila</i>	orally given <i>A. muciniphila</i> WST01 strain powder with maximum live bacteria of $5 \times 10^{10}$ CFU/g (12 weeks)	60 overweight/obese and drug naïve type 2 diabetes patients	Obesity/Type 2 diabetes	Evaluate the effects of <i>A. muciniphila</i> WST01 strain in overweight or obese patients with T2D.	NCT04797442
	<i>A. muciniphila</i>	Daily oral dose of pasteurized <i>A. muciniphila</i> (120 days)	98 hyperglycaemic healthy adults	Dysglycaemia	demonstrate the efficacy of pasteurized <i>A. muciniphila</i> (pAKK) in improving insulin sensitivity	NCT05114018

Table resuming the different validly published studies and the clinical trials in progress that use *A. muciniphila* as a probiotic or prebiotics to enhance its abundance, the cohort, the intervention, the clinical case, the results and the references. †: Increase.

Clinical trials and human studies are essential when assessing the benefits of newly identified prebiotics. Of the many potential prebiotics which have been studied, only a few substrates, including Xylo-oligosaccharides (XOS) and resistant starch (RS) have been validated through human studies. Finegold et al. demonstrated that xylo-oligosaccharides promoted intestinal health by modulating the microbial community: an increase in the levels of *Faecalibacterium* sp. and *Akkermansia* sp. as well as *Bifidobacteria* was detected (155). Moreover, a randomized dietary study by Maier et al. proved that resistant starch increased the levels of *A. muciniphila* in participants who followed a high resistant starch diet (156). Other ongoing clinical

studies involve the use of various prebiotics in different diseases such as T2D, cancer and other diseases in order to uncover their potential in enriching the abundance of *A. muciniphila*.

Other probiotic treatments may also increase the levels of *A. muciniphila*. For example, a fasting programme combined with laxative treatment for one week followed by a six-week probiotic intervention with a probiotic containing several different bacterial strains showed an increase in the abundance of *Akkermansia* (157). Four other clinical studies are in progress about the use of different bacteria as probiotics and their effect on modulation of the intestinal flora in cancer or IBS and, most importantly, on increasing the abundance of *A. muciniphila* (Table 3).

Other than natural components, *A. muciniphila* has been used by Payahoo et al. as a marker to assess the efficiency of a pharmaceutical agent, Oleylethanolamide, for treatment of obese people. This study showed that abundance of *A. muciniphila* bacterium increases significantly in oleylethanolamide group compared to the placebo group and modifies the energy balance (170).

## Conclusion and perspectives

A new area of research is emerging with the study of interbacterial communication, particularly between probiotic bacteria in transit and intestinal bacteria. *A. muciniphila* has been proven to have many beneficial effects in immune and metabolic regulation which can result in stimulating host health and preventing of pathogens. Nowadays, it is considered as a next generation probiotic to treat metabolic disorders such as obesity, diabetes, inflammatory diseases, as well as cancer (Figure 2). It has been reported that *A. muciniphila* in its two forms (live and pasteurized) is safe for use in human trials and two known companies have already started producing *A. muciniphila* probiotics (A-Mansia Biotech and Pendulum). However, there is no significant evidence on the link between this bacteria and malnutrition, reason why more studies should focus on this topic. Finally, more studies and mainly human clinical trials should be carried out in order to assess mechanisms of action and long-term effects of *A. muciniphila* before using for therapeutic applications.

## Author contributions

RI, RW: Writing-original draft preparation. GD: Writing, reviewing and editing. J-CL, DR: Reviewing, supervision. All authors have read and agreed to the published version of the

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgstr.2022.1024393/full#supplementary-material>

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## **Context 2**

### **Review: Ethanol-Producing Microorganisms in Human Gut Microbiota**

## **Article 6**

### **A Catalog of Ethanol-Producing Microbes in Humans**

Babacar MBAYE, **Reham MAGDY WASFY**, Maryam TIDJANI ALOU, Patrick BORENTAIN, Rene GEROLAMI, Jeans Charles DUFOUR and Matthieu MILLION

« [Article submitted to Future Microbiology.](#) »



## A Catalog of Ethanol-Producing Microbes in Humans

Journal:	<i>Future Microbiology</i>
Manuscript ID	FMB-2023-0250.R4
Manuscript Type:	Systematic Review
Keywords:	Endogenous ethanol production, gut microbiome, mycobiome, microbiota, dysbiosis, alcohol, non-alcoholic steatohepatitis, metabolic dysfunction-associated steatohepatitis

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3 **1 Abstract**  
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5 **2 Background:** Endogenous ethanol production emerges as a mechanism of non-alcoholic  
6 steatohepatitis, obesity, diabetes and auto-brewery syndrome. **Methods:** To identify ethanol-  
7 producing microbes in humans, we used the NCBI taxonomy browser and the PubMed  
8 database with an automatic query and manual verification. **Results:** 85 ethanol-producing  
9 microbes in human were identified. *Saccharomyces cerevisiae*, *Candida* and *Pichia* were the  
10 most represented fungi. *Enterobacteriaceae* was the most represented bacterial family with  
11 mainly *Escherichia coli* and *Klebsiella pneumoniae*. Species of the *Lachnospiraceae* and  
12 *Clostridiaceae* family, of the *Lactobacillales* order and of the *Bifidobacterium* genus were  
also identified. **Conclusions:** This catalog will help the study ethanol-producing microbes in  
human in the pathophysiology, diagnosis, prevention and management of human diseases  
associated with endogenous ethanol production.

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3 13 **Keywords:** Endogenous ethanol production, gut microbiome, mycobiome, microbiota,  
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5 14 dysbiosis, alcohol, non-alcoholic steatohepatitis, metabolic dysfunction-associated  
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For Review Only

## 16 **Plain Language Summary**

17 Our bodies are home to a vast community of tiny living organisms like bacteria, viruses, and  
18 archaea, collectively known as the microbiota. These microbes are crucial for our well-being  
19 and contribute to the proper functioning of our bodies. However, certain factors such as  
20 antibiotics and an imbalanced diet can disturb this microbial community, a condition known  
21 as dysbiosis, leading to various diseases. This review focuses on a particular aspect of  
22 dysbiosis related to the production of ethanol, a type of alcohol, within our bodies. While the  
23 disruption of the microbiota has been linked to several health issues, the role of  
24 ethanol production as a contributor is not well-explored. The review aims to shed light on the  
25 microbes involved in this process. By examining numerous studies from the PubMed  
26 database, the researchers found 85 microbes capable of producing ethanol in the human body.  
27 This includes 61 bacterial and 24 yeast species. In conclusion, this review provides a detailed  
28 updated catalog of ethanol-producing microbes in humans *in vitro*. Understanding these  
29 microbes and their role in diseases related to endogenous ethanol production could pave the  
30 way for better diagnostic tools and treatments in the future.

## 31 **Introduction**

32 Under physiological conditions, small quantities of endogenous ethanol are formed in the  
33 body, particularly in the colon, during sugar metabolism [1]. This endogenously produced  
34 ethanol is rapidly eliminated by intestinal and hepatic dehydrogenases [Adh], catalases, and  
35 the microsomal ethanol oxidation system [1]. When ethanol concentration exceeds the  
36 internal metabolic capacity, ethanol accumulates and can lead to complications and even the  
37 onset of certain diseases [2]. This situation is mainly due to an imbalance in the intestinal  
38 microbiota, favoring the proliferation of microbes that produce ethanol from sugars [3]. The  
39 first case of this endogenous ethanol production was described in 1894 in a French subject by  
40 Bouchard [4]. People suffering from this phenomenon experience symptoms like those  
41 associated with acute ethanol intoxication [5]. Various diseases have been associated with this  
42 abnormal production of endogenous ethanol (Table 1), namely auto-brewery syndrome [6-  
43 15], diabetes [15-18], obesity [15,19], and non-alcoholic steatohepatitis (NASH) [7,8,20-22].  
44 Among these diseases, different intestinal [6-11], oral [13] and urinary [14] microbiota have  
45 been reported as ethanol-producing microbes in the literature. Furthermore, even in those who  
46 do not consume alcohol, the phenomenon of endogenous ethanol production might result in  
47 medico-legal issues related to drunk driving [9]. To date, ethanol-producing microbes in  
48 humans have not been fully investigated. Therefore, this review aims to present for the first  
49 time a comprehensive catalog of all known ethanol-producing microbes in humans reported in  
50 the literature. Hence, this catalog could guide the researchers to further study the potential  
51 role of all mentioned ethanol-producing microbes in the pathophysiology, diagnosis, and  
52 management of human diseases associated with endogenous ethanol production.

## 53 **Methods**

54 **Our bibliographic strategy to identify microbes found in humans associated with *in vitro***  
55 **ethanol production is reported in Figure 1.** Regardless of the atmosphere or medium used for  
56 culture, only *in vitro* production of ethanol of a pure microbial culture was considered to  
57 designate an ethanol-producing microbe. Articles that reported the presence of enzymes in the  
58 genome, such as alcohol dehydrogenase, but did not assess ethanol production *in vitro* were  
59 excluded. For this purpose, all the species available on the NCBI taxonomy browser database  
60 were screened [<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi>; Accessed on  
61 11/05/2022]. Only validated species, as confirmed on the List of Prokaryotic names with  
62 Standing in Nomenclature (<https://www.bacterio.net/>) for bacteria, were included. For each  
63 species, a query was used to extract the hits (articles) linked to ethanol production. The search  
64 was carried out without date or language restrictions. PubMed database was used to search the  
65 for all the articles related to ethanol-producing microbes. The following query was used:  
66 "microorganism name" [tw] OR "Synonyms"[tw] AND [ethanol[mesh]] AND [produc\*[tw]  
67 OR endogenous[tw] OR synthesis\*[tw] OR secret\*[tw]] NOT " forensic toxicology" [mesh]  
68 NOT mutant[tw] NOT "DNA, recombinant" [MESH] NOT biotransformation[tw] NOT  
69 biofuels[tw] NOT bioreactor[tw] NOT disinfect\*[tw]."

70 A total of 7,275 articles were extracted and collated in Access® database (Access  
71 2007, Microsoft, France). These articles were initially manually screened according to titles  
72 and abstracts by two of the authors (BM and RMW). If the article seems to be related to  
73 ethanol production, a second screening was conducted by reading the full text to confirm the  
74 presence of ethanol-producing microbes, yielding a total of 5,397 eligible articles. Human  
75 associated microbes were defined based on an updated list of human bacteria [28-30] and  
76 fungi [31] previously published in our center.



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3 77           These articles were all studied to create our catalog of human ethanol-producing  
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5 78   bacteria and fungi. MS Access® software (Access 2007, Microsoft, France) was used to  
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7 79   record the ethanol production of all microbes. For each microbe, after careful reading and  
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9 80   analysis of the articles following the query by two of the authors (BM and RMW), the MS  
10  
11 81   Access® database (Access 2007, Microsoft, Paris, France) was used to notify ethanol  
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13 82   production ("Ethanol production" tab (yes or no)) and the fact that species were verified  
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15 83   members of the human microbiota via the "Human" tab (yes or no). The final output file was  
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17 84   created (Excel 2007, Microsoft, France). It included the following data: taxonomic rank of  
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19 85   each detected microorganism (bacteria, yeasts, and archaea) showing *in vitro* ethanol  
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21 86   production status (yes or no), microbe found in humans (yes or no) and the number of articles  
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23 87   or “hits” (Query\_PMID\_Count) detected for each species.  
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## 88 Results

### 89 Overview of ethanol-producing microbes

90 Among the 931 microbes first identified after automatic query, 36.52% (340/931) were  
91 identified as ethanol producers, including 52.6% (179/340) fungi, 45.8% (156/340) bacteria,  
92 and 1.6% (5/340) archaea. Microbes in human accounted for 14%, 13%, and 0% of fungi,  
93 bacteria, and archaea, respectively, while non-human represented 86%, 87%, and 100% (no  
94 human archaea with *in vitro* ethanol production in the literature), respectively (Supplementary  
95 Tables 1, 2 & 3). Ultimately, 85 ethanol-producing microbes in humans (61 bacteria and 24  
96 fungi) were identified (Table 2 & Table 3) [32-140], however, no archaea found in humans  
97 were reported as producing ethanol *in vitro* (Supplementary Table 3).

98 Among human ethanol-producing bacteria, *Pseudomonadota* (nine species and 846  
99 hits) and *Bacillota* (46 species and 434 hits) were the most represented phyla. At the family  
100 level, *Enterobacteriaceae* (six species and 837 hits), *Lactobacillaceae* (18 species and 126  
101 hits), *Streptococcaceae* (four species and 73 hits), *Clostridiaceae* (nine species and 67 hits),  
102 *Bifidobacteriaceae* (five species and 18 hits), *Oscillospiraceae* (four species and 15 hits) and  
103 *Lachnospiraceae* (five species and five hits) were highly represented.

104 Noteworthy, the *Enterobacteriaceae* family has the highest number of hits (837) but  
105 only 6 species were found as ethanol producers: Strikingly, 4 were *Klebsiella* species (*K.*  
106 *pneumoniae*, *K. oxytoca*, *K. aerogenes* and *K. variicola*), while the two others were  
107 *Escherichia coli* and *Raoultella planticola*. In the corresponding phylum (*Pseudomonadota*,  
108 formerly *Proteobacteria*), only these 6 species and 3 other well-known human pathogens:  
109 *Aeromonas hydrophila*, *Pantoea agglomerans* and *Hafnia alvei*, were found to produce  
110 ethanol *in vitro*. This suggests that these 9 species likely include pathobionts (at the strain  
111 level) specifically associated with endogenous-ethanol production associated diseases, as

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3 112 demonstrated in the seminal paper of Yuan *et al.* [7] linking ethanol-producing *K.*  
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5 113 *pneumoniae* strains and non-alcoholic steatohepatitis.

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8 114 The highly reported genera were *Klebsiella* (four species and 69 hits), *Clostridium*  
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10 115 (seven species and 61 hits), *Lactococcus* (two species and 45 hits), *Limosilactobacillus* (three  
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12 116 species and 32 hits), *Streptococcus* (two species and 28 hits), *Leuconostoc* (three species and  
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14 117 20 hits), *Pediococcus* (three species and 16 hits), *Bifidobacterium* (five species and 14 hits),  
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16 118 *Ruminococcus* (three species and 14 hits) and *Lentilactobacillus* (three species 13 hits)  
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19 119 (Supplementary Table 1).

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21 120 For fungi, *Ascomycota* (23 species and 2593 hits) was the most represented phylum.  
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23 121 Meanwhile, *Debaryomycetaceae* (seven species and 158 hits), *Saccharomycetaceae* (four  
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25 122 species and 2304 hits), *Phaffomycetaceae* (four species and 40 hits), and *Pichiaceae* (four  
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27 123 species and 66 hits) were the most reported among the nine identified families  
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30 124 (Supplementary Table 2). Moreover, 15 ethanol-producing fungal genera were reported, but  
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32 125 the most represented genera were *Candida* (six species and 149 hits), *Pichia* (three species  
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34 126 and 66 hits), *Cyberlindnera* (two species and nine hits), *Meyerozyma* (two species and nine  
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36 127 hits) and *Saccharomyces* (two species and 2143 hits). In addition, the most frequently cited  
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38 128 ethanol-producing genera and species in the literature are shown in Figure 2 and Figure 3. It  
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40 129 was noted that the most frequently cited in ethanol production include both bacteria and  
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42 130 yeasts, namely *Saccharomyces cerevisiae* (2139 hits), *Escherichia coli* (765 hits),  
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44 131 *Kluyveromyces marxianus* (heterotypic synonym *Candida kefyri*) (143 hits), *Staphylococcus*  
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46 132 *aureus* (110 hits), *Candida albicans* (96 hits) and *Pichia kudriavzevii* (57 hits) were the most  
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48 133 represented ethanol-producing microbes in the literature (Figure 3, Table 4 & Table 5).  
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50 134 Strikingly, *S. cerevisiae* and *E. coli* had outstanding numbers of hits compared to all other  
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56 135 species.

### 136 **Human ethanol-producing bacteria**

137 Sixty-one ethanol-producing bacterial species were reported in this review, divided into four  
138 phyla, 15 families, and 30 genera (Table 2, Supplementary Table 1 & Figure 2). In the  
139 following section, we will group and discuss the most described human ethanol-producing  
140 species (represented by more than two species in each genus) based on shared ethanol-  
141 producing pathways.

#### 143 ***Lactobacillales* group**

144 This group comprises 44 ethanol-producing species divided into three families, including  
145 *Lactobacillaceae* (63 %), *Streptococcaceae* (25 %) and *Enterococcaceae* (11 %), and 15  
146 genera including *Streptococcus* (60 %), *Enterococcus* (33 %), *Pediococcus* (27 %),  
147 *Lactobacillus* (27 %) and *Limosilactobacillus* (20%), *Lentilactobacillus* (20 %), and  
148 *Leuconostoc* (20 %). The species of this group use the heterolactic pathway for ethanol  
149 formation. Sugars are degraded via phosphoketolase (pentose-phosphate-pathway) to D-  
150 xylose-5-phosphate, converted to D-glyceraldehyde-3-phosphate (G3P) and acetyl phosphate.  
151 The resulting G3P and the acetyl phosphate are converted into lactic acid and ethanol/or  
152 acetate, respectively. Beyond heterolactic fermentation, some species of the *Streptococcus*  
153 genus can use the hexitol pathway to produce lactate, acetate, formate, and ethanol. This  
154 hexitol pathway uses sugar substitutes such as sugar alcohols (e.g. sorbitol and mannitol).

155 Those *Streptococcus* species are generally associated with the oral microbiota [141].  
156 Their endogenous ethanol production has been correlated with their abundance in non-  
157 alcoholic fatty liver disease (NAFLD) [23]. In this review, nine ethanol-producing  
158 *Streptococcus* species were identified; among them, *S. mutans* and *S. mitis* were reported to be  
159 major causes of dental caries [141]. These two species have been previously reported in oral  
160 auto-brewery disease [13], showing higher ethanol production in co-culture with *Klebsiella*

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2  
3 161 *pneumoniae* [13]. Moreover, the increased abundance of *S. mutans* in NASH patients has  
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5 162 already been described by Naka *et al.* [142]. Interestingly, the administration of a specific  
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7 163 strain (TW871) of *S. mutans* was reported to induce NASH in a mouse model [143].  
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10 164 *Streptococcus* species ferment carbohydrates to produce mainly lactic acid, with minor  
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12 165 amounts of acetic and formic acids, CO<sub>2</sub> and ethanol [144]. In anaerobic conditions,  
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14 166 *Streptococcus* species produce formate, acetate, and ethanol via the pyruvate formate-lyase  
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16 167 (PFL) enzyme. In aerobic conditions, the PFL pathway is inactive and is replaced by pyruvate  
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18 168 oxidase in some *Streptococcus* species, and pyruvate dehydrogenase in others [144]. In  
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20 169 addition, some *Streptococcus* species form lactate which is catalyzed by lactate  
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22 170 dehydrogenase in case of sugar overload [141].  
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26 171 For the genus *Limosilactobacillus*, recently reclassified by Zheng *et al.* [58], several  
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28 172 assigned species have been associated with different pathologies. In this review, only three  
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30 173 species were identified as capable of producing ethanol including *Limosilactobacillus*  
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32 174 *fermentum*, *Limosilactobacillus reuteri* and *Limosilactobacillus panis*. *L. fermentum* was  
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34 175 reported to be associated with NASH [23,146] and liver enlargement in animals [147],  
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36 176 whereas *L. reuteri* was reported in metabolic syndrome and obesity [148,149].  
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#### 42 178 ***Lachnospiraceae* group**

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44 179 Ethanol-producing *Lachnospiraceae* group in our review included three genera and five  
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46 180 species as follows: *Blautia* (*B. coccoides* and *B. hydrogenotrophica*), *Enterocloster* (*E.*  
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48 181 *bolteae* and *E. asparagiformis*) and *Mediterraneibacter* (*M. torques* and *M. gnavus*). The  
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50 182 *Lachnospiraceae* family is one of the most represented bacterial groups in the human  
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52 183 intestinal microbiota, and various studies have demonstrated both beneficial and harmful  
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54 184 effects on human health. *Blautia* and *Enterocloster* ethanol-producing species belonging to  
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56 185 this group use the Embden-Meyerhof pathway (EMP), which metabolizes carbohydrates into  
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3 186 pyruvate, which is oxidized to acetyl-CoA by pyruvate dehydrogenase [50]. This acetyl-CoA  
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5 187 undergoes two successive reactions (acetaldehyde and ethanol) thanks to the bifunctional  
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7 188 enzyme alcohol-acetaldehyde dehydrogenase (AdhE) [50]. Whereas *Enterocloster* species  
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9 189 described in this review convert acetyl-CoA to acetate via the phosphotransacetylase enzyme.  
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11 190 This acetyl-CoA is converted to acetaldehyde by aldehyde dehydrogenase and to ethanol by  
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13 191 alcohol dehydrogenase [46]. Furthermore, *M. torques* and *M. gnavus* were reported to  
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15 192 produce ethanol from a variety of substrates [150], using the same pathways as above, from  
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17 193 pyruvate to acetyl CoA and then to ethanol via the bifunctional enzyme alcohol-acetaldehyde  
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19 194 dehydrogenase.  
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### 26 196 ***Clostridiaceae* group**

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28 197 The *Clostridiaceae* group showed ten ethanol-producing species belonging to *Clostridium*,  
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30 198 *Paraclostridium*, and *Sarcina* genera (Table 2). *Clostridium* species (*C. amylolyticum*, *C.*  
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32 199 *butyricum*, *C. cochlearium*, *C. sporogenes*, *C. tyrobutyricum*, *C. beijerinckii*, *C.*  
33  
34 200 *malenominatum*, and *C. asparagiforme*) can metabolize complex sugars (e.g. inulin, starch,  
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36 201 etc.) into simple sugars (e.g. glucose, fructose) to use them as carbon source. These ethanol-  
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38 202 producing *Clostridium* species use the acetone-butanol-ethanol (ABE) pathway. This pathway  
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40 203 involves glycolysis of carbohydrates (by the EMP pathway), generating organic acids (e.g.,  
41  
42 204 acetic acid and butyric acid) through a series of pyruvate metabolism reactions, and  
43  
44 205 eventually fermenting these organic acids to produce ethanol, acetone, and butanol [37].  
45  
46 206 Moreover, those *Clostridium* species can produce ethanol from pyruvate by transforming the  
47  
48 207 latter into acetyl-CoA via pyruvate synthase enzyme. The resulting acetyl-CoA is then  
49  
50 208 transformed into acetaldehyde [36] dehydrogenase. However, *Paraclostridium bifermentans*  
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52 209 and *Sarcina ventriculi* species use AdhE to produce ethanol directly from acetyl-CoA [40].  
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3 211 ***Oscillospiraceae group***  
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5 212 The ethanol-producing species in this *Oscillospiraceae* group all belong to the genus  
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7 213 *Ruminococcus* including *R. albus*, *R. flavefaciens* and *R. bromii*. *R. albus* was the first species  
8  
9 214 in the *Ruminococcus* genus to be described for ethanol production from starch [151].  
10  
11 215 Additionally, *R. bromii* was reported to produce ethanol in addition to acetate in pure culture  
12  
13 216 with fructose [152]. Moreover, Isotope labeling analysis of carbohydrate fermentation  
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15 217 products proved that the EMP pathway is the main metabolic pathway for *Ruminococcus*  
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17 218 species [153], where pyruvate obtained from sugar metabolism is converted to acetyl-CoA  
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19 219 and ethanol by AdhE [154].  
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26 221 ***Klebsiella group***  
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28 222 Strikingly, four ethanol-producing *Klebsiella* species were reported in our catalog (Table 2).  
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30 223 *Klebsiella* species can live in both aerobic and anaerobic conditions, using different types of  
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32 224 sugars as carbon sources [155], where pyruvate is obtained whatever the carbon source is  
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34 225 [36]. *Klebsiella* species use two fermentation processes: mixed acid fermentation and  
35  
36 226 butylene glycolytic fermentation [156]. In the former, a complex mixture of several acids is  
37  
38 227 produced: formate, lactate, acetate, succinate, and ethanol. The produced ethanol results from  
39  
40 228 the transformation of pyruvate into acetyl-CoA. This acetyl-CoA is, in turn, transformed into  
41  
42 229 acetaldehyde and ethanol by alcohol dehydrogenase. The second process is characterized by  
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44 230 the formation of 2,3-butanediol via acetone. According to Li *et al.*, the 2,3-butanediol  
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46 231 pathway is one of the pathways involved in high ethanol production in *Enterobacteriaceae*  
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48 232 (particularly *Klebsiella pneumoniae*) in both anaerobic and aerobic conditions [157].  
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55 234 ***Bifidobacterium group***  
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3 235 The genus *Bifidobacterium* comprises 118 species and 18 subspecies, some of which are  
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5 236 considered probiotics and often used in dairy products. *Bifidobacterium* species were reported  
6  
7 237 to play an important role in the metabolism of complex sugars in the colon [158]. Five  
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9 238 ethanol-producing *Bifidobacterium* species were identified in our catalog including  
10  
11 239 *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*,  
12  
13 240 *Bifidobacterium longum*, and *Bifidobacterium animalis*. *Bifidobacterium* species tend to  
14  
15 241 depolymerize complex sugars (e.g. oligosaccharides and polysaccharides) into monomers or  
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17 242 simple sugars, which in turn are fed into the fructose-6-phosphate shunt to produce end  
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19 243 products (such as lactic and acetic acids) and minorities (such as formic acid and ethanol)  
20  
21 244 [91]. The ethanol production process involves breaking down sugars into two-carbon and  
22  
23 245 three-carbon molecules such as acetyl phosphate and glyceraldehyde 3-phosphate,  
24  
25 246 respectively. Glyceraldehyde 3-phosphate is converted into pyruvate, whose phosphoroclastic  
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27 247 reaction leads to the formation of acetyl phosphate and formate. The resulting acetyl  
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29 248 phosphate is reduced to ethanol at the expense of acetate production.  
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## 250 **Human ethanol-producing fungi**

251 Ethanol production through fermentation was initially described in yeasts, where  
252 *Saccharomyces cerevisiae* was used for ethanol production [3]. Surprisingly, the most  
253 extensively studied ethanol-producing microbe is *S. cerevisiae*, which is also linked to auto-  
254 brewery syndrome [6,10,14]. However, it is the most used probiotic [159,160]. Moreover, our  
255 catalog showed other ethanol-producing fungi species (Table 3, Supplementary Table 2, and  
256 Figure 2), including some opportunistic species. We retrieved 27 ethanol-producing fungi  
257 species, distributed into two phyla (*Ascomycota* (96 %), and *Basidiomycota* (6%)), and 13  
258 genera (*Candida* (five species), *Pichia* (three species), *Saccharomyces* (two species),  
259 *Meyerozyma* (two species), and *Cyberlindnera* (two species)). *Candida* was the most  
260 represented genus, with five distinct ethanol-producing species: *Candida albicans*, *Candida*  
261 *parapsilosis*, *Candida sanyaensis*, *Candida sojae*, and *Candida tropicalis*. *Candida* species  
262 were associated with some diseases due to their ability to produce endogenous ethanol, such  
263 as auto-brewery syndrome [8,20,26], diabetes [16-18], and NASH [8] (Table 1). Furthermore,  
264 ethanol-producing *Pichia* species in our catalog, including *Pichia kudriavzevii*, *Pichia*  
265 *membranifaciens*, and *Pichia fermentans* have been associated with NAFLD [8,161].

266 Surprisingly, *Pichia kudriavzevii* had been associated with high quantities of  
267 endogenous ethanol in NASH patients (up to 6 g/L) [8]. Yeasts generally use the same  
268 pathway for ethanol production as bacteria via the "pyruvate fermentation to ethanol II"  
269 pathway, according to the MetaCyc database (<https://metacyc.org/>, Accessed on 14/05/2022).  
270 This pathway shows carbohydrate metabolism until a simple sugar is obtained. The latter  
271 enters the glycolysis cycle or the Entner-Doudoroff pathway, with pyruvate as an end product.  
272 Pyruvate decarboxylase further decarboxylates pyruvate into acetaldehyde and CO<sub>2</sub>. Alcohol  
273 dehydrogenase then catalyzes the reduction of acetaldehyde to ethanol.

## 274 Discussion

275 Our study aimed to establish a catalog of known ethanol-producing microbes in human using  
276 software capable of querying Pubmed. A total of 7275 were retrieved, enabling us to identify  
277 85 ethanol-producing microbes. These microbes were classified based on their taxonomy; for  
278 some, name changes were also considered after validation on the List of Prokaryotic names  
279 with Standing in Nomenclature (LPSN) or Taxonomy browser.

280 In fact, the human gut harbors a range of microbes with different roles in health and  
281 disease [162]. These microbes produce various molecules that contribute to digestive  
282 homeostasis and, in the case of dysbiosis, can lead to the onset of certain diseases. Ethanol,  
283 one of the metabolites produced by the microbiota even in healthy individuals, is known as  
284 "physiological ethanol" [163]. However, high ethanol concentrations may contribute to  
285 several diseases, including diabetes [16,17], auto-brewery syndrome [6,7,13], NASH  
286 [7,21,23,146], obesity [20], and possibly liver disease in persons living with HBV virus [48].

287 To our knowledge, this review is the first to report all known human ethanol-  
288 producing microbes in one catalog. Most studies have focused on endogenous ethanol-related  
289 complications [164]. This catalog is consistent with the literature data, as most of our  
290 microbes were at least cited in one or more articles related to endogenous ethanol production.  
291 Concerning NASH, several species have already been described, in particular *K. pneumoniae*  
292 [7,8,157], *L. fermentum* [23,146], and *L. lactis* [146]. Yuan *et al.* showed that administration  
293 of ethanol-hyperproducing *K. pneumoniae* strain, isolated from a patient with NASH, can  
294 induce the disease in a mouse model [7]. Surprisingly, antibiotic intake improved the liver  
295 status in the mice in the previous study [7].

296 Moreover, various fungi species have been associated with the auto-brewery  
297 syndrome, including *S. cerevisiae*, *C. albicans*, *C. parapsilosis*, and *P. kudriavzevii* [8]. On  
298 the other hand, another ethanol-producing *S. boulardii* (a closely related species to *S.*

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3 299 *cerevisiae*) was described as a probiotic for NASH patients [165]. Overall, this highlights the  
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5 300 significance of additional research into the role and processes by which those ethanol-  
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7 301 producing microbes could be linked to the pathophysiology of various human diseases.  
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10 302 This review has several limitations. This review takes into account current taxonomy  
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12 303 when creating this catalog, and considers the concept of "one micro-organism, one name" to  
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14 304 help clinicians identify old and new names for each species. However, it's a real challenge to  
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16 305 take into account the changing names of microbial species over time and the names of species  
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18 306 with numerous synonyms, notably for fungi, to obtain a coherent systematic review. Another  
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20 307 limitations of this review is the use of a single database (PubMed), and only publications  
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22 308 submitted before January 28, 2023, were considered. For future studies, several search  
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24 309 engines should be included. In addition, the use of an automatic query on a single database  
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26 310 does not allow us to ensure exhaustiveness.  
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30 311 However, this work has the advantage of being one of the first to systematically  
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32 312 review all the microbes found in humans capable of producing ethanol *in vitro*. Readers  
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34 313 should be aware that finding a species capable of producing ethanol is not proof of its  
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36 314 pathogenic role. Firstly, within a single species, a strain-dependent effect has been well  
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38 315 described [7]. Secondly, there is certainly a dose effect, as we found, for example, that the  
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40 316 maximum ethanol production level was much greater for yeasts (up to 6 g/L) than for bacteria  
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42 317 (up to 1.2 g/L) [7].  
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46 318 Finally, as the microbiota is a complex system where microbe-microbe interactions  
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48 319 modify the behavior of each microbe, the detection of an ethanol-producing microbe is not  
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50 320 synonymous with a risk of disease associated with endogenous ethanol production. For  
51  
52 321 example, we were surprised to find that *Bifidobacterium adolescentis*, although usually  
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54 322 associated with health, was capable of producing ethanol. Moreover, positive or negative  
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56 323 interactions have been found in co-culture experiment between different microbial species for  
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3 324 the production of ethanol, notably between gut lactic acid bacteria and yeast [166-168].  
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5 325 Noteworthy, *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum* have been found  
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7 326 to decrease the ethanol amount in co-culture with a yeast, *Kluyveromyces marxianus*  
8  
9 327 (*Candida kefyr*) [167]. Strikingly, both these species were previously associated with absence  
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11 328 of obesity in case-control studies while *Limosilactobacillus reuteri*, found associated with  
12  
13 329 ethanol production in the present review, was previously associated with obesity [148,149].  
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17 330 This strongly suggests that microbiotherapy and/or probiotics could be a putative  
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19 331 therapeutic options in diseases associated with endogenous ethanol production. This is  
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21 332 confirmed by a case of auto-brewery syndrome successfully treated by fecal microbiota  
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23 333 transplantation [169]. These discoveries promise new opportunities for healthcare  
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25 334 pharmaceutical development to standardize microbiotherapy [170,171].  
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### 31 336 **Conclusion**

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33 337 Ethanol is a molecule produced naturally in the gut, but its toxic effects are unequivocal when  
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35 338 levels exceed an individual-dependent threshold. Ethanol is only one element among others,  
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37 339 and future studies should clarify the importance of ethanol-producing microbes for the  
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39 340 diagnosis, prevention and/or treatment of diseases associated with endogenous ethanol  
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41 341 production. Overall, this catalog highlights how ethanol-producing microbes should be  
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43 342 considered as non-invasive indicators in disease diagnosis, but also as potential mediators that  
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45 343 can be targeted by future therapeutic options. We hope that this work and catalog will open up  
46  
47 344 new avenues of active research to better understand the mechanism of diseases associated  
48  
49 345 with endogenous ethanol production that have not been fully explained to date, such as  
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51 346 metabolic diseases and notably NASH, recently renamed MASH for Metabolic Dysfunction-  
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53 347 Associated Steatohepatitis. We do believe that understanding endogenous ethanol production  
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55 348 and associated dysbiosis will provide essential keys to human health over the coming decades.  
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3 349 **Summary points**  
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- 5 350 • Various diseases and complications are linked to endogenous ethanol production;  
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7  
8 351 however, little is known about ethanol-producing microbes in human.  
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10 352 • The current catalog describes 85 ethanol-producing microbes in humans, with the  
11  
12 353 predominance of bacterial species.  
13  
14 354 • Among the ethanol-producing species *Saccharomyces cerevisiae*, *Escherichia coli*,  
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16 355 *Kluyveromyces marxianus* (*Candida kefyr*), *Staphylococcus aureus*, *Candida albicans*,  
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19 356 and *Pichia kudriavzevii* remain the most cited in the literature.  
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21 357 • In the future, this catalog could serve as a diagnostic tool and a target for treating  
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23 358 endogenous ethanol production-related diseases.  
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For Review Only

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3 866 **List of figures**  
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5 867 **Figure 1. Flow chart of literature review to identify ethanol-producing microbes in**  
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8 868 **humans**  
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10 869 After an automatic script on the NCBI database, retrieved articles were manually screened for  
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12 870 *in vitro* ethanol production. An Access (Access 2007, Microsoft, Paris, France) database was  
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14 871 used to manage results and identify the number of hits for each ethanol-producing microbial  
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16 872 species. A ‘hit’ was defined as an article mentioning a human-associated microbial species  
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18 873 AND an *in vitro* ethanol production reported in one article. Total 85 microbes (61 bacterial  
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20 874 and 24 fungal species) found in humans were identified with potential *in vitro* ethanol  
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24 875 production.  
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28 877 **Figure 2. Number of articles mentioning *in vitro* ethanol production by bacteria (left)**  
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30 878 **and yeast (right) found in humans at the genus and species level.**  
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32  
33 879 The size of each taxon was correlated with the number of hits. Colors are for visual  
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35 880 presentation and do not represent a color coded information. The Wordart online software was  
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37 881 used (<https://wordart.com/>). For bacteria, *Escherichia* (notably *E. coli*), *Staphylococcus*  
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39 882 (notably *S. aureus*), *Klebsiella* (notably *K. pneumoniae*) and *Lactococcus* (*L. lactis*) were the  
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41 883 most represented. For fungi, *Saccharomyces cerevisiae* was expected (the brewer’s or baker’s  
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43 884 yeast) as *Kluyveromyces marxianus* (also named *Candida kefir*, which correspond to the well-  
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45 885 known beverage with known low levels of ethanol). *Candida albicans*, *Candida glabrata*, and  
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47 886 *Pichia kudriavzevii*, were also identified as major ethanol-producing fungi found in humans.  
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49 887 Strikingly, we recently identified these three yeasts as putative pathobionts in non-alcoholic  
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51 888 steatohepatitis [7].  
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3 890 **Figure 3: Number of hits by microbial species with *in vitro* ethanol production found in**  
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5 891 **humans**

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8 892 Hits corresponded to the number of articles reporting a human associated microbes and  
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10 893 reporting an *in vitro* ethanol production. *Saccharomyces cerevisiae* (2139 hits), *Escherichia*  
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12 894 *coli* (765 hits), and *Kluyveromyces marxianus* (heterotypic synonym *Candida kefir* 143 hits)  
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14 895 were the 3 species with the highest number of hits. The species with at least 20 hits were  
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16 896 represented. Sixteen species were identified and represent the main species to be investigated  
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18 897 as diagnostic biomarker, mediators and putative targets for future therapeutic options in  
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20 898 diseases associated with endogenous ethanol production. Caution must be taken because large  
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22 899 variations in ethanol levels have been observed between species (yeasts > bacteria [7]), and  
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24 900 between strains of the same species [7].  
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3 901 **List of Tables**  
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5 902 **Table 1. Diseases linked to endogenous ethanol production.**  
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7 903 <sup>a</sup>We recently performed a comprehensive review of microbes associated with auto-brewery  
8

9 904 syndrome (49 cases) which is fully detailed in the supplementary data of reference 8.  
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11 905 In this table, we represented the main diseases associated with endogenous ethanol production  
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13 906 and some microbial species reported in the corresponding articles.  
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15 907 **Table 2. Ethanol-producing bacteria in humans.**  
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17 908 For each species, at least 1 reference is cited. Complete details could be found in  
18

19 909 Supplementary Table 1.  
20

21 910 **Table 3. Ethanol-producing fungi in humans.**  
22

23 911 For each species, at least 1 reference is cited. Complete details could be found in  
24

25 912 Supplementary Table 2.  
26

27 913 **Table 4. Most frequently cited yeasts for ethanol production in the literature.**  
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29 914 The number of hits is the number of articles mentioning this species as producing ethanol in  
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31 915 vitro in Pubmed following our automatic query. For instance, we identified *Saccharomyces*  
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33 916 *cerevisiae* as a microbe found in humans, then we found 2139 articles ascertaining its ethanol  
34

35 917 production *in vitro*.  
36

37 918 **Table 5. Most frequently cited bacteria for ethanol production in the literature.**  
38

39 919 The number of hits is the number of articles mentioning this species as producing ethanol in  
40

41 920 vitro in Pubmed following our automatic query. For instance, we identified *Escherichia coli*  
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43 921 as a microbe found in humans, then we found 765 articles ascertaining its ethanol production  
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45 922 *in vitro*.  
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3 923 **Supplementary files**  
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5 924 **Supplementary Table 1. Bacterial species and corresponding hits with PMID number**  
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8 925 The articles reporting *in vitro* ethanol production by microbes in humans can be found with  
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10 926 the PMID number. Query\_PMIID\_count: number of hits for this species. Query\_PMIID\_List:  
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12 927 list of articles with PMID number in which *in vitro* ethanol production was confirmed or not  
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14 928 for this species.  
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16  
17 929 **Supplementary Table 2. Fungal species and corresponding hits with PMID number**  
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19 930 The articles reporting *in vitro* ethanol production by microbes in humans can be found with  
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21 931 the PMID number. Query\_PMIID\_count: number of hits for this species. Query\_PMIID\_List:  
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23 932 list of articles with PMID number in which *in vitro* ethanol production was confirmed or not  
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25 933 for this species.  
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28 934 **Supplementary Table 3. Archaeal species and corresponding hits with PMID number**  
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30 935 The articles reporting *in vitro* ethanol production by microbes in humans can be found with  
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32 936 the PMID number. Query\_PMIID\_count: number of hits for this species. Query\_PMIID\_List:  
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34 937 list of articles with PMID number in which *in vitro* ethanol production was confirmed or not  
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36 938 for this species.  
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**Table 1. Diseases associated with endogenous ethanol production**

Diseases	References	Associated ethanol-producing species
<b>Autobrewery syndrome</b>	<b>[6-15]</b>	<b>[8]<sup>a</sup></b>
Gut cases	[6-11]	<i>Candida albicans</i> <i>Saccharomyces cerevisiae</i> <i>Candida glabrata</i> ( <i>Nakaseomyces glabratus</i> ) <i>Candida tropicalis</i> <i>Pichia kudriavzevii</i> <i>Candida parapsilosis</i> <i>Candida stelloidea</i> <i>Candida guilliermondii</i> <i>Candida kefir</i> ( <i>Kluyveromyces marxianus</i> ) <i>Candida intermedia</i> <i>Klebsiella pneumoniae</i>
Oral case	[13]	<i>Candida albicans</i>
Urinary case	[14]	<i>Candida glabrata</i> ( <i>Nakaseomyces glabratus</i> )
<b>Diseases associated with metabolic syndrome</b>		
Diabetes	[15-18]	
Obesity	[15,19]	
<b>NASH (non-alcoholic steatohepatitis)</b>		
Characterization of gut microbiomes in nonalcoholic steatohepatitis [NASH] patients: A connection between endogenous alcohol and NASH	Zhu, 2013 [20]	<i>Escherichia coli</i>
Fatty Liver Disease Caused by High-Alcohol-Producing <i>Klebsiella pneumoniae</i>	Yuan, 2019 [7]	<i>Klebsiella pneumoniae</i>
Endogenous ethanol produced by intestinal bacteria induces mitochondrial dysfunction in non-alcoholic fatty liver disease	Chen, 2020 [21]	<i>Klebsiella pneumoniae</i>
Endogenous Ethanol and Triglyceride Production by Gut <i>Pichia kudriavzevii</i> , <i>Candida albicans</i> and <i>Candida glabrata</i> Yeasts in Non-Alcoholic Steatohepatitis	Mbaye, 2022 [8]	<i>Pichia kudriavzevii</i> , <i>Candida albicans</i> and <i>Candida glabrata</i>
Microbiome-derived ethanol in nonalcoholic fatty liver disease	Meijnikman, 2022 [22]	<i>Limosilactobacillus fermentum</i> and other <i>Limosilactobacillus</i> species



Increased fecal ethanol and enriched ethanol-producing gut bacteria <i>Limosilactobacillus fermentum</i> , <i>Enterocloster bolteae</i> , <i>Mediterraneibacter gnavus</i> and <i>Streptococcus mutans</i> in nonalcoholic steatohepatitis		<i>Enterocloster bolteae</i> <i>Limosilactobacillus fermentum</i> <i>Mediterraneibacter gnavus</i> <i>Streptococcus mutans</i>
<b>Others findings</b>		
Crohn's disease	[23]	
Chronic intestinal pseudo-obstruction and small intestinal bacterial overgrowth	[24]	<i>Candida albicans</i> and <i>Saccharomyces cerevisiae</i>
<b>Endogenous ethanol production in children</b>		
Rupture of stomach in African child	[25]	
Auto-Brewery Syndrome in a Child with Short Gut Syndrome: Case Report and Review of the Literature	[11]	<i>Candida glabrata</i> ( <i>Nakaseomyces glabratus</i> ) and <i>Saccharomyces cerevisiae</i>
Endogenous ethanol fermentation in a child with short bowel syndrome	[26]	<i>Candida kefyr</i> ( <i>Kluyveromyces marxianus</i> )
<b>General population</b>		
Endogenous Ethanol Production Levels in Saudi Arabia Residents	[27]	

Table 2. Ethanol-producing bacteria in humans

Phylum	Family	Genus	Species	References
<b>Bacillota</b>	<i>Bacillaceae</i>	<i>Weizmannia</i>	<i>Weizmannia coagulans</i>	[32]
	<i>Clostridiaceae</i>	<i>Clostridium</i>	<i>Clostridium amylolyticum</i>	[33]
			<i>Clostridium butyricum</i>	[34]
			<i>Clostridium cochlearium</i>	[35]
			<i>Clostridium sporogenes</i>	[36]
			<i>Clostridium tyrobutyricum</i>	[37]
			<i>Clostridium beijerinckii</i>	[38,39]
			<i>Clostridium malenominatum</i>	[35]
	<i>Enterococcaceae</i>	<i>Paraclostridium</i>	<i>Paraclostridium bifermentans</i>	[40,41]
		<i>Sarcina</i>	<i>Sarcina ventriculi</i>	[42]
		<i>Enterococcus</i>	<i>Enterococcus faecalis</i>	[43,44]
			<i>Enterococcus mundtii</i>	[45]
	<i>Lachnospiraceae</i>	<i>Blautia</i>	<i>Blautia coccoides</i>	[46]
			<i>Blautia hydrogenotrophica</i>	[47]
		<i>Enterocloster</i>	<i>Enterocloster bolteae</i>	[46,48]
		<i>Mediterraneibacter</i>	<i>Mediterraneibacter torques</i>	[49]
			<i>Mediterraneibacter gnavus</i>	[50]
	<i>Oscillospiraceae</i>	<i>Ruminococcus</i>	<i>Ruminococcus albus</i>	[51,52]
			<i>Ruminococcus flavefaciens</i>	[53]
			<i>Ruminococcus bromii</i>	[54]
		<i>Fructilactobacillus</i>	<i>Fructilactobacillus florum</i>	[55]
		<i>Lacticaseibacillus</i>	<i>Lacticaseibacillus casei</i>	[56,57]
			<i>Lentilactobacillus parabuchneri</i>	[58-60]
		<i>Lentilactobacillus</i>	<i>Lentilactobacillus buchneri</i>	[58,59,61]
			<i>Lentilactobacillus senioris</i>	[58,59]
		<i>Leuconostoc</i>	<i>Leuconostoc mesenteroides</i>	[62,63]
			<i>Leuconostoc lactis</i>	[64]
			<i>Leuconostoc pseudomesenteroides</i>	[65]
		<i>Levilactobacillus</i>	<i>Levilactobacillus brevis</i>	[66,67]
		<i>Limosilactobacillus</i>	<i>Limosilactobacillus reuteri</i>	[68-70]
			<i>Limosilactobacillus fermentum</i>	[71]
			<i>Limosilactobacillus panis</i>	[72,73]
	<i>Pediococcus</i>	<i>Pediococcus inopinatus</i>	[74]	
		<i>Pediococcus acidilactici</i>	[75-77]	
		<i>Pediococcus pentosaceus</i>	[78]	
	<i>Schleiferilactobacillus</i>	<i>Schleiferilactobacillus perolens</i>	[79]	
		<i>Schleiferilactobacillus harbinensis</i>	[79]	
	<i>Weissella</i>	<i>Weissella confusa</i>	[80]	
	<i>Paenibacillaceae</i>	<i>Paenibacillus</i>	<i>Paenibacillus polymyxa</i>	[81]
			<i>Paenibacillus macerans</i>	[82,83]
	<i>Staphylococcaceae</i>	<i>Staphylococcus</i>	<i>Staphylococcus aureus</i>	[43]
	<i>Streptococcaceae</i>	<i>Streptococcus</i>	<i>Streptococcus mutans</i>	[84,85]
			<i>Streptococcus agalactiae</i>	[86]
		<i>Lactococcus</i>	<i>Lactococcus lactis</i>	[87-90]
			<i>Lactococcus cremoris</i>	[90]
<b>Actinomycetota</b>	<i>Bifidobacteriaceae</i>	<i>Bifidobacterium</i>	<i>Bifidobacterium adolescentis</i>	[91-92]
			<i>Bifidobacterium bifidum</i>	[93,94]
			<i>Bifidobacterium breve</i>	[95,96]
			<i>Bifidobacterium longum</i>	[97]
			<i>Bifidobacterium animalis</i>	[98,99]
<b>Pseudomonadota</b>	<i>Enterobacteriaceae</i>	<i>Klebsiella</i>	<i>Klebsiella variicola</i>	[100]
			<i>Klebsiella aerogenes</i>	[101]
			<i>Klebsiella oxytoca</i>	[102,103]
			<i>Klebsiella pneumoniae</i>	[7,8]
		<i>Escherichia</i>	<i>Escherichia coli</i>	[104]
	<i>Raoultella</i>	<i>Raoultella planticola</i>	[105,106]	

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	<i>Aeromonadaceae</i>	<i>Aeromonas</i>	<i>Aeromonas hydrophila</i>	[107]
	<i>Erwiniaceae</i>	<i>Pantoea</i>	<i>Pantoea agglomerans</i>	[108]
	<i>Hafniaceae</i>	<i>Hafnia</i>	<i>Hafnia alvei</i>	[109]

For Review Only

**Table 3. Ethanol-producing fungi in humans**

Phylum	Family	Genus	Species	References	
<i>Ascomycota</i>	<i>Saccharomycetaceae</i>	<i>Saccharomyces</i>	<i>Saccharomyces cerevisiae</i>	[6,110]	
			<i>Saccharomyces kudriavzevii</i>	[110]	
		<i>Kluyveromyces</i>	<i>Kluyveromyces marxianus (Candida kefyi)</i>	[111,112]	
		<i>Nakaseomyces</i>	<i>Nakaseomyces glabratus ([Candida] glabrata)</i>	[113,114]	
	<i>Debaryomycetaceae</i>	<i>Candida</i>		<i>Candida sanyaensis</i>	[115]
				<i>Candida sojae</i>	[116,117]
				<i>Candida albicans</i>	[8,118]
				<i>Candida tropicalis</i>	[114,119]
		<i>Meyerozyma</i>	<i>Meyerozyma guilliermondii</i>	[120]	
			<i>Meyerozyma caribbica</i>	[121]	
	<i>Dipodascaceae</i>	<i>Geotrichum</i>	<i>Geotrichum candidum</i>	[122,123]	
	<i>Metschnikowiaceae</i>	<i>Clavispora</i>	<i>Clavispora lusitaniae</i>	[124,125]	
	<i>Phaffomycetaceae</i>	<i>Cyberlindnera</i>	<i>Cyberlindnera fabianii</i>	[126]	
			<i>Cyberlindnera saturnus</i>	[127,128]	
		<i>Wickerhamomyces</i>	<i>Wickerhamomyces anomalus</i>	[129,130]	
	<i>Barnettozyma</i>	<i>[Candida] norvegica</i>	[131]		
<i>Pichiaceae</i>	<i>Pichia</i>	<i>Pichia kudriavzevii</i>	[8,132]		
		<i>Pichia fermentans</i>	[133,134]		
	<i>Pichia membranifaciens</i>	[135,136]			
	<i>Saturnispora</i>	<i>Saturnispora silvae</i>	[137]		
<i>Saccharomycetales incertae sedis</i>	<i>Diutina</i>	<i>Diutina rugosa</i>	[138]		
<i>Trichomonascaceae</i>	<i>Wickerhamiella</i>	<i>Wickerhamiella pararugosa</i>	[139]		
<i>Basidiomycota</i>	<i>Sporidiobolaceae</i>	<i>Rhodotorula</i>	<i>Rhodotorula mucilaginosa</i>	[140]	

**Table 4. Most frequently cited yeasts for ethanol production in the literature**

Species	Number of Hits
<i>Saccharomyces cerevisiae</i>	2139
<i>Kluyveromyces marxianus</i> (heterotypic synonym <i>Candida kefir</i> )	143
<i>Candida albicans</i>	96
<i>Pichia kudriavzevii</i>	57
<i>Candida tropicalis</i>	39
<i>Wickerhamomyces anomalus</i>	30
<i>Nakaseomyces glabratus</i> OR <i>Candida glabrata</i>	18
<i>Clavispora lusitaniae</i> OR <i>Candida lusitaniae</i>	11
<i>Candida parapsilosis</i>	11
<i>Rhodotorula mucilaginosa</i>	10

**Table 5. Most frequently cited bacteria for ethanol production in the literature**

<b>Species</b>	<b>Number of hits</b>
<i>Escherichia coli</i>	765
<i>Staphylococcus aureus</i>	110
<i>Lactococcus lactis</i>	44
<i>Clostridium beijerinckii</i>	43
<i>Klebsiella pneumoniae</i>	39
<i>Enterococcus faecalis</i>	28
<i>Streptococcus mutans</i>	27
<i>Lacticaseibacillus casei</i>	23
<i>Limosilactobacillus fermentum</i>	23
<i>Leuconostoc mesenteroides</i>	16
<i>Klebsiella oxytoca</i>	16
<i>Levilactobacillus brevis</i>	15
<i>Klebsiella aerogenes</i>	13
<i>Lentilactobacillus buchneri</i>	11

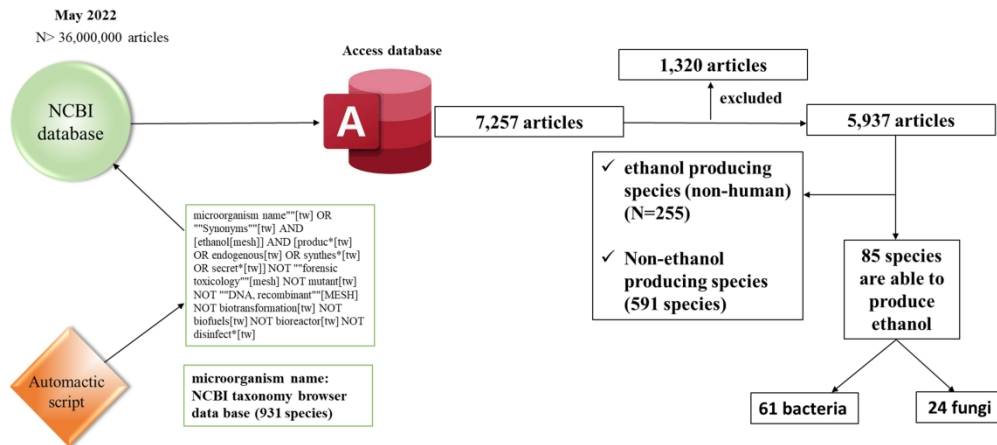


Figure 1: Flow chart of literature review

After an automatic script on the NCBI database, retrieved articles were manually screened for in vitro ethanol production. An Access (Access 2007, Microsoft, Paris, France) database was used to manage results and identify the number of hits for each ethanol-producing human microbial species. A 'hit' was defined as an article mentioning a human-associated microbial species AND an in vitro ethanol production reported in one article. Total 85 human microbes were found to be associated with in vitro ethanol production.

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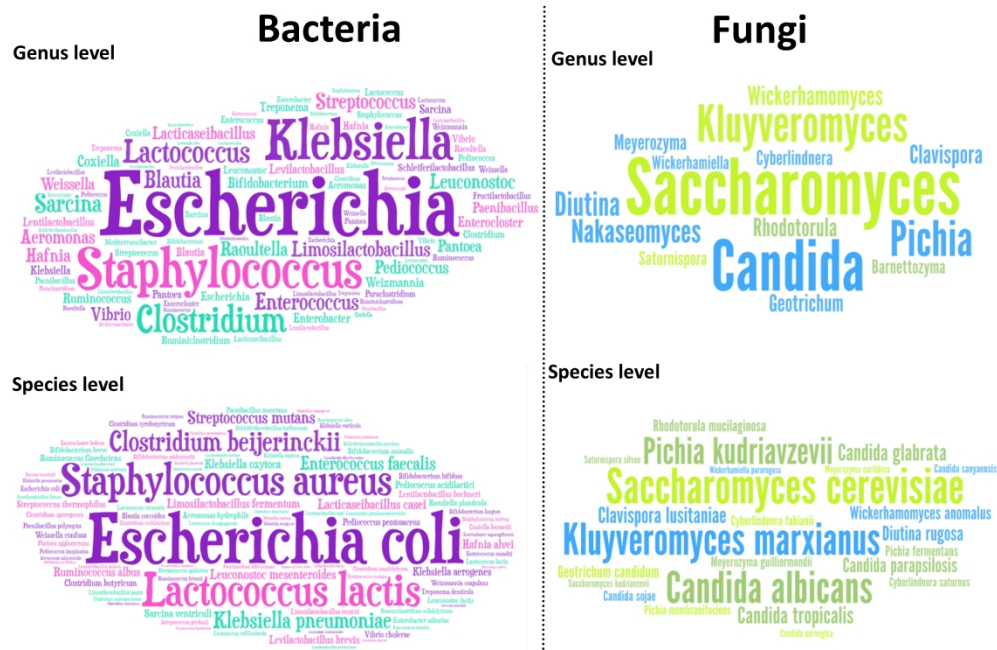


Figure 2: Number of articles mentioning in vitro ethanol production for bacteria (left) and yeast (right) at genus and species level.

The size of each taxon was correlated with the number of hits. Colors are for visual presentation and do not represent a color coded information. The Wordart online software was used (<https://wordart.com/>).

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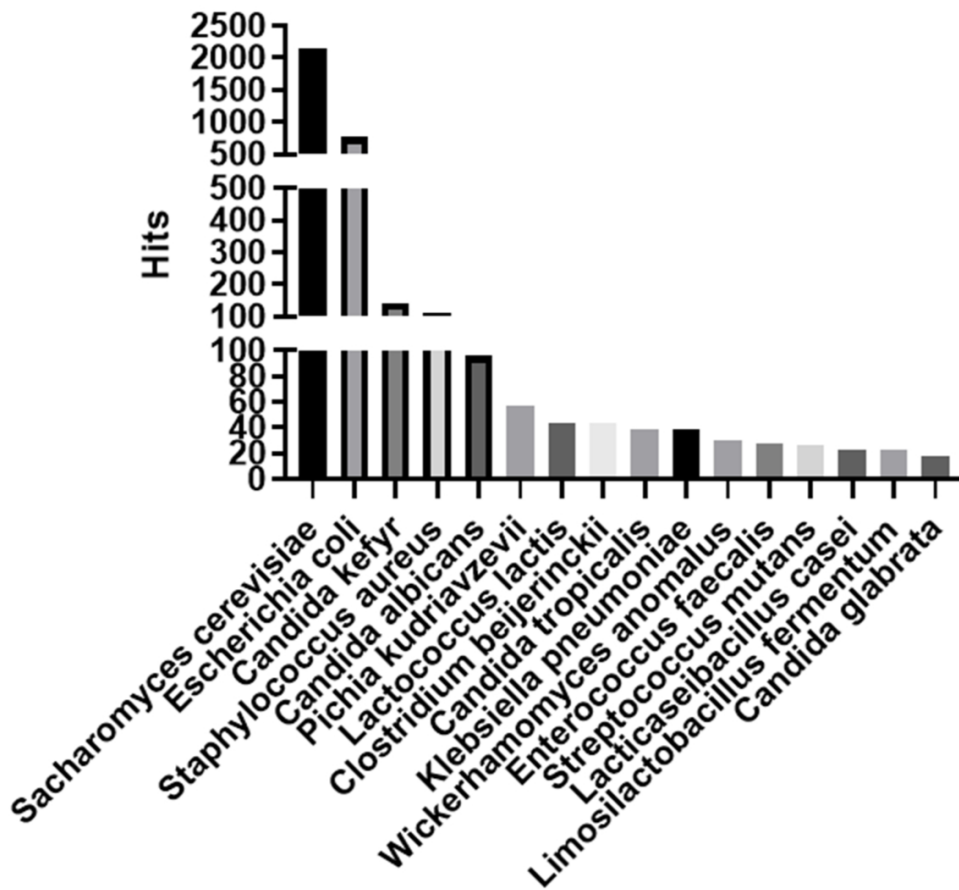


Figure 3: Human microbial species with the higher number of hits for in vitro ethanol production. Hits corresponded to the number of articles reporting a human associated microbes and reporting an in vitro ethanol production. *Saccharomyces cerevisiae* (2139 hits), *Escherichia coli* (765 hits), and *Kluyveromyces marxianus* (heterotypic synonym *Candida kefir* 143 hits) were the 3 species with the higher number of hits.

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Supplementary figure 1: Ethanol production pathway I

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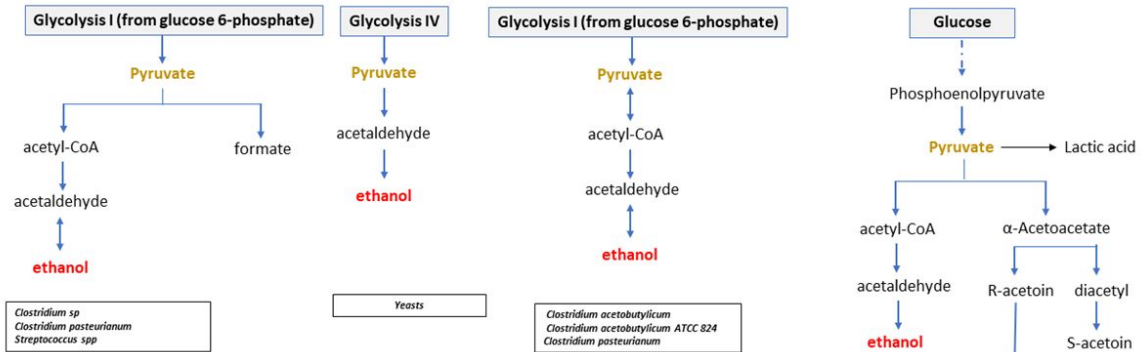


Figure 2. Pyruvate fermentation to ethanol via glycolysis pathway

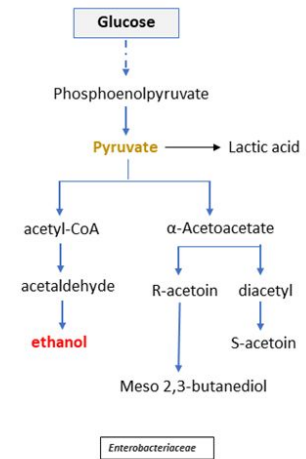
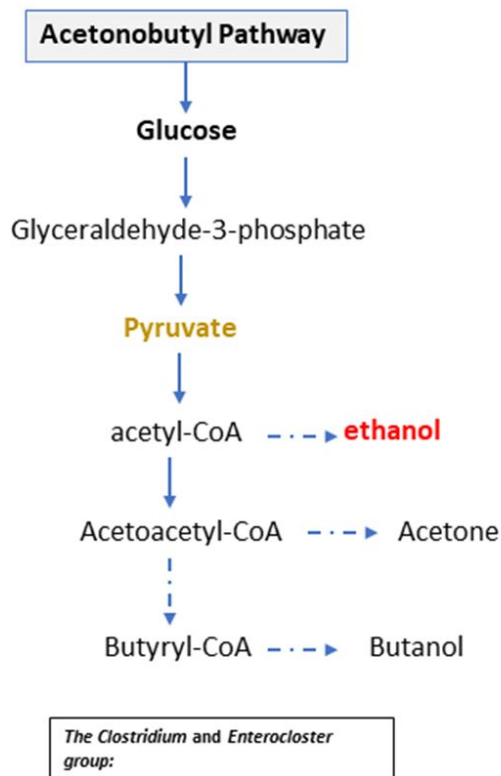


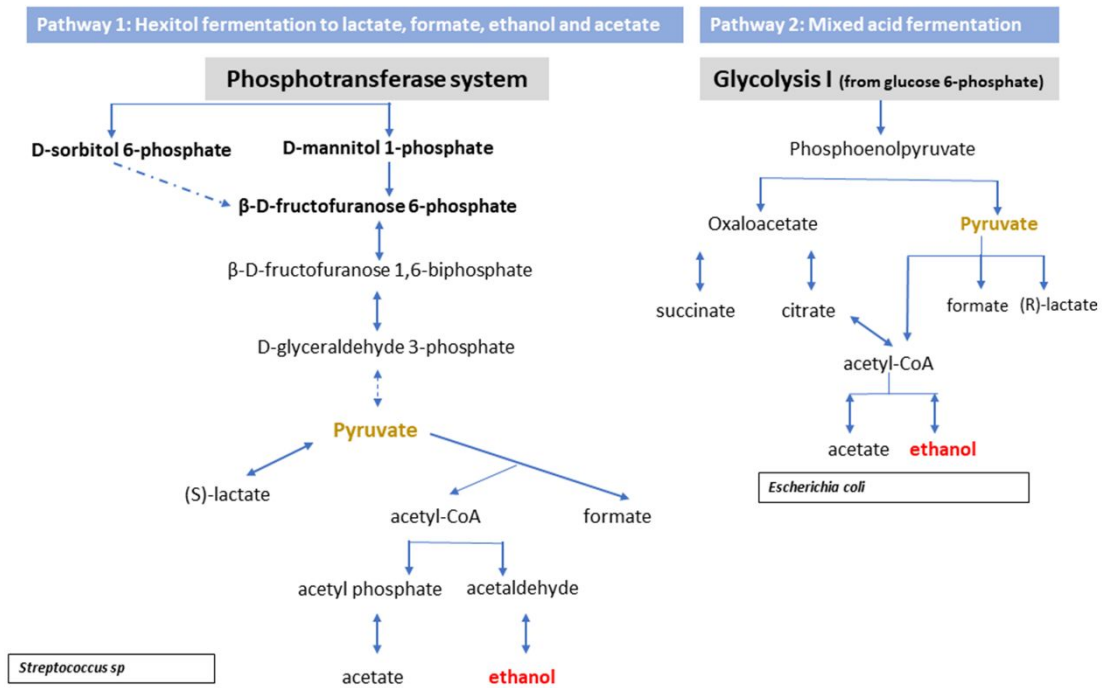
Figure 3. 2,3-butanediol fermentation pathway

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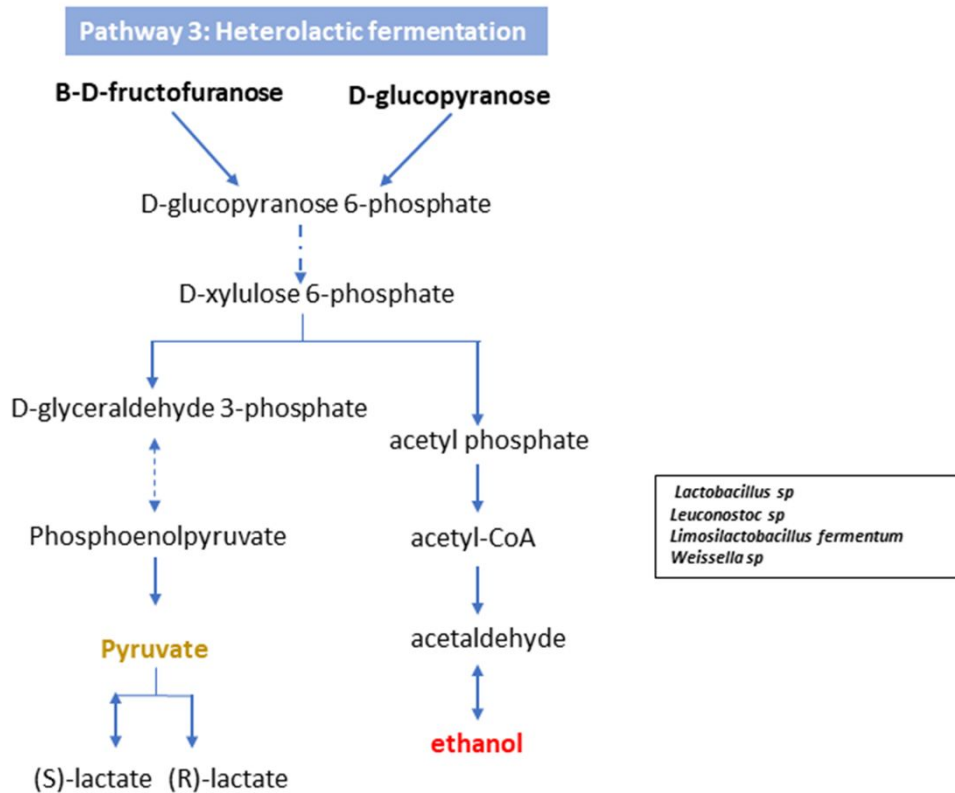


Supplementary figure 2: Ethanol production pathway II

A



B



## **Context 3**

# **Gut Microbiota Characterization in Patients with NASH**

## **Article 7**

### **Endogenous Ethanol and Triglyceride Production by Gut *Pichia kudriavzevii*, *Candida albicans* and *Candida glabrata* Yeasts in Non-Alcoholic Steatohepatitis**

Babacar Mbaye, Patrick Borentain, **Reham Magdy Wasfy**, Maryam Tidjani Alou, Nicholas Armstrong, Giovanna Mottola, Line Meddeb, Stéphane Ranque, René Gérolami, Matthieu Million and Didier Raoult

[« Article published in Cells »](#)

## Article

# Endogenous Ethanol and Triglyceride Production by Gut *Pichia kudriavzevii*, *Candida albicans* and *Candida glabrata* Yeasts in Non-Alcoholic Steatohepatitis

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**Abstract:** Nonalcoholic steatohepatitis (NASH) increases with fructose consumption and metabolic syndrome and has been recently linked with endogenous ethanol production, notably by high alcohol-producing *Klebsiella pneumoniae* (HiAlc Kpn). *Candida* yeasts are the main causes of auto-brewery syndromes but have been neglected in NASH. Here, the fecal ethanol and microbial content of 10 cases and 10 controls were compared. Ethanol was measured by gas chromatography-mass spectrometry. Species identification was performed by MALDI-TOF MS, and triglyceride production was assessed by a colorimetric enzymatic assay. The fecal ethanol concentration was four times higher in patients with NASH (median [interquartile range]: 0.13 [0.05–1.43] vs. 0.034 [0.008–0.57],  $p = 0.037$ ). Yeasts were isolated from almost all cases but not from controls (9/10 vs. 0/10,  $p = 0.0001$ ). *Pichia kudriavzevii* was the most frequent (four patients), while *Candida glabrata*, *Candida albicans*, and *Galactomyces geotrichum* were identified in two cases each. The concentration of ethanol produced by yeasts was 10 times higher than that produced by bacteria (median, 3.36 [0.49–5.60] vs. 0.32 [0.009–0.43],  $p = 0.0029$ ). Using a 10% D-fructose restricted medium, we showed that NASH-associated yeasts transformed fructose in ethanol. Unexpectedly, yeasts isolated from NASH patients produced a substantial amount of triglycerides. *Pichia kudriavzevii* strains produced the maximal ethanol and triglyceride levels in vitro. Our preliminary human descriptive and in vitro experimental results suggest that yeasts have been neglected. In addition to *K. pneumoniae*, gut *Pichia* and *Candida* yeasts could be linked with NASH pathophysiology in a species- and strain-specific manner through fructose-dependent endogenous alcohol and triglyceride production.

**Keywords:** nonalcoholic steatohepatitis; fructose; ethanol; *Pichia kudriavzevii*; *Candida*; yeast; fungi; auto-brewery syndrome; gut microbiota; gut mycobiome; metabolic syndrome; nonalcoholic fatty liver disease; metabolic-associated fatty liver disease; microbial culturomics

## 1. Introduction

Carbohydrate-dependent liver steatosis was first described many years ago [1]. This led to the recent identification of fructose as a major mediator of nonalcoholic fatty liver disease, including nonalcoholic steatohepatitis (NASH) [2]. Carbohydrate-dependent steatosis has increased in parallel with the increase in fructose consumption, particularly

in sweetened beverages and high fructose corn syrup (HFCS), which is correlated with the epidemic of obesity and type II diabetes [2]. Interestingly, carbohydrate-dependent steatosis is also accompanied by a significant increase in plasmatic triglycerides, whose role in liver steatosis has not been perfectly decrypted [2,3].

The other major metabolic liver disease is alcoholic liver disease, which also presents a succession of steatosis, fibrosis, cirrhosis, and eventually hepatocarcinoma [4]. Recently, the auto-brewery hypothesis has been revived by studies of the microbiota of patients with NASH [5,6]. Some studies have shown that NASH and auto-brewery syndromes (ABS) can occur after broad-spectrum antibiotic treatment (Supplementary Table S1). Reviewing 59 ABS cases from the literature, we found yeasts in most cases (*Candida* in 78%, *Saccharomyces* in 19%, and *Pichia* in 5%) and *Klebsiella pneumoniae* in 3% of cases (just 2 cases and only a single case without any gut yeast) ([5], Supplementary Tables S1 and S2, Supplementary Figure S1, Supplementary text).

At the same time, studies have shown a modification of the microbiota in NASH patients, particularly the identification of *K. pneumoniae*, whose in vitro analysis showed its capacity to produce alcohol in NASH patients [5]. This alcohol was also detected in the blood and stools of patients with NASH who, by definition, do not consume alcohol [5,7]. Nonsystematic works have also highlighted the possible association of yeasts with NASH and auto-brewery syndromes [5,8]. This is not surprising given that the first analysis of alcoholic fermentation reported by Lavoisier in 1789 was carried out with yeasts consuming sugar. Yeasts have been at the heart of human alcohol production for hundreds of years [9,10]. In addition to ethanol, triglycerides have been implicated in the pathophysiology of NASH [2,3]. However, in recent works linking yeast and NASH [8], the yeasts were not cultured, and ethanol and triglyceride production by gut yeast strains from NASH patients was not assessed.

In this work, we wanted to highlight the link that could exist between the microbiota, particularly fungi, and their production of alcohol and triglycerides based on fructose by using organisms specifically isolated from NASH patients.

## 2. Materials and Methods

### 2.1. Patients

We performed a case-control study comparing the fecal microbiota of 10 consecutive patients with nonalcoholic steatohepatitis (NASH) and 10 healthy controls. Patients were recruited from the Hepatology Department of Marseille University Hospital (Southeastern France). Healthy controls without liver disease were recruited from the same city using a snowball approach. Alcoholism, defined as drinking more than two alcoholic beverages per day for men and more than one alcoholic beverage per day for women, was an exclusion criterion for both cases and controls. Screening for diabetes, hypertension, elevated cholesterol, and hypertriglyceridemia was routine for cases. A history of diabetes, hypertension, elevated cholesterol, or hypertriglyceridemia were exclusion criteria for controls. In contrast, no blood pressure measurements or blood tests (to exclude diabetes or dyslipidemia) were performed in the controls. For patients, diagnostic criteria included (1) a metabolic syndrome (central obesity, increased triglycerides, reduced HDL cholesterol, high blood pressure, and type 2 diabetes), (2) steatohepatitis defined by the association of steatosis (liver steatosis at echography) with hepatitis (increased alanine aminotransferase (ALAT) more than two times the upper limit of normal and/or severe fibrosis), and (3) exclusion of differential diagnosis (notably alcoholic liver disease (>210 g/week in men or >140 g/week in women), chronic viral hepatitis (HBV, HCV), autoimmune hepatitis, hemochromatosis, and drug-induced liver disease). For cases and controls, exclusion criteria included antibiotics in the previous month, increased alcohol consumption (men  $\geq 30$  g/j, women  $\geq 20$  g/j), liver disease other than NASH, therapy that may cause steatosis (corticosteroid, amiodarone, estrogen, tamoxifen, and HIV protease inhibitors) and refusal or the impossibility of obtaining patient consent.

## 2.2. Measurement of Fecal Ethanol

One gram of stool was suspended in 5 mL of high-performance liquid chromatography (HPLC) water in Headspace glass vials. A calibration range was prepared from an ethanol stock solution in water with concentrations ranging from 0.5 to 100 mM. Standards and samples were spiked with isopropanol at 100 mM (internal standard). Measurements were performed using an HS-GC-MS system (Perkin Elmer, Villebon sur Yvette, France) combining an HS40 headspace injector, a Clarus 500 gas chromatograph, and an SQ8 S mass spectrometer. All vials were positioned onto the headspace sample tray after homogenization by shaking. They were heated one by one at 60 °C for 10 min to vaporize the alcohols that were automatically transferred to the gas chromatography system by overpressure (1 min, 25 psi) followed by a 1.8 s depressurization (needle/transfer line/GC inlet at 70/80/150 °C). Alcohols were introduced into a ZB-BAC2 chromatography column (30 m, 0.32 mm ID, 1.2 µm; Phenomenex, Le Pecq, France) maintained at 40 °C and separated using Helium as a carrier gas at 10 psi. Compounds were individually monitored by mass spectrometry with a selected ion recording (SIR) method: ethanol m/z 31 and isopropanol m/z 45. The MS inlet line and electron ionization source were set at 150 °C. All data were collected and processed using Turbomass 6.1 (Perkin Elmer) software. Internal calibration was calculated using the peak areas from the associated SIR chromatograms.

## 2.3. Selective Yeast Culture

Yeast culture was conducted by inoculating three fungal media by suspending 0.3 g of stool with 1 mL of 1X PBS for each sample. We used two commercial media, Chromoagar Candida agar (Becton Dickinson, Le Pont de Claix, France) and Sabouraud dextrose agar (Becton Dickinson, Le Pont de Claix, France). We also used another medium, the FastFung medium, specifically developed in our center suitable for the growth of fastidious fungi with a higher fungal colony count and lower culture contamination rate [11]. Serial dilutions were performed from 100 µL of the stock solution. For each tube, 50 µL was spread on each agar and incubated at 30 °C for 48 h before identification by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Microflex mass spectrometer (Bruker Daltonics, Leipzig, Germany), as previously reported [12].

## 2.4. Selective Culture of Enterobacteria and Resistance to Ethanol

Following the study of Yuan et al. [5] that highlighted the importance of specific strains of *Klebsiella pneumoniae* that produced an elevated level of ethanol in patients with NASH, we looked at the Gram-negative bacteria growing on MacConkey medium with or without ethanol in the medium. Thus, we chose a selective medium for nonfastidious Gram-negative bacteria: MacConkey agar liquid and solid medium. Two different approaches were used:

In the first approach, direct inoculation of diluted fecal samples was performed on MacConkey agar. Briefly, 0.3 g of stool was resuspended in 1 mL of 1X PBS after 10-fold serial dilution with 100 µL of this suspension, and 50 µL of each dilution was spread on MacConkey agar (Sigma-Aldrich, Saint Quentin Fallavier, France) and aerobically incubated at 37 °C for 24 h.

In the second approach, an enrichment step in a liquid medium with rumen and blood using blood culture bottles previously emptied of their contents was included. Briefly, enrichment was performed by adding 200 µL of the initial suspension (0.3 g of stools in 1 mL PBS 1X) of each sample inoculated into vials of aerobic blood cultures (bioMérieux, Durham, NC, USA) containing 20 mL of MacConkey broth (Dominique Dutscher, Brumath, France) enriched with 4 mL of defibrinated sheep blood and 4 mL of sterile rumen juice for 10 days. At Day 1, Day 3, Day 7, and Day 10, 500 µL of the bottle content was sampled followed by ten 10-fold serial dilutions and inoculation on MacConkey agar (Dominique Dutscher, Brumath, France). This second approach was also performed by adding 5% and 10% ethanol to the bottle to identify alcohol-resistant nonfastidious Gram-negative



bacteria. All colonies were identified using MALDI-TOF mass spectrometry as previously reported [13].

### 2.5. Measurement of Ethanol Production by Microorganisms

Yeast ethanol production was measured by inoculating 1 mL of a yeast suspension at  $1.5 \times 10^4$  cfu/mL into 20 mL of liquid Sabouraud broth (Dominique Dutscher, France) and incubating for 24 h at 30 °C. After incubation, 1 mL of each culture was placed in a glass vial tube to measure the ethanol concentration by headspace GC/MS as described above. All enterobacteria identified in the NASH patients and controls were grown in MacConkey broth (Dominique Dutscher, France) to measure their ability to produce ethanol. For this purpose, 1 mL of a bacterial suspension of  $1.5 \times 10^4$  cfu/mL was inoculated into 20 mL of liquid MacConkey broth and incubated for 24 h at 37 °C.

### 2.6. Ethanol and Triglyceride Production Assay on 10% D-Fructose

Beyond ethanol production by endogenous gut yeasts from NASH patients, we also investigated triglyceride production for all yeast strains isolated from included individuals and for a standard *Candida albicans* strain from our laboratory used as a control. Thus, we inoculated yeast at a cell density of 5 McFarland in triplicate into 5 mL of 10% D-fructose (Sigma-Aldrich) sterile water solution. After three days of incubation at 30 °C, 1000 µL of the supernatant was collected and analyzed on an Atellica<sup>®</sup> Solution Immunoassay and Clinical Chemistry Analyser (Siemens Healthineers, Saint-Denis, France) to measure triglycerides levels, following the manufacturer's instructions.

### 2.7. Statistical Analysis

To compare quantitative variables, the bilateral unmatched Mann-Whitney test was performed with GraphPad Prism version 9 for Windows (GraphPad Software, San Diego, CA, USA). A  $p$ -value  $\leq 0.05$  was considered significant.

## 3. Results

### 3.1. Human Descriptive Results

#### 3.1.1. Characteristics of the Ten Patients with NASH

The mean patient age was 70.3 years ( $\pm 7.6$ ), and 4 out of 10 patients were male (Table 1). The mean body mass index was 27.6 ( $\pm 3.2$ ); seven patients had diabetes, eight had hypertension, and four had dyslipidemia. Two patients had F0/F1 fibrosis assessed by pulse elastometry fibroscan<sup>®</sup> (ECHOSENS, Paris, France). The remaining eight patients had cirrhosis, of which one was assessed by pulse elastometry fibroscan<sup>®</sup>, three patients with compensated cirrhosis were assessed by clinical, biological, and morphological criteria, and four patients with decompensated cirrhosis were evaluated for liver transplantation. The characteristics of the patients and controls are described in Table 1.

**Table 1.** Patients' and controls' characteristics.

Patient	Sex	Age (Years)	Alcoholism <sup>a</sup>	Diabetes	Hypertension	Increased Cholesterol <sup>b</sup>	Hypertriglyceridemia <sup>c</sup>	Weight <sup>d</sup>
Nash1	M	58	No	Yes	Yes	No	No	Obesity
Nash2	M	69	No	Yes	Yes	Yes	No	Overweight
Nash3	F	66	No	Yes	Yes	Yes	No	Obesity
Nash4	M	83	No	No	Yes	No	No	Obesity
Nash5	F	71	No	Yes	Yes	Yes	Yes	Obesity
Nash6	F	92	No	Yes	Yes	No	No	Overweight
Nash7	F	64	No	Yes	Yes	Yes	Yes	Lean
Nash8	F	75	No	Yes	Yes	Yes	No	Overweight
Nash9	F	66	No	No	No	No	No	Lean
Nash10	M	62	No	Yes	Yes	Yes	Yes	Obesity

Table 1. Cont.

Patient	Sex	Age (Years)	Alcoholism <sup>a</sup>	Diabetes	Hypertension	Increased Cholesterol <sup>b</sup>	Hypertriglyceridemia <sup>c</sup>	Weight <sup>d</sup>
Ctrl1	M	24	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl2	M	26	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl3	M	38	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl4	F	30	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl5	M	27	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl6	M	31	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl7	F	44	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl8	F	27	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl9	F	24	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean
Ctrl10	M	39	No	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	No <sup>e</sup>	Lean

<sup>a</sup> Defined as an individual who drank more than two alcoholic beverages a day for men and more than one alcoholic beverage a day for women M: Male, F: Female, <sup>b</sup> Hypercholesterolemia was defined by total cholesterol > 2 g/L or LDL-cholesterol > 1.6 g/L, <sup>c</sup> Hypertriglyceridemia was defined by triglycerides > 1.5 g/L. <sup>d</sup> Obesity: body mass index (BMI)  $\geq$  30 kg/m<sup>2</sup>, Overweight: BMI between 25 and 30 kg/m<sup>2</sup>, Lean: BMI  $\leq$  25 kg/m<sup>2</sup>. <sup>e</sup> No known medical history.

### 3.1.2. Increased Fecal Ethanol Concentration in NASH

The fecal ethanol concentration was four times higher in patients with NASH (median [interquartile range]: 0.13 [0.05–1.43] g/L) than in the 10 healthy controls (0.034 [0.008–0.57], two-tailed Mann-Whitney test,  $p = 0.037$ , Figure 1, Supplementary Table S3).

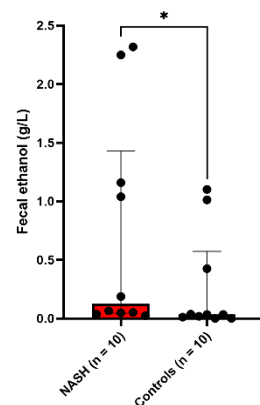


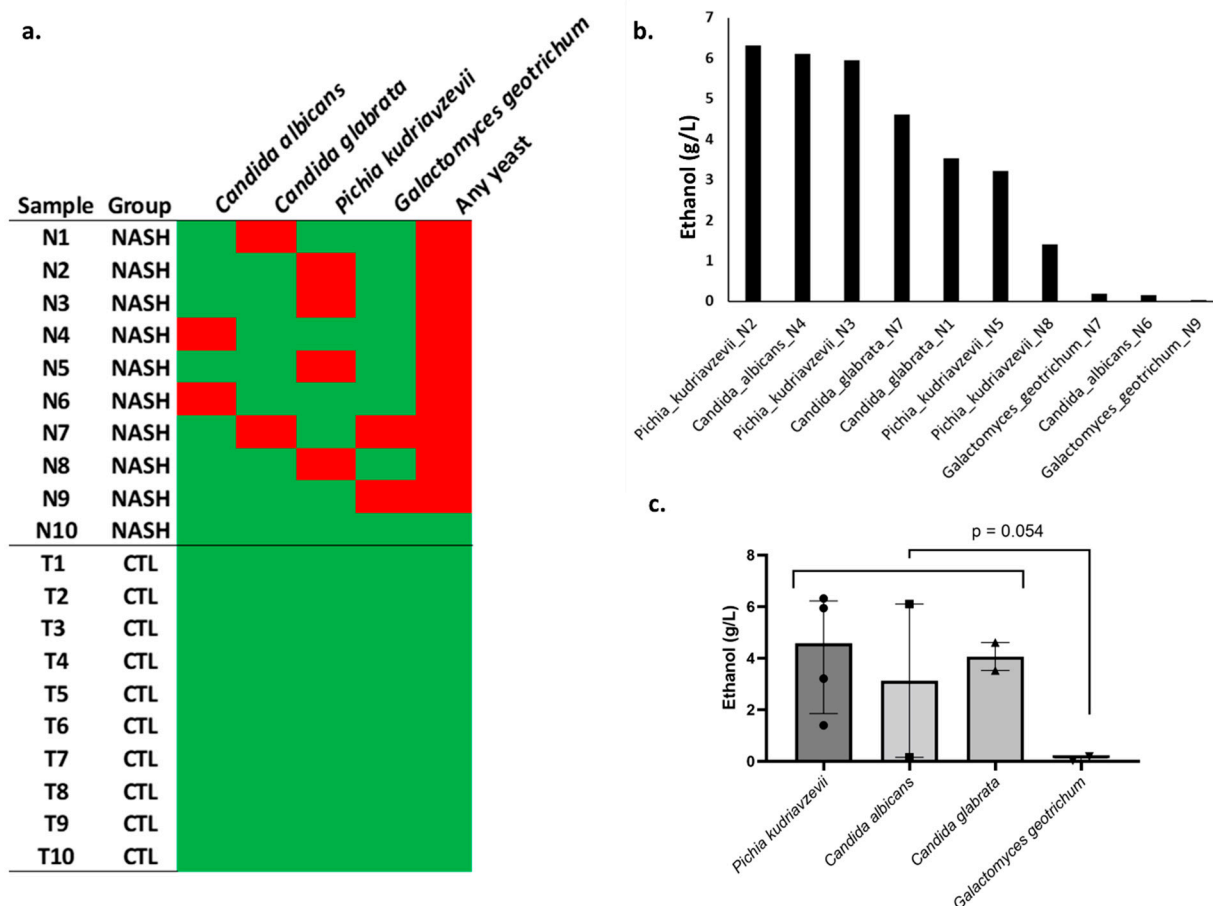
Figure 1. Increased fecal ethanol in NASH patients. \*  $p < 0.05$ .

### 3.1.3. High Prevalence of Gut Yeasts in NASH

In cultures on Chromoagar, FastFung, and Sabouraud media, we did not find any yeast in the 10 controls. However, at least one yeast was cultured in 9 of 10 NASH patients, and this was very significantly different from the controls (9/10 vs. 0/10, two-tailed Fisher test,  $p = 0.0001$ ). One patient had both *Candida* (*C. glabrata*) and *Galactomyces geotrichum*. At the species level, *Pichia kudriavzevii* was the most frequently isolated yeast (four patients), while *Candida glabrata*, *Candida albicans*, and *Galactomyces geotrichum* were identified in two patients (Figure 2a, Supplementary Table S4).

### 3.1.4. Increased Diversity of Potential Gram-Negative Pathogens in NASH

At the individual level, the number of bacteria growing in MacConkey broth enriched with blood and rumen (see methods) and agar with or without ethanol were not different between the two groups (median: two species per individual for both groups). Overall, the diversity of Gram-negative bacteria isolated was higher in patients with NASH. Indeed, 14 distinct species were isolated in the 10 patients with NASH compared with only 6 in the 10 controls (Figure 3, Supplementary Table S4).



**Figure 2.** Isolation and ethanol production by yeast strains in an in vitro experimental model. (a) Yeast species isolated from each sample (N1, Patient Nash1, etc.). NASH: nonalcoholic steatohepatitis, CTL: healthy controls. Red square: isolation of yeast in culture. Green: no yeast isolated. (b) Ethanol production for each strain in an in vitro experimental model (Sabouraud medium, high-performance liquid chromatography); each strain was named with the species and the patient (for instance, *Pichia kudriavzevii*\_N2 is a strain isolated from patient Nash2). (c) Comparison between species.

### 3.2. In Vitro Experimental Models

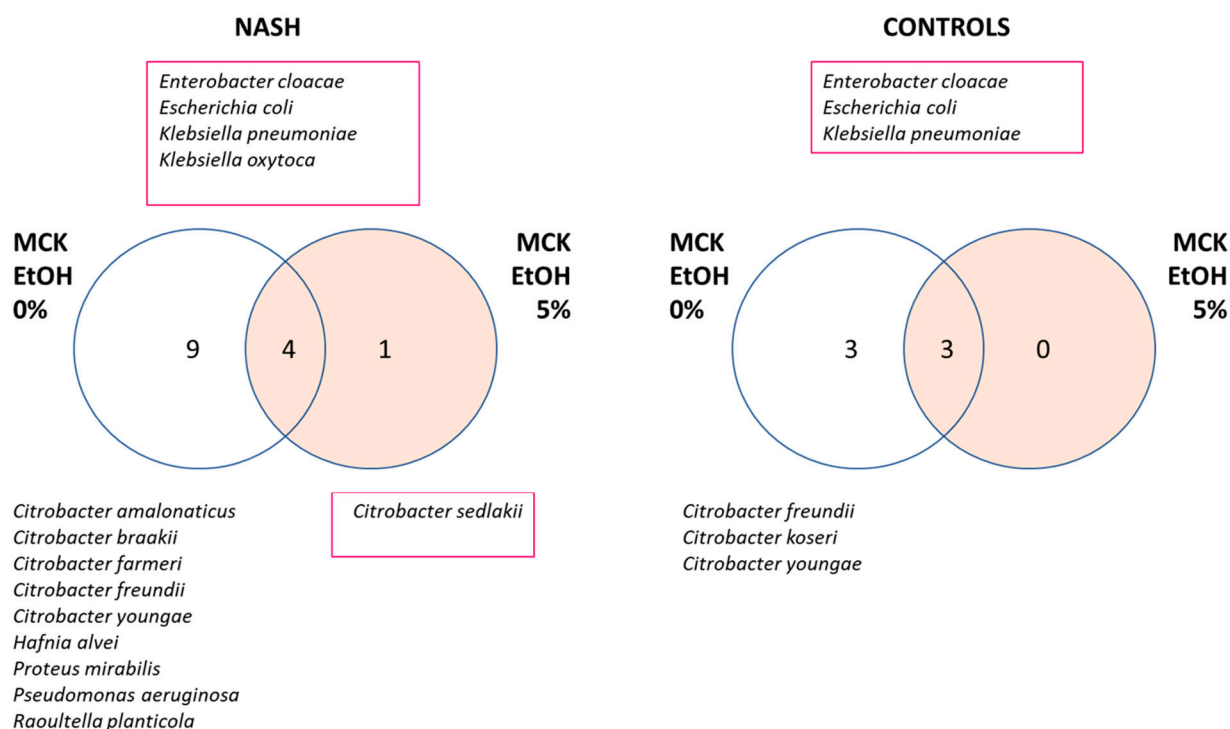
#### 3.2.1. In Vitro Experimental Model to Assess Ethanol Production of Gut Yeasts

In an in vitro experimental model, ethanol production was evaluated on the 10 yeast strains isolated from NASH patients. This production was particularly notable for strains of *P. kudriavzevii*, *C. albicans*, and *C. glabrata* species capable of reaching levels of 6.3 g/L, 6.1 g/L, and 4.6 g/L, respectively. A large variation between strains of the same species was observed. Indeed, for the same species, ethanol production ranged from 1.4 to 6.3 g/L for *Pichia kudriavzevii*, 0.15 to 6.1 for *C. albicans*, and 3.5 to 4.6 for *C. glabrata*, suggesting a considerable strain-dependent effect for ethanol production. In contrast, *G. geotrichum* isolated from two patients produced negligible amounts of ethanol (Figure 2, Supplementary Table S5).

#### 3.2.2. In Vitro Experimental Model Assessing the Dependence and Resistance to Ethanol of Enterobacteria Isolated in NASH

There were more species resistant to 5% ethanol in NASH patients (Figure 3). Indeed, three species were found in both groups able to grow with 5% ethanol in the medium: *Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae*. At the same time, two ethanol-resistant species were found only in patients with NASH: *Klebsiella oxytoca* and *Citrobacter*

*sedlakii*. The latter species grew only in the presence of ethanol and was considered an ‘ethanophile’ strain. No strain was resistant to 10% ethanol.

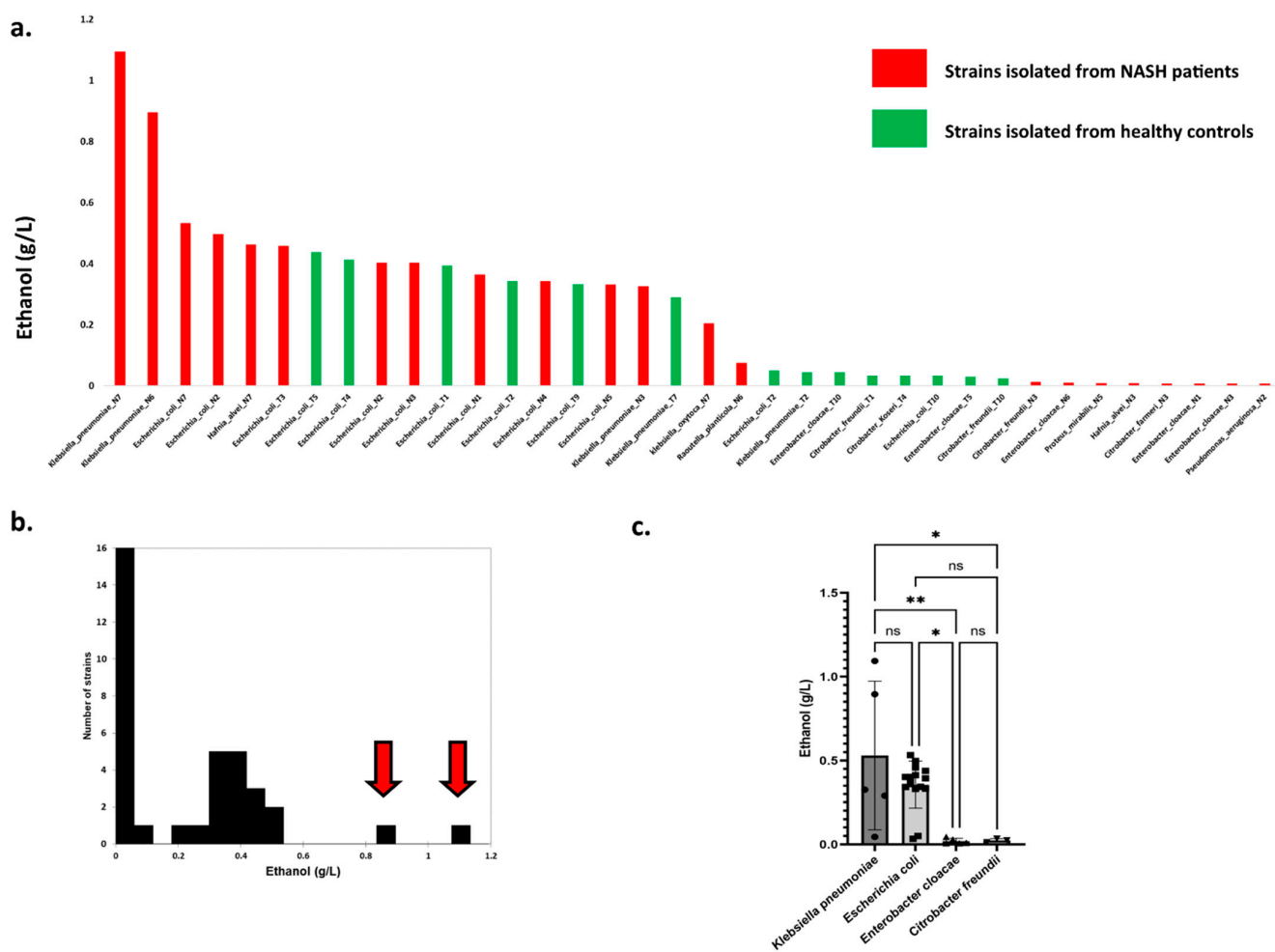


**Figure 3.** Increased diversity of Gram-negative bacteria in NASH patients. Strains were isolated using MacConkey medium. MCK: MacConkey medium, EtOH: ethanol, Species resistant to 5% ethanol were identified by a red square. A higher diversity of Gram-negative bacteria was observed in NASH patients.

Several *Citrobacter* species sensitive to 5% ethanol were found in both NASH patients and controls. In addition, several ethanol-sensitive species were specific to NASH, such as *Hafnia alvei*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Raoultella planticola*. It is interesting to note that *Pseudomonas aeruginosa* and *Proteus mirabilis* are well-known human pathogens, while *Hafnia alvei* and *Raoultella planticola* are increasingly recognized as emerging human pathogens [14,15].

### 3.2.3. In Vitro Experimental Model Assessing the Production of Ethanol Enterobacteria Isolated in NASH

In an in vitro experimental model, ethanol production was assessed for 36 available bacterial strains isolated on MacConkey medium corresponding to 11 Gram-negative species (Figure 4a, Supplementary Table S6). The six strains producing the highest levels of ethanol were all isolated from NASH patients. Surprisingly, two strains producing exceptional amounts of ethanol (Figure 4b, red arrow) were isolated from NASH patients and corresponded to the *Klebsiella pneumoniae* species. At the species level, only four species produced more than 0.2 g/L: *K. pneumoniae*, *E. coli*, *Hafnia alvei*, and *Klebsiella oxytoca*. A visual examination of ethanol production according to the strains (Figure 4a) showed three strain populations, namely, low ethanol producers (<0.2 g/L), strains with high production (>0.2 g/L), and strains with exceptional ethanol production (>0.6 g/L).



**Figure 4.** High ethanol-producing *Klebsiella pneumoniae* in NASH patients. **(a)** Production of ethanol by Gram-negative strains isolated in this study. The 2 strains producing the maximal ethanol concentration were both identified as *K. pneumoniae* from 2 different NASH patients (N6 & N7). **(b)** Distribution of strains according to ethanol production. Red arrows indicate two outliers with exceptional ethanol production. Both strains belong to the *K. pneumoniae* species and were isolated from NASH patients (N6 & N7). **(c)** Ethanol production according to bacterial species (species with at least 3 strains). \*  $p < 0.05$ , \*\*  $p < 0.005$ .

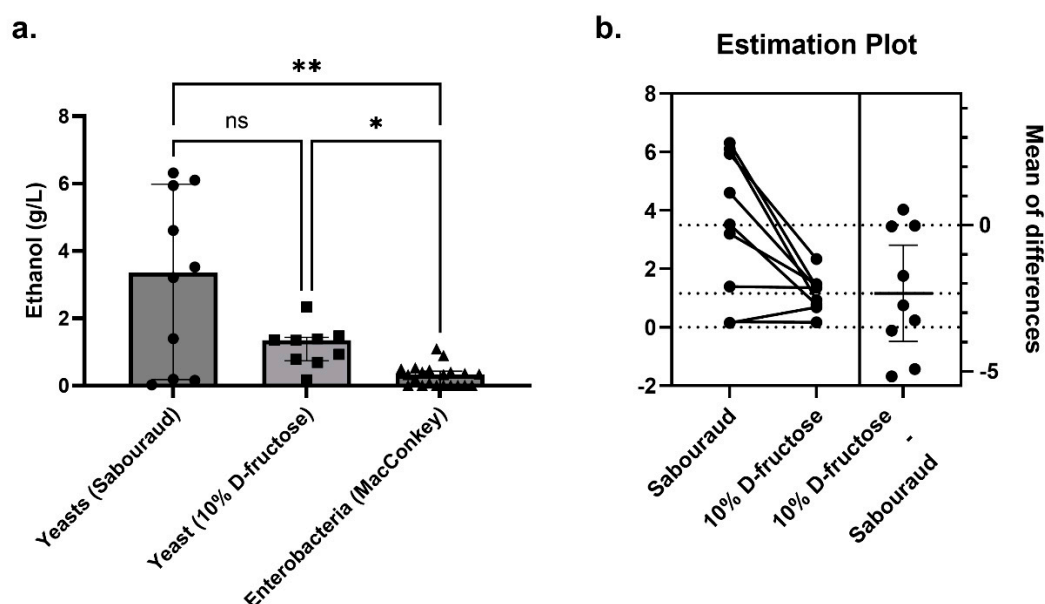
We observed that strains from NASH patients produced more ethanol than strains from controls for *K. pneumoniae*, *K. oxytoca*, and *H. alvei* species (Supplementary Figure S2). However, this was not the case for *E. coli*, for which no difference was observed. Finally, we compared ethanol production for species including at least three strains (Figure 4c). Only *Klebsiella pneumoniae* and *Escherichia coli* were associated with substantial ethanol production (mean between 0.3 and 0.5 g/L), while *Enterobacter cloacae* and *Citrobacter freundii* produced negligible amounts of ethanol. Collectively, these results suggest that among Gram-negative bacteria, *Klebsiella* is the genus most involved in endogenous ethanol production in NASH patients.

### 3.2.4. Comparison of Ethanol Production between Gut Yeast and Enterobacteria In Vitro

Endogenous ethanol production by enterobacteria has also been evaluated extensively and characterized in NASH [5]. Here, we identified yeast only in NASH patients, while enterobacteria were detected in both NASH patients and controls. In an in vitro experimental model, we found high endogenous ethanol production by yeast at high concentrations depending on species and strains. To assess the relative role of yeast and enterobacteria

in endogenous ethanol production in NASH, we compared the concentration of ethanol produced by yeast versus the concentration of ethanol produced by enterobacteria isolated from the 10 NASH patients included in the present study.

In total, 10 yeast strains producing a detectable amount of ethanol were isolated from nine NASH patients, and 21 strains of enterobacteria producing a detectable amount of ethanol were also identified from the NASH patients. The concentration of ethanol produced by yeast was 10 times higher than that produced by bacteria (median, [interquartile range]: 3.36 [0.49–5.60] for yeast versus 0.32 [0.009–0.43] for bacteria, Mann-Whitney test,  $p = 0.0029$ , Figure 5). For 7 of 10 gut yeasts, this production was much higher than that of the *K. pneumoniae* strain with the maximal ethanol production (1.09 g/L). This was strain dependent as one *C. albicans* strain produced a negligible amount of ethanol (Candida\_albicans\_N6, 0.15 g/L). In this patient, a high alcohol-producing *K. pneumoniae* strain (HiAlc Kpn) was identified (Klebsiella\_pneumoniae\_N6, 0.90 g/L, Figure 4a). In the one NASH patient who did not have yeast (Nash10), two enterobacteria were isolated: *Citrobacter youngae* and *Escherichia coli*. Unfortunately, the ethanol production of these two strains could not be evaluated.

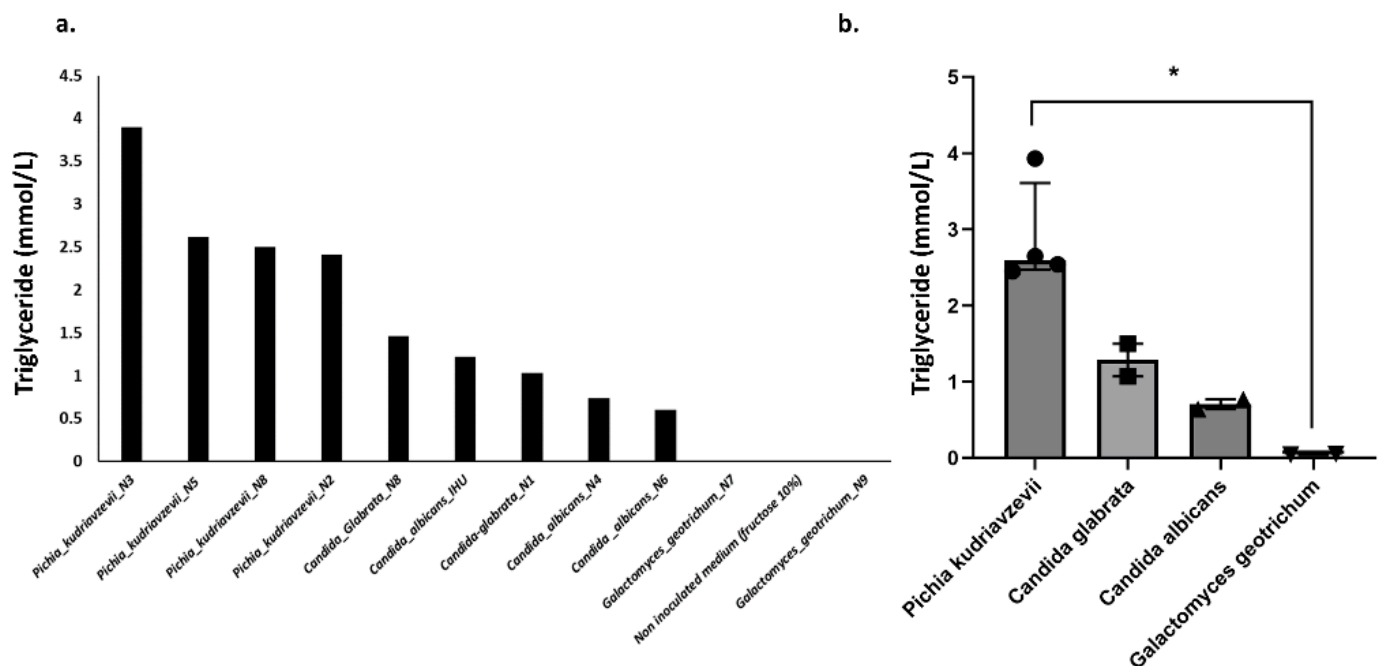


**Figure 5.** Ethanol production by yeast compared to enterobacteria isolated from NASH patients. (a) In Sabouraud's medium ( $n = 10$  yeast strains), the concentration of ethanol produced by yeast was 10-fold higher than that produced by enterobacteria (MacConkey's medium,  $n = 21$  bacterial strains), including *Klebsiella pneumoniae*, which produces a lot of alcohol (HiAlc Kpn, 1.09 g/L for the strain with maximum ethanol production). In a secondary analysis using 10% D-fructose restricted medium ( $n = 9$  yeast strains, Galactomyces\_geotrichum\_N9 was lost before this secondary experiment), substantial ethanol production was observed. \*  $p < 0.05$ , \*\*  $p < 0.005$ . (b) For the nine strains for which ethanol production could be assessed on both Sabouraud's and 10% D-fructose, ethanol production was significantly higher on Sabouraud's medium (paired two-tailed  $t$ -test,  $p = 0.015$ ).

Finally, Takahashi et al. [16] reported a synergistic effect of a strain of EtOH-producing enterobacteria, *Klebsiella pneumoniae*, and an EtOH-producing yeast, *Candida albicans*, isolated from an ABS patient. In this study, the coculture of *C. albicans* and *K. pneumoniae* produced considerably more alcohol than *C. albicans* alone [16]. Strikingly, our patient with the highest fecal ethanol concentration (Nash7, fecal EtOH = 2.3 g/L) harbored a high ethanol-producing *Candida glabrata* (*C. glabrata*\_N7, EtOH 4.6 g/L, Figure 2b) as well as the highest ethanol-producing bacterial strain, *Klebsiella pneumoniae* (*K. pneumoniae*\_N7, EtOH 1.09 g/L).

### 3.2.5. In Vitro Experimental Model Assessing Triglyceride Production by Gut Yeasts

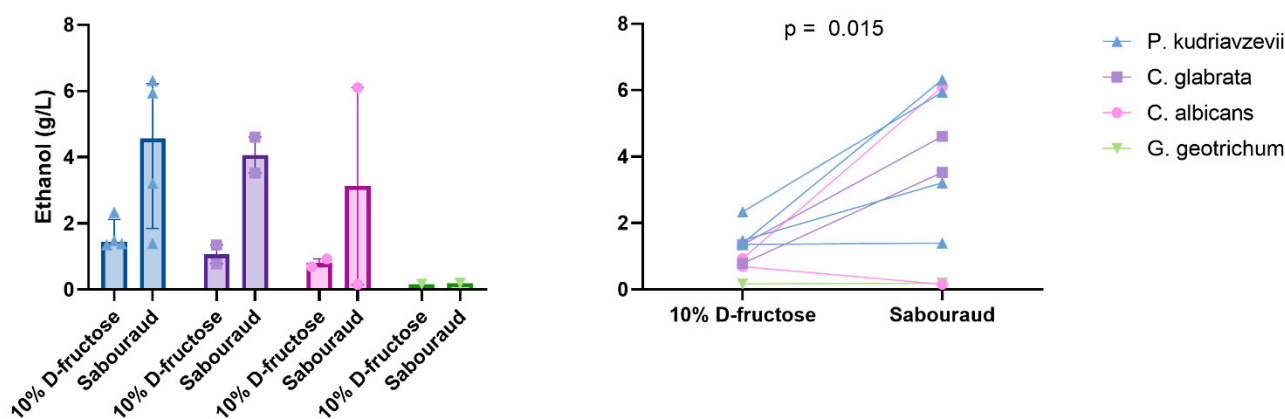
The species most involved in this triglyceride production were *Pichia kudriavzevii*, *C. glabrata*, and *C. albicans*, reaching levels of 3.9 mmol/L, 1.5 mmol/L, and 0.7 mmol/L, respectively. A large variation in triglyceride production between strains of the same species was observed (Figure 6). The production was highest for *Pichia kudriavzevii* species, ranging from 2.4 to 3.9 mmol/L, while *C. glabrata* produced 1.1 to 1.5 mmol/L, and *C. albicans* produced 0.6 to 0.8 mmol/L. In contrast, the isolated *Galactomyces geotrichum* strains produced low amounts of triglycerides (Figure 6, Supplementary Table S7). These results suggest that among the isolated yeasts, *Pichia kudriavzevii* appears to be the most involved in triglyceride production in NASH patients.



**Figure 6.** Triglyceride production by yeast strains. A value of 0.036 mmol/L was measured for ultrapure water and considered the background value. The values provided in the figure took into account this background value. (a) Triglyceride production for each strain, (b) Comparison between species. \*  $p < 0.05$ .

### 3.2.6. In Vitro Experimental Model Assessing Fructose as a Specific Substrate for Ethanol Production

We then tested the functional link between fructose consumption, specifically associated with NASH in the literature [2], and ethanol production by an in vitro model. In this model, strains were grown on a medium containing only water and 10% D-fructose. Only nine strains were studied. Indeed, the *Galactomyces geotrichum*\_N9 strain was lost despite several culture attempts. We observed that 10% D-fructose and water were sufficient for *Candida* and *P. kudriavzevii* to produce ethanol levels higher than the best HiAlc *K. pneumoniae* (up to >2 g/L, Figure 5, Supplementary Table S5). In particular, all four strains of *P. kudriavzevii* produced more than 1 g/L of ethanol from this fructose-restricted medium. For the nine strains for which ethanol production could be assessed on both Sabouraud's medium and 10% D-fructose medium, ethanol production was significantly higher on Sabouraud's medium (mean  $\pm$  SD,  $3.49 \pm 2.46$  g/L vs.  $1.16 \pm 0.61$  g/L, paired two-tailed  $t$ -test,  $p = 0.015$ , Figure 7).



**Figure 7.** Ethanol production by gut yeasts according to species and medium. Median and interquartile are shown. Paired two-tailed *t*-test.

#### 4. Discussion

This work allowed us to ascertain that patients with NASH commonly carry yeast in the gut and that these organisms are significantly associated with the presence of alcohol in the stool. Alcohol has very rarely been measured in human stool [5]. In this work, we were able to show that in controls without NASH, ethanol was not detected, whereas ethanol is present in the stool of patients with NASH. This simple measure could be a crucial element in the diagnosis and follow-up of patients with NASH. Thus, the association between yeast-related ABS and NASH seems significant. It is likely that the association between yeast and alcohol production is a key factor in the cause of NASH cases. Yeasts consume sugars, in particular glucose and fructose [17], while producing alcohol as has been known for centuries [9,10]. Furthermore, the production of triglycerides associated with NASH may also result from the fermentation of fructose by yeast [18].

Our results regarding the presence of yeasts suggest the possibility of eradicating yeast in the gastrointestinal tract as a novel method for managing NASH, emphasizing the need to determine whether such treatment could allow the elimination of alcohol from the gut. The link between fructose, yeast, and endogenous alcohol production suggests a link between the global explosion of fructose consumption in sweetened beverages [19] and the increase in the incidence of NASH [2]. Confirming such an association would require further epidemiological studies focusing on the fructose–NASH ratio and the fructose–endogenous ethanol ratio. It is possible that increased antibiotic use also promotes yeast colonization, and this is consistent with the fact that auto-brewery syndromes have been observed following various antibiotic therapies [20–25].

Recent reports have demonstrated the role of ethanol-producing bacteria in NASH, particularly enterobacteria such as *K. pneumoniae* [5] and *Citrobacter* [26]. These results reinforce the validity of the data replicated in our study. However, yeasts, clearly identified as a cause of ABS (Supplementary Tables S1 and S2, Supplementary text), have been completely neglected in most recent studies of NASH [5,26], even though they are microorganisms with a higher capacity for producing ethanol, even on a 10% D-fructose restricted medium, than bacteria (Figure 5). In the literature, two cases combining ABS and NASH proven by liver biopsy have been reported [5,27]. In addition to the study of Yuan et al. mentioned above [5], we found one case with the identification of the *Candida parapsilosis* yeast [27]. Gut yeasts could have a more important role than *K. pneumoniae* in endogenous ethanol production as we found that most gut *Candida* and *Pichia* yeast produced much higher ethanol levels (up to 6-fold) than the *K. pneumoniae* strain with the maximal ethanol production.

The study we report here is strengthened by the diversity of microorganisms that we included, involving both yeasts and bacteria. This seems all the more relevant, as we found two patients in the literature with ABS harboring both yeast and *Klebsiella pneumoniae* [16,23].



In addition, a synergistic effect was previously reported between *Klebsiella pneumoniae* and *Candida albicans* in an ABS patient [16].

Microbiological culturing was critical to the assessment of strain-dependent ethanol production. While recent studies report preliminary findings linking *Candida* yeasts and NASH using serology and metagenomics [8], the present study allowed the identification of viable and active bacteria and yeasts from living patients being treated for NASH. Accurate and rapid culture and identification of fungal species were essential to characterize yeast species and antifungal susceptibility. Our center was the first to offer matrix-associated laser desorption ionization spectrometry-time-of-flight for routine microbial identification in the diagnostic laboratory [13]. It is also a reference diagnostic laboratory for fungi for Southeastern France. Based on 6192 clinical yeast isolates, we have implemented fungal diagnostics using a comprehensive and updated MALDI-TOF MS database [12]. We also developed an optimized medium (the 'FastFung' medium) for the culture of fastidious fungi [11]. This allows us to rapidly, accurately, and confidently culture and identify the intestinal yeast species found in the present study.

The link between NASH and endogenous ethanol and triglyceride production was not demonstrated in the present study. However, our study is a strong argument for further testing this hypothesis. To demonstrate this link, the Bradford-Hill criteria could be considered as follows. (1) Strength: We found a dramatic effect size here as 9 out of 10 of the cases had intestinal yeast compared to none of the controls. Moreover, ethanol levels produced by yeasts were much higher than those observed with *K. pneumoniae*, recently linked with NASH in humans [5]. (2) Consistency/reproducibility: An association with intestinal yeast has already been found in other studies from other centers in other countries [8]. However, new studies using high-throughput fungal culture methods similar to those used in the present work [11–13] should be conducted in other centers and clarify if yeast is present in all or specific NASH patient types. Clearly, these preliminary results need to be replicated in larger studies with multivariate analyses taking into account more potential confounding factors, and appropriate corrections for multiple comparisons. (3) Specificity: The fungal species and strains must be identified. Indeed, one of the intestinal yeast species (*Galactomyces geotrichum*) and one of the two *C. albicans* strains isolated in the present study did not produce ethanol. (4) Temporality: This criterion could be evaluated in future in vitro or in vivo experimental models. (5) Biological Gradient: Future studies should evaluate a quantitative association between gut yeast concentration and ethanol and fecal triglycerides in NASH patients. (6) Plausibility and consistency with current knowledge are already demonstrated for endogenous ethanol production. (7) Analogy is provided in the present work with the auto-brewing syndrome. Indeed, in this disease, gut yeasts cause (neurological) disease through endogenous ethanol production, and antifungal treatment cures the disease. The analogy is also supported by the recent study linking ethanol-producing *K. pneumoniae* and NASH [5]. (8) Finally, the last criterion, reversibility, will be the most important: can antimicrobial treatment, based on the isolation of an ethanol-producing fecal microbe, reverse endogenous ethanol production and cure NASH patients? In addition to the study by Yuan et al. [5], our study is a call to action for active collaborations between nutritionists, liver disease specialists, microbiology laboratory, and infectious disease specialists.

Our preliminary results on endogenous triglyceride production from fructose by gut yeasts in NASH are completely new and decipher new hypotheses for gut microbiome-associated metabolic diseases. Future studies should better characterize acyl chain lengths and lipid droplets potentially produced by gut yeasts, and their role in the host's health and metabolic diseases. For instance, the mechanism of lipid droplet formation by the yeast *Saccharomyces cerevisiae* and its Sei1/Ldb16 Seipin complex has been only recently decrypted [28]. Maximal cholesterol assimilation has been evidenced for *Pichia kudriavzevii* yeast [29] but the possible impact on human lipid metabolism has not been investigated. Studies are needed to test if antifungal treatment could influence plasmatic triglyceride levels and liver fat.

Here, we present an original observational human study using multi-domain (bacterial and fungal) microbial culturomics [30] with in vitro experimental findings. Previous studies on the link between yeast and NASH used DNA sequencing and serological approaches [8]. The unparalleled advantage of culture (culturomics) over DNA or RNA sequencing (metagenomics) is to obtain strains. Isolation of patient strains in pure culture is the best way to characterize them metabolically (ethanol and triglyceride production), decipher the strain-specific effect [31], and investigate their susceptibility to antimicrobial treatment, but also to demonstrate their effect in vivo on liver fat regulation in future studies.

To confirm the putative instrumental role of gut yeasts for fatty liver disease and NASH through ethanol and triglyceride production, human studies remain of foremost importance. However, in vitro and animal models could help decipher the mechanism and are critical for causal inference, and to test reversibility. Here, we developed several in vitro models to better characterize ethanol resistance, dependence, and production by gut enterobacteria and yeasts. We isolated an 'ethanophile' strain suggesting that future 'microbial culturomics' studies on NASH should include culture media enriched in ethanol. In addition to fructose, several media using simple and/or complex carbohydrates will be helpful to decipher the substrate specificity for ethanol production. Indeed, this could have a major impact on the therapeutic diet for NASH patients. Lastly, a recent study showed that a hyperglycemia-stimulating diet induces liver steatosis in sheep [32]. Such animal models will be another opportunity to test and confirm the functional evidence of the putative link between NASH and endogenous ethanol and triglyceride production. Chiu et al. [33] recently reported an in vivo experimental study with fecal microbiota transplantation of control and NASH human feces to germ-free C57BL/6JNarl mice. Mice fecal content was analyzed by culturomics. Yeasts were reported in mice receiving NASH feces but not in those receiving CTL feces. The difference was significant. Strikingly, this result was not even discussed as the authors focused only on bacteria. This confirms that it is time to stop overlooking yeasts in NASH.

We have previously published a paper on the specific effect of *Lactobacillus* strain on weight regulation [31]. Again, in the present study, in the context of another nutritional and metabolic disease (NASH), we decipher a specific microbial effect of intestinal yeast on ethanol and triglyceride production in NASH patients (*P. kudriavzevii* > *C. glabrata* = *C. albicans* > *G. geotrichum*). However, even though the sample size is small, our results strongly suggest that the resolution at the species level is not sufficient. Indeed, among different strains of the same species, the ability to transform Sabouraud medium (40% glucose + peptone) or 10% D-fructose into ethanol is different. In patient 'NASH\_6', we identified a *C. albicans* strain (Candida\_albicans\_N6) with a low capacity to transform D-fructose into ethanol. However, his *Klebsiella pneumoniae* strain (K\_pneumoniae\_N6) produced more ethanol (0.90 g/L) than his yeast (*C. albicans*\_N6, 0.16 g/L on Sabouraud medium and 0.69 g/L on 10% D-fructose medium). This is the only yeast strain for which ethanol production was higher on D-fructose medium than on Sabouraud medium, but both were very low compared to high alcohol-producing yeasts. Experimental variability or strain specificity could explain this discrepancy. Nevertheless, the specific observation (*K. pneumoniae* > *C. albicans*) in this patient suggests that neither domain (yeast nor bacteria) can be overlooked in NASH.

Further studies are needed, but the strain-specific effect is critical for two reasons. First, it highlights the limitations of 16 s amplicon sequencing studies whose resolution is not capable of distinguishing strains of the same species. Indeed, species determination based on 250 bp (as is typically done in Illumina MiSeq instruments) is equivocal for polyphyletic taxonomic groups. Second, strain specificity is a definitive argument for culturomics to complement metagenomic studies. Indeed, only culturomic studies allow the domestication of live strains then available for full characterization, including for ethanol production.

The limitations of our study include a limited number of patients, the fact that the controls were younger than the cases, the use of only two culture media (Sabouraud and 10% D-fructose), and the incomplete analysis of metabolites produced beyond ethanol

and triglycerides. However, our study opens a new field. Future studies should include more patients, better matched, and more comprehensively investigate the substrates and products of NASH-associated yeasts. However, fructose and ethanol seem to be the most relevant substrate and product, respectively, given the state of knowledge and recent literature on NASH [2,5,7,8].

We can speculate that in the future, culturomics focused on ethanol-producing microbes (yeast and bacteria) could be part of the diagnostic workup of any patient with NASH. Once ethanol-producing strains are identified, precision and personalized medicine will allow clinicians to treat patients with appropriate antimicrobial molecules based on in vitro susceptibility testing and/or probiotics that interfere with ethanol production. For example, it has been demonstrated in vitro that *Lactobacillus paracasei*, a probiotic marketed worldwide and widely consumed, is able to decrease ethanol production by *Candida kefyri* in a species-specific manner [34]. If it is confirmed that microbial endogenous ethanol and triglyceride productions play a critical role in the pathophysiology of NASH, future management of NASH patients will necessarily include evaluation of the fecal microbiota (fungal and bacterial) and strain-specific treatment. Inspired by the study of Yuan et al. [5], our study confirmed the putative role of HiAlc strains of *K. pneumoniae* but expanded the field to the HiAlc fungi strains to offer great hope to NASH patients.

In conclusion, this work links the presence of *Candida*-type yeasts, alcohol, and triglyceride production in the digestive tract of living NASH patients. It opens avenues for the therapeutic management and prevention of this syndrome, whose very rapid increase seems to be linked to changes in dietary behavior. In addition, our findings suggest the possibility of specific, inexpensive therapeutics targeting yeasts producing alcohol as a method for treating ABS and NASH.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11213390/s1>, Figure S1: Repartition of species in 53 cases of auto-brewery syndrome in which at least 1 yeast and/or 1 bacterium was identified, Figure S2: Comparison of ethanol production between NASH and controls for each species; Table S1: Details and references of 59 cases of auto-brewery syndrome found in 55 articles from the literature, Table S2: Species associated with auto-brewery syndrome, Table S3: Fecal ethanol measurements, Table S4: Results of fungal and Gram-negative bacterial culture, Table S5: Ethanol production of yeasts, Table S6: Ethanol production of Gram-negative bacterial strains, Table S7: Triglyceride production by Yeast strains from NASH patients. References [35–38] are cited in the supplementary materials

**Author Contributions:** B.M. investigation, formal analysis, writing—original draft, visualization; P.B. investigation, formal analysis, writing—review & editing; R.M.W. investigation, formal analysis; M.T.A. writing—review & editing; N.A. formal analysis; G.M. investigation, formal analysis, writing—review & editing; L.M. methodology; S.R. investigation, supervision, validation; R.G. investigation, supervision writing—review & editing, project administration; M.M. conceptualization, supervision, formal analysis, writing—review & editing, project administration, visualization; D.R. conceptualization, supervision, writing—review & editing, project administration. All authors have read and agreed to the published version of the manuscript.

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### Abbreviations

ABS	Auto-brewery syndrome
ALD	Alcoholic liver disease
EtOH	Ethanol
GC-MS	Gas chromatography—mass spectrometry
HPLC	High-performance liquid chromatography
MALDI-TOF MS	Matrix assisted laser desorption ionization—time of flight mass spectrometry
MAFLD	Metabolic associated fatty liver disease
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

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## **Article 8**

### **Increased Fecal Ethanol and Enriched Ethanol-producing Gut Bacteria *Limosilactobacillus fermentum*, *Enterocloster bolteae*, *Mediterraneibacter gnavus* and *Streptococcus mutans* in Nonalcoholic Steatohepatitis**

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# Increased fecal ethanol and enriched ethanol-producing gut bacteria *Limosilactobacillus fermentum*, *Enterocloster bolteae*, *Mediterraneibacter gnavus* and *Streptococcus mutans* in nonalcoholic steatohepatitis

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**Background:** Non-alcoholic steatohepatitis (NASH) has become a major public health issue as one of the leading causes of liver disease and transplantation worldwide. The instrumental role of the gut microbiota is emerging but still under investigation. Endogenous ethanol (EtOH) production by gut bacteria and yeasts is an emerging putative mechanism. Microbial metagenomics and culture studies targeting enterobacteria or yeasts have been reported, but no culturomics studies have been conducted so far.

**Aim:** To assess fecal EtOH and other biochemical parameters, characterize NASH-associated dysbiosis and identify EtOH-producing gut microbes associated with the disease, fecal samples from 41 NASH patients and 24 controls were analyzed. High-performance liquid chromatography (HPLC) was used for EtOH, glucose, total proteins, triglyceride and total cholesterol. Viable bacteria were assessed with microbial culturomics. Microbial genetic material was assessed using 16S metagenomics targeting the hypervariable V3V4 region.

**Results:** Fecal EtOH and glucose was elevated in the stools of NASH patients ( $p < 0.05$ ) but not triglyceride, total cholesterol or proteins. In culturomics, EtOH-producing *Enterocloster bolteae* and *Limosilactobacillus fermentum* were enriched in NASH. V3V4 16S rRNA amplicon sequencing confirmed the enrichment in EtOH-producing bacteria including *L. fermentum*, *Mediterraneibacter gnavus* and *Streptococcus mutans*, species previously associated with NASH and other dysbiosis-associated diseases. Strikingly, *E.*

*bolteae* was identified only by culturomics. The well-known *Lactococcus casei* was identified in controls but never isolated in patients with NASH ( $p < 0.05$ ).

**Conclusion:** Elevated fecal EtOH and glucose is a feature of NASH. Several different EtOH-producing gut bacteria may play an instrumental role in the disease. Culturomics and metagenomics, two complementary methods, will be critical to identify EtOH-producing bacteria for future diagnostic markers and therapeutic targets for NASH. Suppression of EtOH-producing gut microbes and *L. casei* administration are options to be tested in NASH treatment.

#### KEYWORDS

non-alcoholic steato-hepatitis, metabolic-associated fatty liver disease, endogenous ethanol, gut microbiota, enterocloster bolteae, limosilactobacillus fermentum, mediterraneanibacter granovus, streptococcus mutans

## Introduction

Non-alcoholic fatty liver disease (NAFLD), including its most severe form, non-alcoholic steatohepatitis (NASH), is becoming a major public health issue (Younossi et al., 2016). In a recent meta-analysis, the prevalence of NASH was estimated between 1.50% and 6.45% of the general population. Overall, mortality incidence rates ranged from 11.77 (range 7.10-19.53) for NAFLD to 25.56 (range 6.29-103.80) per 1,000 person-years for NASH (Younossi et al., 2016). According to the 2018 Global Burden of Disease study, the prevalence of NAFLD increases exponentially up to 25% worldwide alongside metabolic syndrome and diabetes (Younossi et al., 2018) and will soon be the leading cause of liver transplantation worldwide (Haldar et al., 2019). Although viral hepatitis remains the leading cause of mortality, NAFLD is the fastest-growing cause of liver morbidity and mortality (Paik et al., 2020). For example, age-standardized mortality rates are falling for HBV, HCV, and ALD (alcohol liver disease), but this rate is rising for NAFLD (Paik et al., 2020).

Dysbiosis of the gut microbiota has already been associated with metabolic diseases including diabetes (Wu et al., 2023), obesity (Million et al., 2012), dyslipidemia (Guo et al., 2022) but its involvement in NAFLD and more specifically NASH is not clearly established. Recent studies have shown using metagenomics a dysbiotic profile in NASH patients characterized by an increase in *Proteobacteria* at the phylum level (Zhu et al., 2013; Yuan et al., 2019), *Lactobacillaceae* (Meijnikman et al., 2022) and *Enterobacteriaceae* (Zhu et al., 2013; Yuan et al., 2019) at the family level, and *Lactobacillus*, *Clostridium* and *Escherichia* (Zhu et al., 2013; Yuan et al., 2019; Jin and Xu, 2023) at the genus level.

Endogenous ethanol (EtOH) production by gut microbes has recently (Zhu et al., 2013) been identified as one of the mechanisms underlying the role of the gut microbiota in the pathogenesis of NASH (Zhu et al., 2013; Yuan et al., 2019). Zhu et al. found high concentrations of EtOH in the blood of NASH patients, confirmed in patients' stools by Yuan et al. (Zhu et al., 2013; Yuan et al., 2019). Case-control studies have shown the involvement of specific microorganisms in endogenous EtOH production, notably yeasts of the *Candida* and *Pichia* genus (Mbaye et al., 2022) and bacteria including *Klebsiella pneumoniae*, *Limosilactobacillus fermentum* and *Lactococcus lactis* (Mbaye et al., 2023; Yuan et al., 2019).

The *Clostridium* genus, which was previously associated with NASH (Zhu et al., 2013; Jin and Xu, 2023), has recently been reclassified into several new genera including the *Enterocloster* genus that consists of six species (Haas and Blanchard, 2020). *Enterocloster* spp. have already been associated with diseases. *Enterocloster citroniae* was associated with type 2 diabetes (Ruuskanen et al., 2022), *Enterocloster clostridioformis* with fatty liver (Ruuskanen et al., 2021) and *Enterocloster bolteae* with autism, diabetes, and fatty liver (Ruuskanen et al., 2021; Frame et al., 2023). Two species of this genus, *E. bolteae* and *Enterocloster asparagiformis*, are known to produce EtOH *in vitro* (Mohan et al., 2006).

To date, no culturomics studies have been carried out to characterize the microbiota of NASH patients. This untargeted omics method remains essential and complementary to metagenomics for exploring the microbiota. Indeed, this approach yields different but complementary results for gut microbial profiling. Indeed, it is a high throughput culture approach which is based on the multiplication of culture conditions with a variation of physico-chemical parameters such as culture media, atmosphere, and temperature, combined with MALDI-TOF MS for identification of the generated colonies. It thus allowed the isolation of over 300 previously unknown bacterial species in the human gut including species that were considered uncultivable using conventional culture methods (Lagier et al., 2016). This method is invaluable as only culture-based approaches provides living bacterial strains from

**Abbreviations:** NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; CPR, Candidate Phyla Radiation; OUT, Operational taxonomic units.



complex samples (Lagier et al., 2012a; Tidjani Alou et al., 2017) which can be studied further and potentially be used as probiotics for targeted bacteriotherapy (Cao et al., 2023; Song et al., 2023).

In this study, we assessed biochemical parameters specially focusing on fecal EtOH concentration. Culturomics was used for the first time to characterize the composition of the gut microbiota of NASH patients compared with that of healthy controls. 16S rRNA targeted amplicon sequencing was used as a complementary method. Comparison between the two methods was performed, expected to yield different but overlapping results, as previously published (Lagier et al., 2012b).

## Methods

### Participants

We performed a case-control study comparing the fecal EtOH and microbiota using culturomics and metagenomics methods. Participants were recruited in the hepatology department where they were monitored. Cases were diagnosed with NASH according to recruitment criteria described in our previous study (Mbaye et al., 2022) whereas controls were individuals with no diagnosed liver disease. Stool collection occurred between January and June 2022. It is noteworthy that no blood samples were collected for this study. Exclusion criteria for both groups were antibiotics within the month prior to sampling, excessive alcohol consumption (men  $\geq 30$  g/d, women  $\geq 20$  g/d), steatosis-inducing drugs, and refusal of consent by the participant. Participants were not instructed to fast or abstain from drinking alcohol prior to submitting stool samples.

### Ethical considerations

This study was conducted upon obtaining the approval of the Local Ethics Committee and “Comité de Protection des Personnes” (CPP: 21.04391.000046 - 21075). The informed and written consent of each participant was collected in compliance with the Declaration of Helsinki (World Medical Association., 2013).

### Samples

As we previously showed that culturomics efficiently discriminates cases from controls in another nutritional disease (severe acute malnutrition (Pham et al., 2019)) and only highlights viable bacteria (Bellali et al., 2019), we primarily focused on culturomics results (14 NASH and 10 controls). Not all samples could be analyzed by culturomics as it is a time-consuming method compared to V3V4 16S rRNA amplicon sequencing (culturomics ~ 6 weeks per sample with ~ 5,000 colonies per sample, V3V4 16S rRNA sequencing ~ 1 week for 96 samples). Therefore, the number of samples analyzed using culturomics simultaneously was limited (a maximum of three samples due to the extensive workload) and we aimed at analyzing at least one control for one or two cases simultaneously. Thus, only the first 24 samples (14 NASH and 10

controls) were analyzed using culturomics whereas all samples (41 NASH and 24 controls) were analyzed using V3V4 16S amplicon sequencing simultaneously. No sample was common to our previous studies (Mbaye et al., 2022; Mbaye et al., 2023) allowing us to test reproducibility of our previous findings using different methods (specific yeast and enterobacterial microbial culture were used in our previous study but not untargeted culturomics). Characteristics of the population in this study are described in Table 1.

### Measurement of fecal ethanol

For biochemical measurements, 0.05g of stool was suspended in 1.5 mL of high-performance liquid chromatography (HPLC) water

TABLE 1 Baseline characteristics.

	Controls (n = 24)	NASH (n = 41)
<b>Demographic characteristics</b>		
Age: mean $\pm$ SD	63.8 $\pm$ 7.6	64.2 $\pm$ 9.0
Female ratio (%)	14 (58%)	18 (44%)
<b>Comorbidities</b>		
Alcohol consumption	8 (33%)	5 (12%)
EtOH intake < 20 g/j	8 (33%)	2 (5%)
EtOH intake $\geq$ 20 g/j	0 (0%)	3 (7%)
Body Mass Index (kg/m <sup>2</sup> ): mean $\pm$ SD	26.0 $\pm$ 2.5	30.0 $\pm$ 5.0
Normal weight (18.5 < BMI < 24.9)	12	4
Overweight (25.0 $\leq$ BMI < 29.9)	11	21
<b>Obesity</b>	1 (4%)	16 (39%)
Moderate obesity (30.0 $\leq$ BMI < 34.9)	1	10
Severe obesity (35.0 $\leq$ BMI < 39.9)	0	5
Morbid obesity (BMI $\geq$ 40)	0	1
Diabetes mellitus (%)	0 (0%)	24 (59%)
Hypertension (%)	4 (17%)	26 (63%)
Dyslipidemia (%)	0 (0%)	19 (46%)
Smoking (%)	0 (0%)	6 (15%)
<b>Liver fibrosis stage</b>		
F1	0 (0%)	6 (15%)
F2	0 (0%)	6 (15%)
F3	0 (0%)	8 (19%)
F4	0 (0%)	17 (41%)
No information on fibrosis stage	0 (0%)	1 (2%)
Hepatocellular carcinoma	0	8 (19%)
Ascitis	0	3 (7%)

BMI, Body mass index; NASH, Nonalcoholic steatohepatitis; SD, standard deviation.

in Eppendorf tubes. The tubes were homogenized and centrifuged at 250 rpm for 15 to 20 minutes. 1 mL of the supernatant was collected after filtration at 0.22  $\mu$ m in freeze-drying tubes to perform the measurement of biochemical parameters (EtOH, glucose, triglycerides, total proteins and cholesterol) using the Atellica® Solution Immunoassay and Clinical Chemistry Analyser (Siemens Healthineers, Saint-Denis, France) according to the manufacturer's instructions.

## Bacterial isolation using culturomics

Microbial culturomics was used to explore the bacterial diversity of our stool samples generating a large number of colonies due to the various culture conditions used which identification is facilitated by the use of the MALDI-TOF MS technology as explained below.

For that purpose, direct inoculation of the stool sample was performed with 0.3 g of stool resuspended in 1 mL of 1x PBS. Ten serial dilutions of this suspension were realized, and 50  $\mu$ L of each dilution was spread on 5% Columbia (COS) agar enriched with sheep blood (bioMerieux Marcy l'Etoile, France) and modified YCFA (yeast extract, casein hydrolysate, fatty acids) agar ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium1611.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1611.pdf)). Inoculated agar plates were incubated in aerobic and anaerobic atmospheres at 37°C for 48 hours.

Additionally, enrichments were made in liquid broths. Anaerobic culture conditions consisted of 200  $\mu$ L of each sample inoculated in anaerobic blood culture bottles (bioMerieux, Durham, NC, USA) and YCFA medium, both supplemented with 5% of defibrinated sheep blood and 5% of 0.22  $\mu$ m filtered rumen fluid. Serial dilutions of each culture were inoculated on COS agar and YCFA agar over a one-month period at different timepoints (24h, day 3, day 7, day 10, day 15, day 21, day 30). The colonies obtained were sub-cultured after a 48 hour-incubation at 37°C using Zip bag (Oxoid, Dardilly, France) containing an anaerobic generator, gasPak (Becton Dickinson, Le Pont de Claix, France). An aerobic condition was also performed with 200  $\mu$ L of stool inoculated into an aerobic blood culture bottle (BioMerieux, Marcy l'Etoile, France) medium supplemented with 5% of defibrinated sheep blood and 5% of 0.22  $\mu$ m filtered rumen fluid. Serial dilutions were inoculated on COS agar over a one-month period at different timepoints (24h, day 3, day 7, day 10, day 15, day 21, day 30) and incubated for 24 hours at 37°C in an aerobic atmosphere. Colonies were sub-cultured in the same conditions. Purified colonies were identified using MALDI-TOF MS and genome sequencing when necessary (Lagier et al., 2012a). Briefly, isolated colonies were then plated on a 96 MSP plate with 2  $\mu$ L of pre-prepared matrix solution containing saturated  $\alpha$ -cyano-4-hydroxycinnamic acid, 50% acetonitrile and 2.5% trifluoroacetic acid. Spectra were recorded in positive linear mode. Data were automatically acquired using FlexControl v.3.4 and MALDI Biotyper Compass v4.1 software for assay preparation and biotyping analysis. Spectra from different species were compared with the MBT Compass BDAL v.11 library (Bruker) containing 10,833 spectra references (3,893 species), as well as with our culturomics laboratory database containing 9,973 spectra

references (2,186 bacterial species). Identification at the species level was obtained for a spectrum matched with a score higher than 1.9. Colonies unidentified using MALDI-TOF MS (score < 1.9) were identified using genome sequencing (Fournier et al., 2015).

## V3V4 16S rRNA amplicon sequencing

### DNA extraction was realised with two different protocol

Method 1 DNA was extracted using the E.Z.N.A Tissue DNA kit (omega BIO-TEK, 400 Pinnacle Way suite 450, Norcross, GA 30071, USA). Method 2 fecal oeses were put into a tube that contains 500  $\mu$ L of PBS, glass powder and glass beads, to perform mechanical lysis using fastprep (90 seconds, 6.5m/sec). After decanting for 1min, the supernatant is collected. The latter was then centrifuged at 12000rpm for one minute and the supernatant was eliminated. On the base, 19  $\mu$ L of mixture containing 2  $\mu$ L glycoprotein denaturation buffer 10x (New England Biolabs) and 17  $\mu$ L H<sub>2</sub>O were added to denature, heat to 100°C for 10 min.

Deglycolization of the samples was achieved by adding a mixture containing 2  $\mu$ L of reaction buffer G3 10X (New England Biolabs), 2  $\mu$ L of EndoHf (New England Biolabs), 2  $\mu$ L of cellulase (SIGMA, France) and 16  $\mu$ L H<sub>2</sub>O Incubate at 37°C overnight. The next day, 25  $\mu$ L of proteinase K (Euromedex, 24 Rue des Tuileries, 67460 Souffelweyersheim) and 180  $\mu$ L of buffer G2 (Qiagen, Hilden, Germany) were added to the tubes. After incubation for 1 hour at 56°C and centrifugation for 1min at 1100g, 200  $\mu$ L of supernatant was collected in an EZ1 flat-bottomed tube for the EZ1 tissue protocol from Qiagen (Hilden, Germany).

For each extraction protocol, genomic DNA was amplified for the 16S "V3-V4" regions by PCR for 45 cycles, using the Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Inc, Wilmington, MA, USA), we use V3\_V4 primers with adapters (FwOvAd\_341FTCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG; RevOvAd\_785RGTCTCGTGGGCTCGGAG ATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). After purification on CleanNGS (CleanNA Coenecoop 75 2741 PH Waddinxveen, the Netherlands), the concentration was measured with High sensitivity Qubit technology (Life technologies, Carlsbad, CA, USA). Library of each extraction method were dilute at 3,5 ng/ $\mu$ L and pooled (1:1). Illumina sequencing adapters and dual-index barcodes were added to amplicons by pcr. After purification on CleanNGS (CleanNA

Coenecoop 75 2741 PH Waddinxveen, the Netherlands), this library was pooled with others multiplexed samples. The global concentration was quantified by a Qubit with the high sensitivity kit (Life technologies, Carlsbad, CA, USA). Pooled amplicons were dilute to obtain a 8 pM library and we added 15% of PhiX control. The 16S rRNA were sequenced on MiSeq technology (Illumina, Inc, San Diego CA 92121, USA) on 500 cycle cartridges. Automated cluster generation and paired-end sequencing with dual index reads were performed in single 39-hours run in a 2x250bp. The paired reads were filtered according to the read qualities. Taxonomic assignment was performed using the MetaGX pipeline (developed in our center) as previously described (Bellali et al., 2021).

## Comparison of culturomics and V3V4 16S amplicon sequencing results

To understand why some species were found to be associated with NASH in culture but not in V3V4 16S amplicon sequencing, we used blastN to match the complete reference sequence of the 16S rRNA gene of each species significantly associated with NASH (or absence of NASH) with the assembled 16S amplicon sequencing datasets prior to the initial taxonomical assignment. The reference sequence of each species was found using LPSN (<https://www.bacterio.net/>, last accessed October 23<sup>rd</sup>, 2023). BLASTn was applied to the 65 samples with the following thresholds: 97% identity and 95% coverage.

### Statistical analysis

To compare quantitative variables such as fecal EtOH concentration, the bilateral Mann-Whitney test was performed with GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). For fecal ethanol and glucose, visual examination clearly showed that several samples exceeded the maximum value of healthy controls (upper normal value). This prompted us to check whether the proportion of samples with abnormal values could be due to chance. Barnard's two-tailed exact test was used. A  $p$ -value < 0.05 was considered significant. To compare the microbiota profile obtained by culturomics, the detection frequency difference of each species was calculated (as relative abundance is not assessed by this technique). For that purpose, the occurrence of each species in both groups was estimated based on its presence/absence in each sample. Once the frequency of each species in each group was determined, the difference was calculated to determine which species are enriched in each group. The bilateral Chi-squared test was used to compare frequency differences. To compare the microbiota profile obtained by illumina MiSeq V3V4 16S gene sequencing, we used three statistical approaches: 1) frequency comparison within each group, which takes into account only the presence or absence of each species and most closely resembles culturomics, 2) linear discriminant analysis using the LefSE pipeline the Lefse pipeline (Afgan et al., 2018) in Galaxy (<https://huttenhower.sph.harvard.edu/galaxy/>) and finally, and 3) linear discriminant analysis using the Microbiome analyst pipeline (Chong et al., 2020) (<https://www.microbiomeanalyst.ca/>). Alpha and beta-diversity were also assessed with this last pipeline.

## Results

### Participants

A total of 65 individuals were recruited for this study including 41 NASH patients and 24 healthy controls. For NASH patients, the mean age was  $64.2 \pm 9.0$  years with a sex ratio of 1.3 (23/18 (M/F)), and the mean body mass index was  $29.5 \pm 5.0$ . Among the NASH patients, 9.75% had a normal weight, 51.2% were overweight, 24.4% were moderately obese, 12.2% were severely obese and 2.4% were

morbidly obese. Among the NASH patients, 53.7% were diabetic and 28.8% had high blood pressure. For healthy controls, the mean age was  $63.8 \pm 7.6$  years with a sex ratio of 10/14 (M/F), and the mean body mass index was  $25.2 \pm 2.5$ .

### Increased fecal ethanol in NASH

The fecal EtOH concentration was significantly higher in the stools of NASH patients compared to healthy controls ( $p$ -value = 0.0145, Figure 1). By setting a threshold at 0.12 g/L (maximal value of healthy controls), 11/41 NASH presented abnormal values (vs 0/24,  $p = 0.0028$ ). There was a trend for increased fecal glucose concentration ( $p = 0.098$ ) with 7/41 abnormal values (vs 0/24,  $p = 0.028$ ). In contrast, none of the other biochemical parameters were significantly different between the two groups (Figure 1; Supplementary Table 1).

### Culturomics results

Culturomics was performed on 24 samples (14 NASH and 10 controls). A total of 34,560 colonies (mean 1,440 colonies per sample) were isolated allowing to identify 358 different bacterial species. These bacterial species were distributed across 11 phyla and 143 genera. 168 species were common to both groups (NASH and controls) whereas 57 species were specific to the control group and 163 to the NASH group (Supplementary Table 2). Among the 358 identified species, only 16 (4.5%) were associated with a significant detection frequency difference including 12 significantly more frequent in the NASH group and 4 more frequent in the control group (Figure 2). As we focused on EtOH-producing microbes, we observed that, among the 12 species detected more frequently in the NASH group, two were known to produce EtOH: *Limosilactobacillus fermentum* and *Enterocloster bolteae*. Moreover, eight increased species in NASH patients were specific to the group (not detected in 10 controls) with the most represented being *Enterocloster bolteae*, *Flacklamia hominis* and *Holdemanella bififormis* (all detected in 6/14 (42.9%) of NASH patients vs 0/10 (0%) controls,  $p=0.013$ ). Similarly, among the four significantly increased species in controls, three species were specific to the group (not detected in the 14 cases, Figure 2), two of which, *Lactilactobacillus casei* and *Phascolarctobacterium faecium*, were known to be consistently associated with health (Wu et al., 2017; Martens et al., 2022). Interestingly, *Alistipes obesi* (renamed *Alistipes communis*), a species previously discovered using the culturomics approach in our center (Hugon et al., 2013), was the most enriched in controls (3/14 vs 8/10,  $p= 0.006$ ) and has recently been associated with health (Yuan et al., 2023).

### Culturomics identified six new species

Unlike amplicon sequencing, culturomics has the unequaled advantage to isolate hitherto unknown species. In this study, we isolated six new species, including 2 from new genera, the description of which is ongoing. For these species, two were

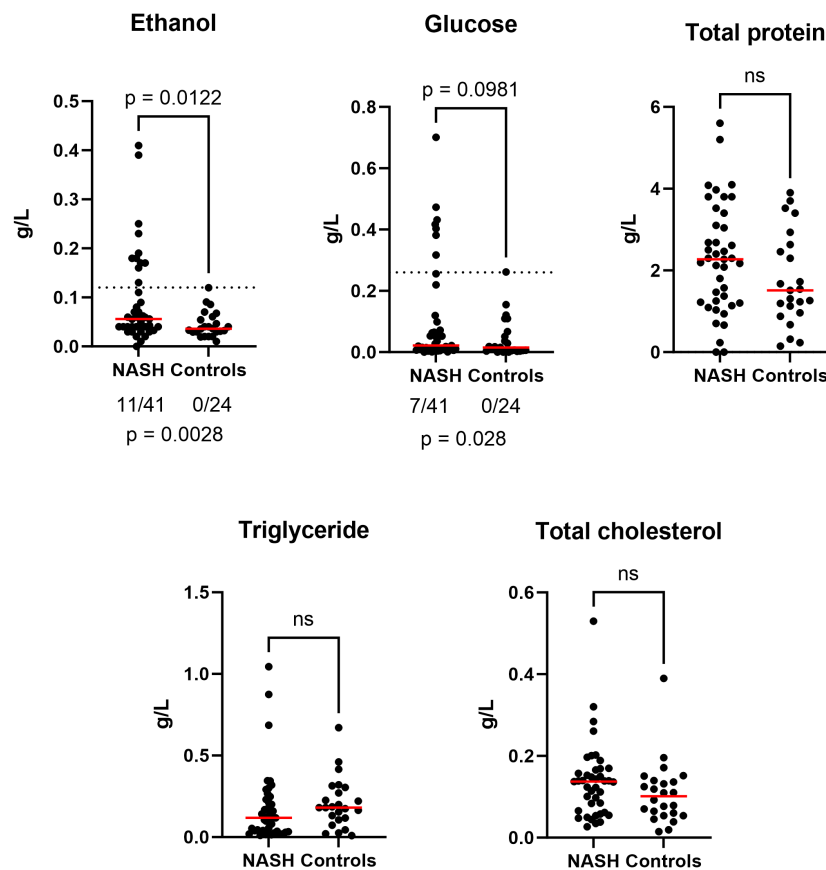


FIGURE 1

Anormal increased fecal ethanol and glucose concentration in NASH ( $n = 65$ ). Bilateral Mann-Whitney test was used to estimate significance for all graphs (ns : not significant). Additionally, for fecal ethanol and glucose, we evaluated if the proportion of anormal values was due to chance using bilateral Barnard's test (see p-value below). Raw data provided in [Supplementary Table 1](#).

isolated from two different NASH patients (*Lancefieldella massiliensis* sp. nov. and *Faecibacter massiliensis* gen. nov. sp. nov.) and four from a single control sample (*Lachnospira massiliensis* sp. nov., *Ligilactobacillus massiliensis* sp. nov., *Phamibacteria massiliensis* gen. nov. sp. nov. and *Pusillibacter massiliensis* sp. nov.). However, these species were not discriminant in both groups.

## V3V4 16S rRNA amplicon sequencing results

After usual Illumina sequencing targeting the V3V4 region of the 16S rRNA gene ([Supplementary Table 3](#), bioproject number PRJEB62828), the diversity was assessed using the Microbiome analyst pipeline ([Chong et al., 2020](#)). Alpha-diversity Simpson index was significantly lower in NASH patients. Strikingly, 7 NASH patients had very low Simpson indexes ([Supplementary Figure 1a](#)). Beta-diversity evidenced an increased heterogeneity of NASH samples compared to controls ([Supplementary Figure 1b](#)). Dendrogram of microbiota profile at the species level did not show a clear distinction between cases and controls ([Supplementary Figure 1c](#)). This prompted us to focused on species with a

significant difference of frequency or abundance between the two groups.

Using the Microbiome analyst pipeline ([Chong et al., 2020](#)) again, a linear discriminant analysis (LDA) between the two groups (41 NASH and 24 controls) was performed at different taxonomic levels (phylum, family, genus, and species). At the phylum level, the LDA showed a significant increase of *Candidatus* Saccharibacteria (a superphylum belonging to the Candidate Phyla Radiation (CPR) division) and *Actinobacteria* in NASH patients ([Supplementary Figure 2a](#)). At the family level, we found an enrichment in *Streptococcaceae*, *Coriobacteriaceae* and *Lachnospiraceae* ([Supplementary Figure 2b](#)). Consistently, the genera *Streptococcus* (*Streptococcaceae*) and *Blautia* (*Lachnospiraceae*) were also increased ([Supplementary Figure 2c](#)).

Conversely, the phyla *Bacteroidetes* and *Euryarchaeota* were decreased in cases whereas at the family level a decrease of *Bacteroidaceae*, *Methanobacteriaceae*, *Rikenellaceae* and *Eubacteriaceae* was observed. At the genus level, *Bacteroides*, *Methanobrevibacter*, *Alistipes*, *Paraoscillibacter*, *Romboutsia* and *Phascolarctobacterium* were associated with controls ([Figure S2a, b and c](#)).

At the species level, we primarily used the LefSE pipeline ([Afgan et al., 2018](#)) to perform the linear discriminant analysis ([Figure 3](#)) but also the Microbiome analyst ([Chong et al., 2020](#)) at the species level ([Figure S3](#)). The NASH group was associated with

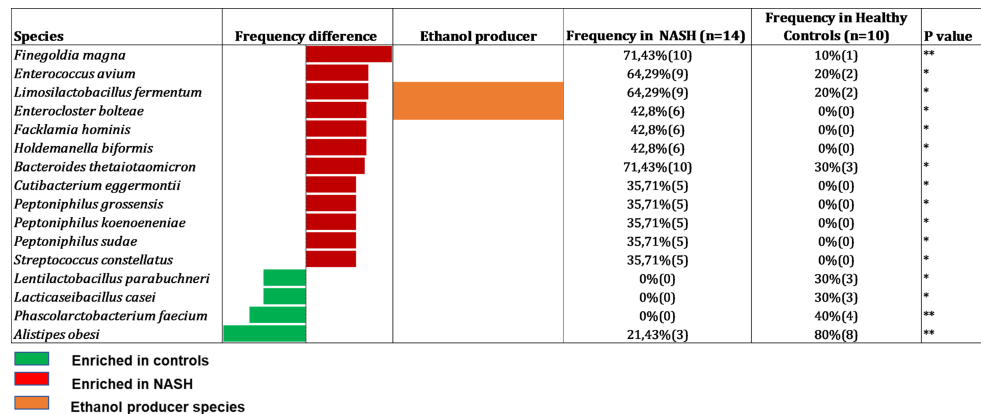


FIGURE 2

Species with a significant frequency difference between NASH and Healthy controls in Culturomics (n = 24). In red species enriched in NASH, in green species enriched in controls and in orange: species capable to produce ethanol according to the literature. Raw data provided in Supplementary Table 2.

*Streptococcus* spp., *Blautia* spp., *Thomasclavelia ramosa* (formerly *Clostridium ramosum* then *Erysipelatoclostridium ramosum*), *Limosilactobacillus fermentum* and *Faecalibacterium timonensis* (Supplementary Figure 3). Healthy controls presented an increased abundance of species, previously associated with health in the literature, namely *Methanobrevibacter smithii*, *Bacteroides* spp. (8 OTUs corresponding to at least 8 *Bacteroides* species, *Romboutsia timonensis* and *Phascolarctobacterium faecium*, Figure 3). Interestingly, the syntrophic taxa, *Methanobrevibacter* spp. and *Bacteroides* spp. were consistently associated with controls in our study, at all taxonomic levels (Samuel and Gordon, 2006; Djemai et al., 2022). The consistency of the depletion of the association between *Methanobrevibacter smithii* and *Bacteroides* species is consistent with the loss of the archaeal-bacterial mutualism reported several years ago (Samuel and Gordon, 2006). Finally, only the LefSE pipeline allow us to identify 3 EtOH-producing species, all enriched in NASH, namely *Mediterraneibacter gnavus* (formerly *Ruminococcus gnavus*), *Streptococcus mutans* and *Limosilactobacillus fermentum* (Figure 3).

## Comparison of culturomics V3V4 16S amplicon sequencing results

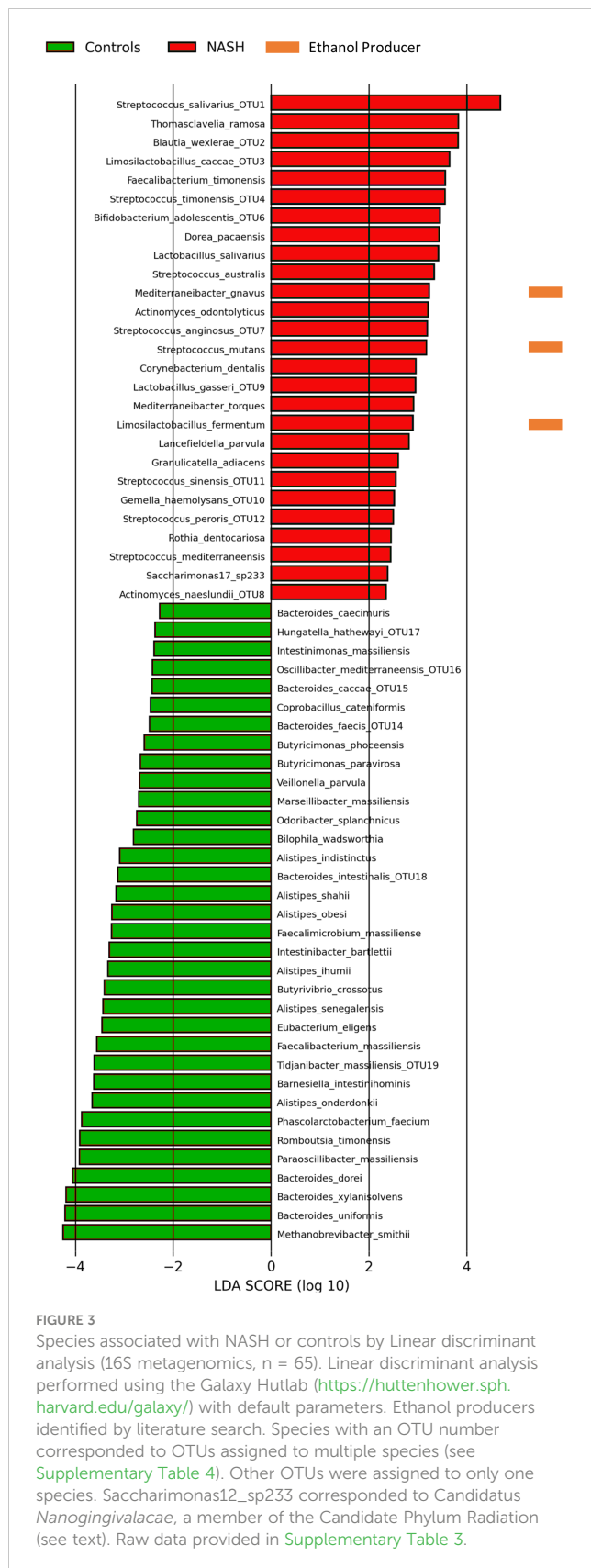
The data obtained using culturomics and 16S amplicon sequencing were analyzed using two different analyses: detection frequency difference (which considers the presence/absence of the species in the sample) performed the culturomics and amplicon sequencing data and LDA (linear discriminant analysis, which considers the relative abundance of species) performed only for the amplicon sequencing datasets. By comparing these two methods, only three species were common for culturomics and 16S metagenomics: the EtOH-producing *Limosilactobacillus fermentum* enriched in NASH patients whereas two species were enriched in controls namely *Phascolarctobacterium faecium* and *Alistipes obesi* (rename *Alistipes communis*) in controls (Figure 2 & 3 and Figure S5).

Interestingly, lactic acid bacteria represented 48% (12/25) of species enriched in NASH patients, the most represented (present in more than 50% of patients) being *Streptococcus salivarius* (40/41), *Streptococcus anginosus* (25/41) and *Streptococcus australis* (23/41). Species enriched in NASH also included *Mediterraneibacter [Ruminococcus] gnavus*, *Thomasclavelia ramosa*, *Gemella haemolysans*, *Faecalibacterium timonensis* and *Granulicatella adiacens* (Figure 3). It is noteworthy that among the species enriched in the NASH group, seven were totally absent in controls: *Actinomyces naeslundii*, *Bifidobacterium adolescentis*, *Limosilactobacillus fermentum*, *Limosilactobacillus caccae*, *Lactobacillus salivarius*, *Streptococcus sinensis* and an unknown member of the Candidate Phyla Radiation (CPR, called saccharimonas17 sp233) classified with a new unclassified family *Candidatus Nanogingivalaceae* (Figure 3).

Conversely, 36 species were found to have strong associations with controls with a predominance of the *Bacteroides* genus (23 species) and health-associated species previously found with the LDA and/or culturomics: *P. faecium*, *R. timonensis* and *A. obesi* (Figure 3). These species also included the main human gut archaeal *M. smithii*, as well as several members of the *Bacteroides* genus for which syntrophic interactions are well described.

## Four EtOH-producing bacteria associated with NASH identified by culturomics and 16S metagenomics

Endogenous EtOH production is an emerging mechanism described in the pathophysiology of NASH (Zhu et al., 2013; Yuan et al., 2019; Mbaye et al., 2022; Mbaye et al., 2023). Among all the species with a significant difference between NASH patients and controls, only four were known to produce EtOH and 4/4 (100%) were enriched in NASH patients (binomial test,  $p = 0.025$ ) by culture and/or sequencing (Table 2; Figure 2, 3). In contrast, none of the species enriched in the controls were known to produce EtOH (Figure 3). These four species included a member of the



**FIGURE 3**  
 Species associated with NASH or controls by Linear discriminant analysis (16S metagenomics, n = 65). Linear discriminant analysis performed using the Galaxy Hutlab (<https://huttenhower.sph.harvard.edu/galaxy/>) with default parameters. Ethanol producers identified by literature search. Species with an OTU number corresponded to OTUs assigned to multiple species (see Supplementary Table 4). Other OTUs were assigned to only one species. Saccharimonas12\_sp233 corresponded to Candidatus *Nanogingivalacae*, a member of the Candidate Phylum Radiation (see text). Raw data provided in Supplementary Table 3.

recently reclassified *Limosilactobacillus* genus (Zheng et al., 2020), *L. fermentum*, a EtOH producer according to the literature (Elshaghabe et al., 2016), was found enriched in both culture

and sequencing and has previously been associated with EtOH production in NASH (Mejnikman et al., 2022; Mbaye et al., 2023) (Table 2). In addition, *S. mutans*, a species of the genus *Streptococcus* able to produce EtOH (Takahashi et al., 1991), was associated with NASH in the amplicon sequencing datasets. The *Streptococcus* genus was previously associated with NASH (Naka et al., 2014) and includes *S. mutans*, a known cariogenic species (Alaluusua and Renkonen, 1983). The other two EtOH-producing species, *E. bolteae* (which ethanol production has been shown *in vitro* with strains enriched in hepatitis B patients (Magdy Wasfy et al., 2023)) and *Mediterraneibacter gnavus* (Hynönen et al., 2016), were found to be associated with NASH for the first time in this study. Specifically, *E. bolteae* was found enriched in NASH patients only using the culturomics approach. This species was recently associated with autism and hepatic steatosis (Ruuskanen et al., 2021; Frame et al., 2023). *Mediterraneibacter gnavus*, reclassified by Togo et al. (Togo et al., 2018), has been associated with several pathologies including obesity (Chen et al., 2021; Lin et al., 2023), intestinal inflammation (Hall et al., 2017), allergies in children (Chua et al., 2018) and non-alcoholic steatosis (Hullar et al., 2021). These four species (Table 2) should be added to the repertoire of gut microbes possibly instrumental in NASH through endogenous ethanol production. As *E. bolteae* was only found using culture-based approaches as well as other species of interest (Table S5) and the V3V4 region of the 16S rRNA gene does not consistently allow unambiguous taxonomic assignment of OTUs, we conducted a BLASTn matching the complete reference sequence of the 16S rRNA gene (Table S4) of the 15 species of interest highlighted using culturomics against the 16S amplicon datasets (Figure S4, Table S5). This analysis showed that *E. bolteae* was also detected using 16S amplicon sequencing although it was depleted in NASH patients whereas *L. fermentum* was also found enriched in NASH patients.

## Discussion

In this study, we confirmed the intestinal dysbiosis associated with NASH using culture-based as well as culture-independent approaches. We first showed an increase in fecal EtOH concentration in NASH patients, confirming results already described in the literature. The NASH-associated dysbiosis found in this study confirms those obtained in previous studies. It was characterized by an increase of *Streptococcaceae* (Vernekar et al., 2018; Mejnikman et al., 2022) and *Lachnospiraceae* at the family level and *Streptococcus*, *Limosilactobacillus* and *Blautia* at the genus level (Abdugheni et al., 2022; Mejnikman et al., 2022). Interestingly, EtOH-producing species (*E. bolteae*, *M. gnavus*, *L. fermentum*, and *S. mutans*) were enriched in NASH patients. The association of *T. ramosa* and *L. fermentum* with NASH confirms our previous findings (with different patients) and other reports in the literature (Loomba et al., 2019; Mejnikman et al., 2022; Mbaye et al., 2023).

Our study evidence a plausible association of *E. bolteae* with NASH through endogenous EtOH production. The bacterium was first isolated from autistic patients (Song et al., 2003) and subsequently repeatedly associated with this disease (Cox et al.,

TABLE 2 Four EtOH-producing species found enriched in NASH according to microbiological and statistical methods.

	Culturomics	V3V4 16S amplicon sequencing			
	N = 24	Step 1 (n = 24)		Step 2 (n = 65)	
	Frequency difference	Frequency difference	Linear discriminant analysis	Frequency difference	Linear discriminant analysis
<i>Limosilactobacillus fermentum</i> <sup>EtOH</sup>	X	X	X	X	X
<i>Mediterraneibacter gnavus</i> <sup>EtOH</sup>				X	X
<i>Streptococcus mutans</i> <sup>EtOH</sup>				X	X
<i>Enterocloster bolteae</i> <sup>EtOH</sup>	X				

Table comparing technique (culturomics & 16S metagenomics) or statistical (frequency difference or linear discriminant analysis) methods, and according to samples (24 analyzed both by culturomics and metagenomics (Step 1) and 41 analyzed only by 16S metagenomics). Step 2 included all samples. *L. fermentum* was the only species found by culturomics and 16S metagenomics, whatever the group of samples included (24 analyzed both by culturomics and metagenomics or 65 analyzed by 16S metagenomics). *Enterocloster bolteae* was identified only by culturomics. Its instrumental role is highly suspected based on several reports associating this species with dysbiosis related diseases (see Discussion). These four species should be added to the repertoire of gut microbes possibly instrumental in the disease through endogenous ethanol production.

2021; Pandit et al., 2021; Frame et al., 2023). It is strict anaerobic, gram-positive, rod-shaped and measures 1.0-1.2  $\mu\text{m}$  in diameter. Acetate and lactate are the main end products of glucose metabolism. Other sugars metabolized by the bacterium include arabinose, fructose, sucrose, glycerol, maltose, mannose, melezitose, sorbitol, trehalose and xylose. It is sensitive to kanamycin and colistin sulfate; intermediate to bacitracin, cefoxitin and clindamycin; and resistant to ampicillin, piperacillin, ticarcillin and vancomycin (Song et al., 2003). Its versatility to consume multiple sugars knowing that it produces ethanol makes it a suitable pathogen candidate. Indeed, a very frequent endogenous production of ethanol can be expected in humans who carry this bacterium even with varied sugar diets.

A 2019 metagenomics study found that this species was the best for classifying NAFLD according to the stage of fibrosis (Loomba et al., 2019). Moreover, *E. bolteae* has already been associated with intestinal dysbiosis in autistic patients (Frame et al., 2023) and chronic inflammatory bowel disease (Chen et al., 2023). This species, found only in the culture-based analysis in this study, is an EtOH producer. Endogenous EtOH production is potentially involved in the pathophysiology of the disease (Zhu et al., 2013; Yuan et al., 2019). *E. bolteae* was the only species found by culture and not by V3V4 16S rRNA amplicon sequencing. The lack of detection of *E. bolteae* in V3V4 16S amplicon sequencing might be due to the fact that these hypervariable regions of the 16S rRNA gene cannot discriminate species of the *Enterocloster* genus. In fact, this genus was recently created subsequently to multiple reclassifications of the *Clostridium* genus (Song et al., 2003). In fact, all OTUs classified within this genus were multi-assigned in the metadata (see Supplementary table S3). However, culturomics allowed the unambiguous identification of *Enterocloster* species: *Enterocloster bolteae* (6 in NASH vs. 0 in controls), *Enterocloster aldenensis* (3 in NASH vs. 0 in controls), *Enterocloster citroniae* (2 in NASH vs. 1 in controls), *Enterocloster clostridioformis* (2 in NASH vs. 0 in controls) and *Enterocloster lavalensis* (1 in NASH vs. 0 in controls). According to the microbial culturomics (Lagier et al., 2012a) approach, the media used for culture were not specific for any specific microbial population, but rather mimicked the sample

environment to explore the bacterial population of the sample as exhaustively as possible. For instance, YCFA medium, rumen and anaerobic atmospheres were used to reproduce the physiologic environment (Tidjani Alou et al., 2020). This suggests the complementarity between these methods to explore gut microbiome (Lagier et al., 2018).

Future studies are needed to understand why the association between *E. bolteae* and NASH was detected using culturomics and shotgun metagenomic, and not 16S amplicon sequencing. Future experimental studies and *in vitro* tests will improve its instrumental role in the pathophysiology of NASH. Our results suggest the potential association of endogenous EtOH production by digestive microorganisms and NASH, and the use of new therapeutic avenues such as antibiotics (*E. bolteae* is expected to be sensitive to rifaximin which is effective in NASH in some studies (Abdel-Razik et al., 2018 2018; Boehme et al., 2023 2023), fecal transplantation or the use of phage therapy targeting *E. bolteae* as described by Yuan et al. with *Klebsiella pneumoniae* (Gan et al., 2023). Future studies will determine ethanol production by the 12 species (81 strains) enriched in NASH patients. In addition, the pathogenic role of these species in the pathophysiology of NASH could be explored in a murine model (Yuan et al., 2019).

In this study, we showed frequency difference did not represent the most relevant parameter that is predictive value. Indeed, even if rare, a microbial species is of particular interest if it is always associated with a disease or always associated with absence of the disease. Even if sample size was quite low due to microbial culturomics fastidious process, we were struck by the fact that the EtOH producing *E. bolteae* had a 100% positive predictive value, in contrast the probiotic *L. casei* has a 100% negative predictive value, suggesting that *E. bolteae* is a pathobiont (Lagier et al., 2018; Abdel-Razik et al., 2018 2018) and *L. casei* a probiotic potentially protective against NASH (Lee et al., 2020). And the probiotic effect of *L. casei* is known for around a century. Strikingly, this species was shown to interfere with ethanol production in co-culture with yeast (Spanhaak et al., 1998). Future studies are strongly needed to test if *L. casei* may have a beneficial effect on NASH.

One of the limitations of this study may be linked to the discrepancy in the number of samples analyzed using culturomics (24 samples) and 16S amplicon sequencing (65 samples) due to the extensive workload associated with the culturomics method.

Testing antimicrobial therapies for NASH without identifying instrumental gut microbes that might include both yeast (Demir et al., 2022; Mbaye et al., 2022) and bacteria is expected to be unsuccessful. Current and previous studies from our center (Mbaye et al., 2022; Mbaye et al., 2023) and others strongly suggest that accurate profiling of the individual gut microbiota using both culturomics and metagenomics for yeasts, bacteria and archaea is likely to be a critical step for precision medicine using microbe-targeted therapies for NASH management in the future.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## Ethics statement

This study involving humans was approved by the ethics committee of the IHU Méditerranée Infection under number 2020-004 and the “Comité de Protection des Personnes” under number CPP: 21.04391.000046 - 21075. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants’ legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)’ legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

## Author contributions

BM: Formal analysis, Visualization, Writing – original draft, Investigation. RMW: Investigation, Writing – review & editing. PB: Resources, Writing – review & editing. MTA: Writing – review & editing. GM: Writing – review & editing, Data curation, Investigation. VB: Investigation, Data curation, Writing – review & editing. AC: Investigation, Data curation, Writing – review & editing. RG: Investigation, Resources, Writing – review & editing, Project administration, Supervision, Validation. MM:

Conceptualization, Project administration, Validation, Formal analysis, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2023.1279354/full#supplementary-material>

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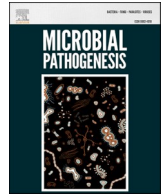
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## **Article 9**

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Million

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## *Limosilactobacillus fermentum*, *Lactococcus lactis* and *Thomasclavelia ramosa* are enriched and *Methanobrevibacter smithii* is depleted in patients with non-alcoholic steatohepatitis

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### ABSTRACT

Non-alcoholic fatty liver (NAFLD), and its complicated form, non-alcoholic steatohepatitis (NASH), have been associated with gut dysbiosis with specific signatures. Endogenous ethanol production by *Klebsiella pneumoniae* or yeasts has been identified as a potential physio-pathological mechanism. A species-specific association between *Lactobacillus* and obesity and metabolic diseases has been reported. In this study, the microbial composition of ten cases of NASH and ten controls was determined using v3v4 16S amplicon sequencing as well as quantitative PCR (qPCR). Using different statistical approaches, we found an association of *Lactobacillus* and *Lactococcus* with NASH, and an association of *Methanobrevibacter*, *Faecalibacterium* and *Romboutsia* with controls. At the species level, *Limosilactobacillus fermentum* and *Lactococcus lactis*, two species producing ethanol, and *Thomasclavelia ramosa*, a species already associated with dysbiosis, were associated with NASH. Using qPCR, we observed a decreased frequency of *Methanobrevibacter smithii* and confirmed the high prevalence of *L. fermentum* in NASH samples (5/10), while all control samples were negative ( $p = 0.02$ ). In contrast, *Ligilactobacillus ruminis* was associated with controls. This supports the critical importance of taxonomic resolution at the species level, notably with the recent taxonomic reclassification of the *Lactobacillus* genus. Our results point towards the potential instrumental role of ethanol-producing gut microbes in NASH patients, notably lactic acid bacteria, opening new avenues for prevention and treatment.

### 1. Introduction

NAFLD (non-alcoholic fatty liver disease) has become the most common cause of chronic liver disease and affects approximately 20% of the world's population. It is increasingly common and has the same stages of progression as alcohol-related liver disease (ALD), from fibrosis to hepatocarcinoma. NAFLD is one of the main causes of liver transplantation in the world [1] because to date no aetiological treatment has been found. It is often related to metabolic syndrome disorders [2] but also to a diet rich in sugar, and particularly fructose [3]. Non-alcoholic steatohepatitis (NASH) is a disease classified as an NAFLD and characterized by an excess of fat in the liver (hepatosteatosis) associated with

inflammation (hepatitis) in individuals who do not consume excessive amounts of alcohol (non-alcoholics).

The role of gut dysbiosis in NAFLD has been a hot research topic [4, 5]. This dysbiosis has been characterised by an increase in *Proteobacteria* [4–6] at the phylum level, *Enterobacteriaceae* [4,5] and *Lactobacillaceae* [7] at the family level, and *Clostridium*, *Escherichia* and *Lactobacillus* at the genus level [4,7]. Several mechanisms suggesting the involvement of the microbiota in the pathogenesis of the disease via the gut-liver axis and the portal vein have been described: 1) an increase in intestinal permeability that promotes the passage of lipopolysaccharides (LPS) to the liver to induce inflammation [8]; 2) an overactivation of lipogenesis following a diet rich in carbohydrates promoting steatosis that evolves

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into NAFLD [9]; and 3) an increased production of endogenous ethanol by intestinal microorganisms inducing very high levels in the portal vein and subsequent hepatic injury [4,5].

Focusing on the role of endogenous ethanol production, many arguments strongly suggest its instrumental role in the pathogenesis of the disease [4,5]. First, high levels of ethanol have been detected in the blood and stools of NASH patients who did not consume alcohol [4,5,10], suggesting its endogenous production. Second, NASH has been associated with the auto-brewery syndrome [5,6], which is already known to be related to intestinal yeast and endogenous ethanol production [7]. Several case-controlled studies have associated ethanol-producing microbes with fatty liver, including *Candida* yeast [7,10], *Pichia* yeast [10], and ethanol-producing *Klebsiella pneumoniae* bacterial strains [5]. Experimental evidence has been reported with the production of ethanol *in vitro* by strains isolated from NASH patients and animal studies [11]. All these findings are consistent with the fact that endogenous ethanol production is probably a critical pathophysiological mechanism in NASH.

Other microbes besides yeasts, notably specific lactic acid bacteria can produce ethanol from sugars [12]. *Limosilactobacillus fermentum* has been shown to produce more ethanol from glucose than from fructose [13] and *Lactococcus lactis* has been known to be involved in wine fermentation processes [14].

Several studies have recently analysed the microbial signature associated with NASH through case-controlled studies with 16S amplicon sequencing [4,5,15]. Most of the results associate the disease with a signature characterised by an increase in Gram-stain negative pathogenic bacteria [4,5], certain bacteria of the genus *Streptococcus* [16], *Blautia* [17] and *Lactobacillus* [7,18]. Conversely, the phylum *Bacteroidetes*, the family *Ruminococcaceae* and the genus *Faecalibacterium* are decreased in NASH patients [15,19].

Despite the different results obtained in the literature, only few studies in human have been performed in France [20]. Accordingly, the objective of this study is to compare the microbial profile of French NASH patients to healthy controls by 16S amplicon sequencing.

## 2. Patients and methods

### 2.1. Patients

Here, we performed a case-controlled study comparing the faecal microbiota of ten patients with non-alcoholic steatohepatitis (NASH) and ten healthy controls. Patients were recruited as previously described [10].

### 2.2. 16S rRNA amplicon sequencing

All 20 samples were sequenced as previously described [21]. Taxonomic assignment was performed as previously reported using the MetaGX pipeline developed in our centre to include culturomics species and putative new species [21]. To identify the microbial signature of non-alcoholic steatohepatitis, we sought to identify species associated with cases and controls using three different methods. First, we simply compared the presence and absence of each species without considering the relative abundance by reporting the difference in detection frequency. Second, we used previously published linear discriminant analysis (LDA) (LEfSe pipeline in Galaxy, a platform for accessible, reproducible and collaborative biomedical analyses [22]), as well as an online integrated approach assessing relative abundance using the Microbiome Analyst pipeline [23] (<https://www.microbiomeanalyst.ca/>).

### 2.3. Measurement of *Methanobrevibacter smithii* by quantitative PCR

The presence of *M. smithii* was assessed using quantitative PCR as previously reported [24]. For *Limosilactobacillus fermentum*, quantitative

PCR was also performed as previously reported [25].

### 2.4. Statistical analysis

To test for significant detection frequency difference, we used two-sided Fisher's exact test using SPSS version 20.0 (IBM, Paris, France). In addition, a principal component analysis based on relative abundance with default parameters was performed using XLSTAT v2022.1.2 (Addinsoft, Paris, France). The presence of *M. smithii* in qPCR, and the number of reads of the 1,879 operational taxonomic units (OTUs) were identified by sequencing the v3v4 hypervariable region of the 16S rRNA gene amplicon. For the figures, OTUs not assigned to a cultured species have been removed to improve readability.

## 3. Results

### 3.1. 16S rRNA amplicon sequencing

Sequencing yielded 1,150,699 good quality reads for the 20 individuals included in our study. The number of reads per sample was no different between the two groups ( $44.673 \pm 12.820$  in NASH and  $42.051 \pm 11.598$  in healthy controls, unpaired *t*-test,  $P = 0.64$ ). 1,879 OTUs were identified with more than 20 reads of which 1,115 were not assigned (59.3%) and 764 were assigned (40.7%). Of these 764 OTUs assigned to a species, 505 were assigned to putative species assigned at least at the family rank and 261 were assigned to at least one cultivated species (Table S1).

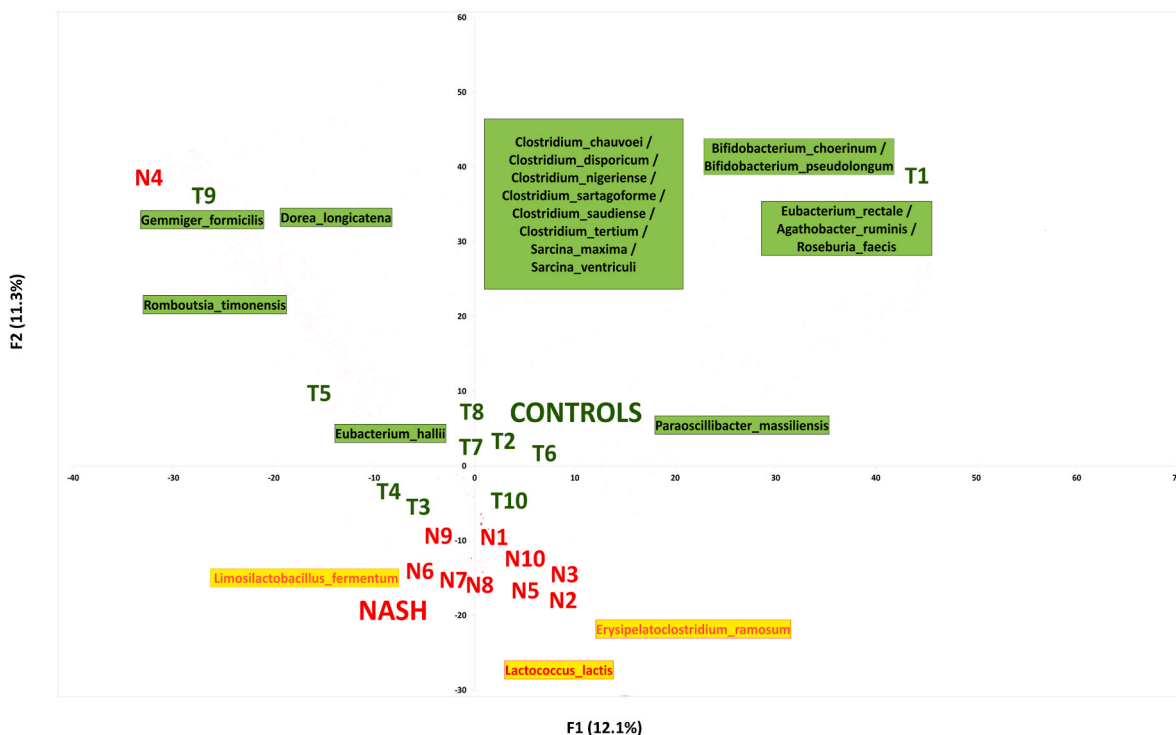
### 3.2. Principal component analysis

A principal component analysis was performed as mentioned in the methods section to provide an overview of the findings. The resulting figure (Fig. 1) clearly shows that the NASH patients cluster almost perfectly (9/10), except for patient N4. Controls also clustered together with two outlier (T1 and T9). Three OTUs associated with the NASH group were also found by other methods (Figs. 2 and 3b), namely *Thomasclavelia ramosa*, the new name in 2023 of the former *Erysipelatoclostridium ramosum* (2013), and previously named *Clostridium ramosum* (1971) or *Bacillus ramosus* (1898). *Limosilactobacillus fermentum* and *Lactococcus lactis*. In contrast, seven OTUs were associated with the controls, namely *Romboutsia timonensis*, *Eubacterium hallii*, *Gemmiger formicilis*, *Dorea longicatena*, *Paraosclibacter massiliensis*, and two multi-assigned OTUs: one corresponded to *Eubacterium rectale/Agathobacter ruminis/Roseburia faecis*, another to *Clostridium chauvoei/Clostridium disporicum/Clostridium nigeriense/Clostridium sartagoforme/Clostridium saudiense/Clostridium saudii/Clostridium tertium/Sarcina maxima/Sarcina ventriculi* (Fig. 1). Beyond these significant associations, it can be observed that several other lactic acid bacteria were associated with NASH including *Lactobacillus salivarius* and streptococci (*S. salivarius*, *S. mutans*). Within the *Bifidobacterium*, *B. longum* and *B. breve* were associated with NASH, whereas *Bifidobacterium adolescentis* was associated with controls (Supplementary Fig. 1).

### 3.3. Comparative analysis of the diversity of the faecal microbiota of NASH patients and controls

The detection frequency of each OTU assigned to a putative or cultured species was compared between each group using a two-tailed Fisher's exact test to identify significant frequency differences. Thus, we identified five OTUs and nine OTUs assigned to cultured species that were significantly more frequent in NASH patients and controls, respectively (Fig. 2).

Of the five increased OTUs in the NASH group, all were assigned to cultured species including four lactic acid bacteria. Two were related to the *Lactobacillus* genus, one to the newly created *Limosilactobacillus* genus and one to the *Streptococcus* genus. Of these lactic acid bacteria,



**Fig. 1.** Principal component analysis based on relative abundance of each species highlights two distinct groups: NASH patients and healthy individuals. Red indicates species clustering with the NASH group and black indicates species clustering with the control group. N: patients with non-alcoholic steatohepatitis. T: controls. Species associated with NASH or controls by using another method (frequency difference, Fig. 2 and/or linear discriminant analysis, Fig. 3b) are identified with a coloured box. For clarity, only species associated with NASH or controls by another statistical approach (frequency difference, relative abundance and/or qPCR) are shown. The complete figure is provided in the supplementary data. *Thomasclavelia ramosa* is the new name in 2023 of the former *Erysipelatoclostridium ramosum* (2013), mentioned in the figure, and previously named *Clostridium ramosum* (1971) or *Bacillus ramosus* (1898).

only one OTU was unambiguously assigned to a cultured species; the ethanol-producing *Limosilactobacillus fermentum* species. The only OTU not matching a lactic acid bacterium was unambiguously assigned to *T. ramosa*. *L. fermentum* and *T. ramosa* were not detected in controls, suggesting a possible NASH-specific signature.

None of these OTUs were detected in all ten NASH patients (Fig. 2b – highest detection frequency 6/10 for *T. ramosa* and *Streptococcus* spp.) suggesting the absence of a NASH-associated core microbiome. Conversely, all NASH patients except one had at least one of these five species (Fig. 2b).

Among the OTUs associated with healthy controls, three were found in the ten controls, namely *Eubacterium hallii*, IHU\_PS\_FamilyXIII\_212 and *Faecalibacterium prausnitzii*. This suggests the existence of a health-associated core microbiome. Among the OTUs with an increased prevalence in healthy controls, only one was related to the former *Lactobacillus* genus, *Ligilactobacillus ruminis*. This species, a homofermentative (unable to produce ethanol) lactic acid bacterium, has been recently reclassified [26], and has been repeatedly associated with health.

Interestingly, similar results were obtained after a linear discriminant analysis (LDA) for NASH patients (Fig. 3) which accounts for relative abundance.

Using the Microbiome analyst server [23], we confirmed the enrichment of the lactic acid bacterial genera *Lactobacillus* and *Lactococcus* in NASH samples, and several strict anaerobic genera (*Romboutsia*, *Faecalibacterium*) with increased relative abundance in controls, previously highlighted by the detection frequency difference and LDA analyses (Fig. 3a). This approach also identified a predominance of the phyla *Proteobacteria* and *Verrucomicrobia* as well as the families *Gammaproteobacteriaceae* and *Verrucomicrobiaceae* in NASH patients. Interestingly, this method was the only one to highlight an enrichment of methanogenic archaea (genus *Methanobrevibacter*) in controls.

At the genus level, *Lactobacillus* and *Lactococcus* genera were

associated with NASH (Fig. 3a). At the species level (Fig. 3b), the four species associated with NASH when examining detection frequency difference were reproducibly found with linear discriminant analysis, including *L. fermentum* and *T. ramosa*. In contrast, the association of *Lactococcus lactis* with NASH was found only by this approach.

In the controls, LDA identified a similar signature previously found by frequency difference. However, some OTUs were found to be increased in relative abundance only using LDA, notably *Romboutsia timonensis* and *Eubacterium rectale*.

A specific qPCR system, developed and validated in our centre [25], was available for *L. fermentum* alone (see methods). Using qPCR, we thus confirmed the significant association between *L. fermentum* and NASH (5/10 vs. 0/10, Mid-P exact test,  $P = 0.02$  – Table S2).

At the species level, we also found that *M. smithii* was decreased in NASH samples, using both the detection frequency difference and the mean rank comparison based on relative abundance although the difference was not significant. We thus tested *M. smithii* using qPCR and found that this species was less frequent in NASH patients (4/10 vs. 8/10 in controls, one-sided mid-P exact test,  $P = 0.047$  – Table S3). Overall, the 16S metagenomic and specific qPCR data were consistent with an association between the absence of *M. smithii* and NASH (Table S3).

#### 4. Discussion

In the present study, we found that the genera *Lactobacillus* and *Lactococcus* and, more specifically, the three species *L. fermentum*, *L. lactis* and *T. ramosa* were associated with NASH. In addition, species often associated with health in the literature were highly enriched in controls: *Eubacterium hallii* [27,28], *Faecalibacterium* spp. [29], *Gemmiger formicilis* and *Romboutsia timonensis* [27]. Our results confirm a dysbiosis signature with the depletion of the human methanogenic archaea *M. smithii* [30], a ubiquitous inhabitant of the human intestinal

a. Frequency difference

Species	Uncultured species	Healthy controls		Frequency difference	p-value	Legend
		NASH n = 10	n = 10			
<i>Faecalibacillus intestinalis</i>		3	9		**	Species found in ≥ 90% controls
IHU_PS_96_Neglecta_55731		2	8		**	
<i>Eubacterium sulci</i>		0	6		**	Species found in ≤ 10% controls
IHU_PS_91_Coriobacteriaceae_2544		1	7		**	
IHU_PS_91_Streptococcaceae_182		0	6		**	
IHU_PS_95_Romboutsia_375		1	7		**	
<i>Eubacterium hallii</i>		5	10		*	
IHU_PS_91_Peptostreptococcaceae_90		1	6		*	
IHU_PS_92_Bifidobacteriaceae_1530109		3	8		*	
IHU_PS_92_Family_XIII_212		5	10		*	
IHU_PS_94_Oscillospiraceae_1126		0	5		*	
IHU_PS_94_Peptostreptococcaceae_448		1	6		*	
IHU_PS_95_Bifidobacterium_1698		1	6		*	
IHU_PS_96_Gemmiger_2850		1	6		*	
IHU_PS_96_Prevotella_102845		0	5		*	
<i>Faecalibacterium prausnitzii</i>		5	10		*	
<i>Parasaccharibacter massiliensis</i>		4	9		*	
IHU_PS_92_Bifidobacteriaceae_289		2	7		*	
<i>Alkalihalophilus pseudofructum</i>		0	4		*	
<i>Gemmiger formicilis</i>		6	1		*	
IHU_PS_87_Bacteria_2019		0	4		*	
IHU_PS_92_Coriobacteriaceae_2880		0	4		*	
IHU_PS_95_Roseburia_2126		0	4		*	
IHU_PS_96_Gemmiger_1147		0	4		*	
<i>Ligilactobacillus ruminis</i>		0	4		*	
<i>Rosellomorea aquimaris</i> / <i>Bacillus halotolerans</i> / <i>Bacillus mojavensis</i> / <i>Bacillus nakamurai</i> / <i>Bacillus paralicheniformis</i> / <i>Cytobacillus solani</i> / <i>Bacillus subtilis</i> / <i>Bacillus tequilensis</i>		0	4		*	
<i>Limosilactobacillus fermentum</i>		4	0		*	
<i>Lactobacillus acidophilus</i> / <i>Lactobacillus amylovorus</i> / <i>Lactobacillus crispatus</i> / <i>Lactobacillus gallinarum</i> / <i>Lactobacillus helveticus</i> / <i>Lactobacillus kitasatonis</i> / <i>Lactobacillus ultunensis</i>		4	0		*	
<i>Lactobacillus gasseri</i> / <i>Lactobacillus hominis</i> / <i>Lactobacillus johnsonii</i> / <i>Lactobacillus taiwanensis</i>		5	0		*	
<i>Streptococcus anginosus</i> / <i>Streptococcus intermedius</i> / <i>Streptococcus periodonticum</i>		6	1		*	
<i>Erysipelatoclostridium ramosum</i>		6	0		**	

b. Detection of species associated with NASH in each patient

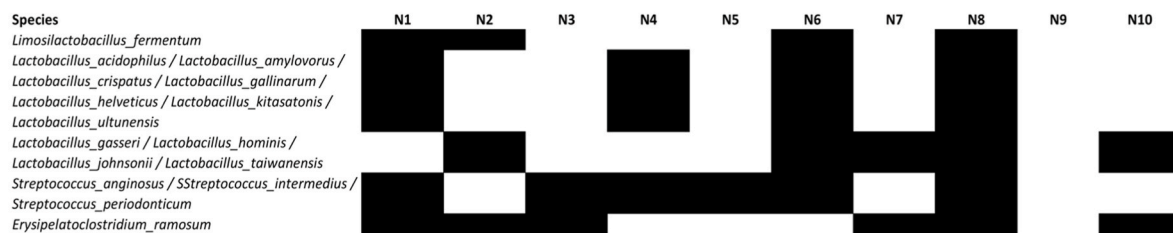


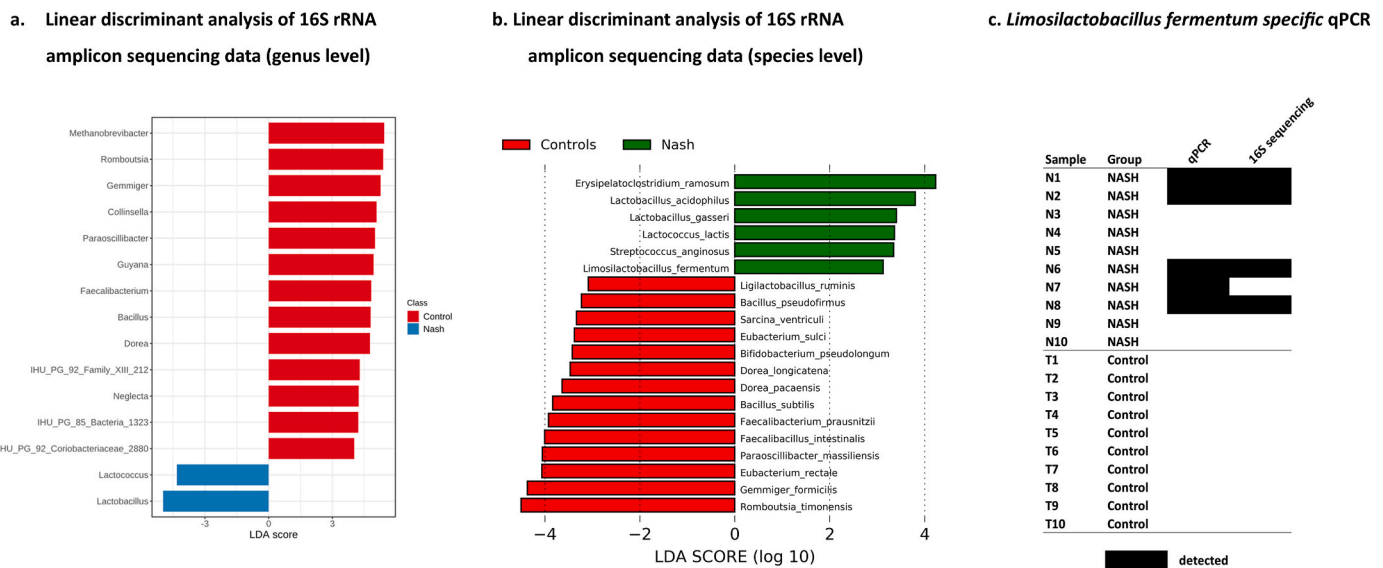
Fig. 2. Species associated with NASH or controls NASH: non-alcoholic steatohepatitis, \*P < 0.05, \*\*P < 0.005, Operational taxonomic units corresponding to uncultured species were named as follows: IHU\_PS\_96\_Neglecta\_55731, for putative species, 96% similarity with the closest phylogenetic cultivated neighbour that belong to the Neglecta genus, number 55731. The difference of frequency is shown. Two-tailed Fisher's exact test. Thomasclavelia ramosa is the new name in 2023 of the former Erysipelatoclostridium ramosum (2013), mentioned in the figure, and previously named Clostridium ramosum (1971) or Bacillus ramosus (1898).

microbiome [31]. We previously reported this archaeal dysbiotic signature in obese people [25,32].

Endogenous ethanol production has been identified as a putative pathophysiological mechanism in NASH [5,7,10]. The first report incriminated *Klebsiella pneumoniae* [5]. In our first study [10] based on a culturomics approach performed on the same study groups, ethanol was significantly higher in the stools of NASH patients than in the stools of healthy controls. This difference in ethanol production could be attributed to the presence of *Klebsiella pneumoniae* and yeast [10]. However, it should be noted that only enterobacteria and yeasts were investigated by specific culture in this first study. The present study showed a concurrent increase in ethanol-producing lactic acid bacteria in patients, notably *L. fermentum* [13] and *L. lactis* [14]. This result is concordant with those recently reported by Meijnikman et al. [7] showing a positive correlation between endogenous ethanol and lactic acid bacteria, thus supporting our results.

The only acid lactic bacteria which was significantly different between the two groups and which was clearly assigned in NASH without ambiguous taxonomic assignment is *L. fermentum* (*Lactobacillus* reclassified in 2020 [26]). Both qPCR and 16S rRNA amplicon sequencing results were consistent with an absence of *L. fermentum* in controls. This corresponds to a perfect positive predictive values in our small sample size. This heterofermentative lactic acid bacterium (i.e. one which is

capable of producing ethanol, acetic acid and carbon dioxide (CO2) in addition to lactic acid) can produce high amounts of ethanol in the presence of glucose but not fructose [13] and its positive correlation with endogenous ethanol production in NASH patients has already been reported [7]. *L. fermentum* is a member of a group of mucosal lactobacilli recently renamed *Limosilactobacillus* [26]. Several species of this group (*L. fermentum*, *L. ingluviei*, *L. mucosae* and *L. reuteri* [26]) have previously been associated with metabolic diseases in human case-controlled studies: *L. fermentum* [7] and *L. mucosae* with NASH [33], and *L. reuteri* with metabolic syndrome and obesity [25,32] (Table 1). In animals, Khan et al. reported, as early as 2007, that inoculating chickens with *L. ingluviei* and *L. fermentum* resulted in a significant increase in liver and body weight in chickens [34]. This has been reproduced with the same species in mice, chicks and ducks [35,36]. Overall, the *Limosilactobacillus* genus [26] is consistently associated with metabolic diseases such as obesity and/or NASH in humans and weight gain in animals. It is notable that a strain-dependent effect has been evidenced in animal studies (Table 1). High levels of *Lactobacillus* have been found in dairy beverages and functional foods in the United States and the same levels have been found in functional foods promoting weight gain in animals [37]. Further studies should clarify whether *Limosilactobacillus* spp. are found in functional foods, and whether consumption of such foods could be associated with metabolic diseases.



**Fig. 3.** Linear discriminant analysis of 16S rRNA amplicon sequencing data and qPCR of *L. fermentum*. a. Genus level using MicrobiomeAnalyst, b. Species level using LefSe pipeline (<https://huttenhower.sph.harvard.edu/lefse/>) taking into account the relative abundance of each OTU. Some OTUs corresponded to several species (see Table S1). For clarity, only one clinically relevant species is represented on the graphic. *Limosilactobacillus fermentum*, *Lactococcus lactis* and *Thomasclostridium ramosum* were unambiguously identified (OTU corresponding to only one cultured species). c. For *L. fermentum*, a specific qPCR system developed in our centre and validated for specificity was available (see methods section). This qPCR confirmed the significant association between *L. fermentum* and NASH (5/10 vs 0/10, Mid-P exact test,  $P = 0.02$ ). *Thomasclostridium ramosum* is the new name in 2023 of the former *Erysipelatoclostridium ramosum* (2013), mentioned in the figure, and previously named *Clostridium ramosum* (1971) or *Bacillus ramosus* (1898).

We found an enrichment of the genera *Lactococcus* and *Streptococcus* in the NASH patients we studied. Interestingly, *L. lactis* can also produce remarkable amounts of ethanol [38] and is used in the beer fermentation process [14]. We found an enrichment of *Streptococcus* in NASH patients previously associated with NASH and inflammation [39]. This increase in lactic acid bacteria could be one of the factors related to weight gain and endogenous ethanol synthesis.

The other species associated with NASH patients was *T. ramosa*, designated as the causative agent of several types of infections in humans [40–42]. This bacterial species has also been shown to be obesogenic in mice [43] and humans [44]. A study on diabetic women showed that its abundance in patients could be related to the development of metabolic syndrome symptoms [45,46]. This was shown in a mouse model to promote the accumulation of fat in the liver and deposits through high glucose uptake, which could potentially lead to steatosis [43]. Using 16S rRNA amplicon sequencing, Vernekar et al. showed an increased relative abundance of the genus *Streptococcus* as well as that of *T. ramosa* in NASH compared to healthy controls [39].

Our study included only a small number of participants (ten cases, ten controls), especially compared to previous studies (Vernekar, India,  $N = 147$ , 2018 [39] - Meijnikman, Belgium,  $N = 146$ , 2022 [7]). However, our study was performed in a different country (France) than other similar studies and highlighted the same signature (*L. fermentum* [7] and *T. ramosa*, formerly called *Clostridium ramosum* [39]) in only one study, supporting the reproducibility of the association. The decrease of the *Faecalibacterium* genus in NASH patients has also been described in other studies (Wong, China,  $N = 38$ , 2013 [15] - Özul, Turkey,  $N = 84$ , 2017 [18] - Demir, Germany,  $N = 111$  [19]). The significant difference found in the present study, albeit with a limited number of participants, suggests a very strong association which is detectable even with low numbers. Although the increase of *L. fermentum* and *T. ramosa* in NASH patients had been previously reported [7,39], to our knowledge this is the first time the association between *L. lactis* and NASH has been identified. Further culturomics studies with genomic and phenotypic/metabolic characterisation of *L. fermentum*, *L. lactis* and *T. ramosa* strains isolated from NASH patients would be necessary to confirm this. Isolation from NASH patients is essential as a strain-dependent effect has

been clearly reported for *Limosilactobacillus* species and metabolic diseases (Table 1). This would allow the development of experimental animal models to confirm their involvement in the pathophysiology of this disease.

Our work presents limitations. In the present study, we present only observational data obtained by two different molecular techniques on a limited number of patients and controls. The absence of a culture method, such as the microbial culturomics developed in our center [47], does not allow us to obtain isolates for experimental studies. However, the important point is that we were able to verify the enrichment of the NASH microbiota in *Limosilactobacillus fermentum* by two different techniques: 16S amplicon sequencing and specific PCR, with a very high predictive value (100% since this species was not detected in controls). This is one of the strengths of the present study. Indeed, this specific PCR was developed and validated in our center and allowed us to associate the *Limosilactobacillus* genus with metabolic diseases (*L. reuteri* and obesity [25,32]). Such a high predictive value should be confirmed in future larger studies.

Additionally, using the same techniques, we also demonstrated the depletion of *Methanobrevibacter smithii* in NASH patients. The specific PCR used for *M. smithii* was developed and validated in our center [31]. Similarly, we were the first to associate *M. smithii* depletion in metabolic diseases [25,32] but also in severe acute malnutrition [24]. Enrichment with *L. fermentum* has already been described in a very recent study [7]. In contrast, we describe here for the first time the depletion of *M. smithii*.

Another limitation of our study is its small sample size. In fact, the sample size determines the statistical power to identify a statistically significant difference. A small sample size may lack the power to detect a small difference (small effect size). On the contrary, if a difference is statistically significant with a small sample size, it means that the difference (size effect) and the strength of the association are important with strength of association being the first Bradford Hill criterion for causality [48].

In addition to the *strength of the association*, the results presented here and the literature support an instrumental causal role for *L. fermentum* according to several Bradford Hill criteria [48]: *consistency* (reproducibility) with previously published studies [7]; *specificity* equating the



**Table 1**

Association of species of the new *Limosilactobacillus* genus [26] with metabolic diseases in humans and body and liver weight gain in animals.

<i>Limosilactobacillus</i> species		References
<b>Humans</b>		
<i>L. fermentum</i>	Enriched in NASH patients	Present study Meijnikman, 2022 [7]
<i>L. mucosae</i>	Enriched in NASH patients	Nobil, 2018 [33]
<i>L. reuteri</i>	Enriched in obese patients	Million, 2012 [25] Million, 2013 [32] Million, 2013 [50]
	Associated with acquired obesity following vancomycin administration	
<b>Animals</b>		
<i>L. fermentum</i>	Increases liver volume in chickens Increases weight gain in pigs Increases weight gain in chickens Increases weight gain in piglets Increases weight gain in chickens	Khan, 2007 [34] Yu, 2008 [51] Weis, 2010 [52] Wang, 2012 [53] Capcarova, 2020 [54]
<i>L. ingluviei</i>	Increases weight gain in animals Increases weight gain in chickens	Million, 2012 [55] Khan, 2007 – strain A4 [34]
	Increases weight gain in mice	Angelakis, 2012 [36]
	Increases weight gain in chicks and ducks	Angelakis, 2010 [35]
<i>L. reuteri</i>	Strain dependent effect of <i>L. reuteri</i> on weight gain in animals (Strain L6798 associated with weight gain, strain ATCC4659 protected against diet-induced obesity in mice) Strain dependent effect of <i>L. reuteri</i> on weight gain in animals (Strain L10 associated with weight gain, strain L3 protected against diet-induced obesity in mice) <i>L. reuteri</i> of Peyer's patches is significantly decreased in obesity-prone mice compared with control or obesity-resistant mice. Peyer's patches of high-fat diet-fed mice are enriched with oxidative-resistant <i>L. reuteri</i> with proinflammatory cytokine-inducing ability. Alleviates metabolic syndrome (strain FGSZY33L6)	Fåk, 2012 [56] Qiao, 2015 [57] Sun, 2016 [58] Zheng, 2021 [59]

bacterial species (genus and species-dependent effect, opposite effect to *L. ruminis* although taxonomically close); *plausibility* with the potential role of endogenous ethanol production in the pathophysiological mechanism of liver damage; *temporality* with experimental evidence of *L. fermentum* administration on the liver weight gain [34]; *coherence* between observational (present study and the study by Meijnikman [7]) and *experimental* evidence [34]; and finally *analogy* between the different species of the genus *Limosilactobacillus* and metabolic diseases in humans or weight gain in animals (Table 1).

Ultimately, our study expands the number of ethanol-producing microorganisms that can be detected in NASH patients. It suggests a synergistic mechanism between bacteria and yeasts in the synthesis of endogenous ethanol already reported in the auto-brewery syndrome [49]. Further studies should assess a possible synergistic effect of *L. fermentum* and/or *L. lactis* with *Candida* or *Pichia* yeasts for ethanol production. Our results support the critical importance of taxonomic resolution at the species level, notably with the recent taxonomic reclassification of the *Lactobacillus* genus. Our results reinforce the potential instrumental role of ethanol-producing gut microbes in NASH, notably lactic acid bacteria, opening new avenues for prevention and treatment.

## Ethical considerations

This study was conducted after obtaining the approval of the Comité de Protection des Personnes (CPP: 21.04391.000046 - 21075). The informed and written consent of each participant in the study was collected in compliance with the Helsinki declarations (World Medical Association, 2013). For each participant, stool samples were collected after consent had been obtained.

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## Institutional review board statement

This study was conducted after obtaining the approval of the local ethics committee (IHU Méditerranée Infection, 2020-004) and the approval of the Comité de Protection des Personnes (CPP: 21.04391.000046-21075). The informed and written consent of each participant in the study was collected in compliance with the Helsinki Declaration. For each participant, stool samples were taken after consent had been obtained.

## Informed consent statement

Informed consent was obtained from all subjects involved in the study.

## CRedit authorship contribution statement

**Babacar Mbaye:** Writing – original draft, Visualization, Investigation, Formal analysis. **Reham Magdy Wasfy:** Investigation. **Maryam Tidjani Alou:** Writing – review & editing, Supervision. **Patrick Bortentain:** Writing – review & editing, Resources, Investigation. **Claudia Andrieu:** Investigation. **Aurelia Caputo:** Formal analysis. **Didier Raoult:** Validation, Supervision, Funding acquisition, Conceptualization. **Rene Gerolami:** Writing – review & editing, Supervision, Resources, Conceptualization. **Matthieu Million:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DIDIER RAOULT reports a relationship with Hitachi Ltd that includes: consulting or advisory.

## Data availability

I have shared the link to my data at the Attach File step

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2023.106160>.

## Abbreviations

ABS	Auto-brewery syndrome
ALD	Alcoholic liver disease
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
LDA	Linear discriminant analysis
LPS	lipopolysaccharides
OTU	Operational taxonomic units

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## **Context 4**

### **Description of New Bacterial Species by Taxonogenomics**

## **Article 10**

### ***Streptococcus bouchesdurhonensis* sp. nov. Isolated from a bronchoalveolar lavage of a patient with pneumonia**

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# *Streptococcus bouchesdurhonensis* sp. nov. isolated from a bronchoalveolar lavage of a patient with pneumonia

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## Abstract

Strain Marseille-Q6994 was isolated from a 72-year-old patient with pneumonia from Bouches-du-Rhône department, in France. Cells were Gram positive, non-motile, catalase and oxidase-negative cocci. The major fatty acids were hexadecanoic (47.4%) and tetradecanoic acids (28.3%). 16S rRNA gene sequence comparison suggested that strain Marseille-Q6994 was affiliated to the *Streptococcus* genus. *GroEL* phylogenetic analysis separated strain Marseille-Q6994 in a distinct branch from the closely related *Streptococcus*-type strains with standing in nomenclature. Whole genome sequencing-based methods (OrthoAverage Nucleotide Identity, digital DNA–DNA hybridization and pangenome analysis) supported the classification of the strain into a novel species. Therefore, based on the phenotypic, genomic, and phylogenetic analyses, we propose the name *Streptococcus bouchesdurhonensis* sp. nov. for which strain Marseille-Q6994<sup>T</sup> (CSUR Marseille-Q6994 = DSMZ 113892) is the type strain.

**Keywords** *groEL* phylogenetic analysis · *Streptococcus bouchesdurhonensis* · Bouches du Rhône · Pneumonia · Whole genome sequencing

## Introduction

*Streptococcus* species are Gram positive, catalase-negative cocci that belong to the *Streptococcaceae* family, within the order *Lactobacillales* (Martínez-Lamas et al. 2020). The list of prokaryotic names with Standing in Nomenclature contains 114 *Streptococcus* species validly published at the time of writing <https://lpsn.dsmz.de/genus/streptococ>

*cus*. Many classification schemes were proposed over the years to better describe the genus (Hardie and Whiley 1997; Ferretti and Köhler 2016). In 1995, *Streptococcus* species were categorized into six phylogenetic groups based on 16S rDNA sequences: *S. pyogenes*, *S. anginosus*, *S. salivarius*, *S. mitis*, *S. bovis*, and *S. mutans* (Kawamura et al. 1995). The *Mitis* group contains twenty species with validly published names including one of the leading pathogens, *S. pneumoniae*, which is a major agent of pneumonia (Park et al. 2019; Lannes-Costa et al. 2021). However, several misidentifications were reported within the group, especially between the commensal species *S. mitis*, *S. pseudopneumoniae* and the pathogenic *S. pneumoniae* (Kilian et al. 2008; Kikuchi et al. 1995; Sadowy et al. 2020). Due to the high similarities in 16S rDNA sequences, several housekeeping genes, *sodA*, *rpoB* and *groEL*, were reported to be helpful for *Streptococcus* species identification (Tian et al. 2019; Mühldorfer et al., 2020). The *groEL* gene showed the highest discriminatory power for inter- and intra-species differentiation (Glazunova et al. 2009). More recently, whole genome sequencing-based methods including Average Nucleotide Identity (ANI), OrthoAverage Nucleotide Identity (OrthoANI), and digital DNA–DNA Hybridization (dDDH) (Goris et al.

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2007) provided an important advance in the genomic field and served to delineate a large number of species within the Mitis group. However, these tools also suffered limitations due to the intra-species variation of the group (Jensen et al. 2016). In this study, we used a polyphasic approach based on culturomics and whole genome sequencing analysis to define a novel *Streptococcus* species.

## Materials and methods

### Strain isolation and MALDI-TOF identification

Strain Marseille-Q6994 was isolated from a bronchoalveolar lavage (BAL) of a 72-year-old male admitted to the Timone hospital on January 1st, 2022. The patient suffered from respiratory diseases. He gave an informed consent for this study. BAL culture enabled detection of monomorphic colonies. Six individual colonies were screened by the matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a Microflex spectrometer (Bruker Daltonics, Leipzig, Germany) as previously described (Carbannelle et al. 2011).

### Phenotypic and biochemical characterization

Culture assays were carried out on 5% sheep blood-enriched Columbia agar. Various temperatures were tested (25, 28, 37, 45, and 56 °C) in aerobic, anaerobic and microaerophilic atmospheres using GENbag systems (bioMérieux) within 24 h of incubation. Bacterial growth was also tested in various NaCl concentrations [5, 5.5, 7.5, 10, 15, and 20%] and pH levels [5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5]. Morphological features were observed using scanning electron microscope (Hitachi, Tokyo, Japan). Biochemical characteristics of strain Marseille-Q6994 were determined using different API strips, API 50CH, API ZYM and API 20 STREP according to the manufacturer's instructions (bioMérieux). The fatty acid methyl ester (FAME) analysis was explored by gas chromatography/mass spectrometry (GC/MS), as previously reported (Sasser 2006). FAMES were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500-S8S, PerkinElmer, Courtaboeuf, France). Spectra were compared with those in repertory databases using MS Search 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg, USA) and the FAMES mass spectral database (Wiley, Chichester, UK).

### DNA extraction and sequencing

Genomic DNA (gDNA) was extracted using the EZ1 advanced XL biorobot (Qiagen, Hilden, Germany) using the EZ1 DNA Tissue kit. In early steps of extraction, glass beads

(Sigma, France) were added to the lysis buffer G2 (Qiagen-EZ1 DNA tissue kit) containing one colony of the strain and required a previous acid wash using a FastPrep-24™ 5G Grinder instrument (mpBio) at maximum speed. Then, the sample was treated with lysozyme for 30 min to make the DNA more accessible. Quantification was assessed by a Qubit assay with the high-sensitivity kit (ThermoFisher Scientific). gDNA of strain Marseille-Q6994 was sequenced on a MiSeq sequencer (Illumina Inc, San Diego, CA, USA) using the Nextera XT DNA sample prep kit (Illumina) (Ravi et al. 2018). Paired-end library was prepared by tagmentation and then normalized on specific beads (Beckman Coulter Inc, Fullerton, CA, USA) according to the Nextera XT protocol (Illumina). Sequencing was performed in a single 39-h run in 2×250-bp with the Miseq reagent Kit (V2-500 cycles-Illumina). To improve Miseq assembly, a run was performed with a GRIDION sequencer (Oxford Nanopore Technologies) using the 1D genomic DNA by ligation kit (Oxford Nanopore Technologies). The library was constructed without fragmentation and was loaded on a flow cell Mk 1 Spot-ON Pk.1 via the Spot-ON port.

### Housekeeping genes phylogenies

Phylogenies were inferred from several housekeeping genes including superoxide dismutase [Mn] (*sodA*), RNA polymerase subunit Beta (*rpoB*), DNA gyrase subunit Alpha (*gyrA*), and the gene coding for the chaperonin 60 kDa (*groEL*), and from the 16S rRNA gene (Glazunova et al. 2009). 16S rRNA sequences were extracted from genomes using the online Contamination Estimator by 16S (Contest16S) algorithm (Lee et al. 2017). For the other genes, sequences were extracted from annotated genomes. Phylogenetic trees were inferred using the Molecular Evolutionary Genetic Analysis (MEGA) software version 7.0.26. Sequences were aligned using MUSCLE. Trees were rooted using the maximum likelihood method. The phylogenetic distances between sequences were calculated using Tamura-Nei's model. *S. agalactiae* NCTC 8181<sup>T</sup> was used as outgroup for the Mitis group within the *Streptococcus* genus (Sitkiewicz and Hryniewicz 2010).

### Genomic annotation and comparison

The genome from strain Marseille-Q6994 was assembled using Spades version 3.14.0 (Bankevich et al. 2012). All *Streptococcus* type strain genomes were downloaded from Genbank (<https://www.ncbi.nlm.nih.gov/>) and annotated using Prokka version 1.14.5 (Seemann 2014). Pangenome analysis was inferred using the Roary software (Page et al. 2015). The detail of RNA genes was obtained using ARAGORN version 1.2 (Laslett and Canback 2004). The Orthologous ANI tool (OAT) was used to calculate

OrthoANI values by reciprocal comparison between strain Marseille-Q6994 and the related type strains (Lee et al. 2016). Digital DNA–DNA hybridization values were obtained by the Genome to Genome Distance Calculator (GGDC) under distance formula 2 available in Type Strain Genome Server (TYGS) platform (<https://ggdc.dsmz.de>). Accession numbers of *Streptococcus*-type strains are presented in Table 1

## Results

### Identification and phylogeny

Using Maldi-TOF MS, strain Marseille-Q6994 was identified as *S. mitis*. Blast of its 16S rRNA sequence against those deposited in Genbank confirmed that it belonged to the *Streptococcus* Mitis group. It shared more than 99% sequence similarity with *S. mitis*, *Streptococcus oralis*, *S.*

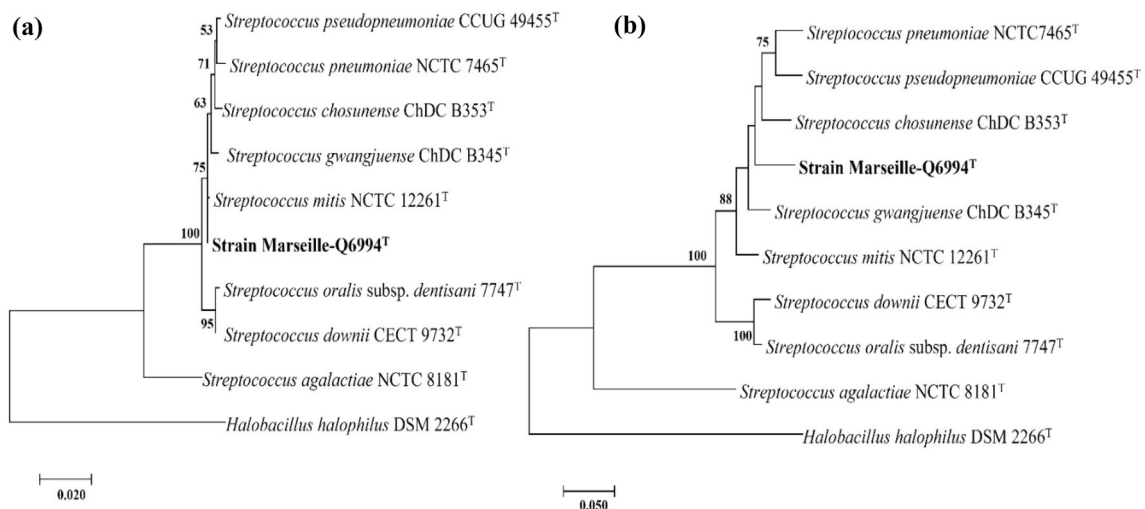
*pseudopneumoniae*, *S. pneumoniae*, *Streptococcus downii*, *Streptococcus gwangjuense*, and *Streptococcus chosunense*. Nucleotide sequences of its housekeeping genes *groEL*, *rpoB*, *sodA*, and *gyrA* were also highly similar to the corresponding genes from *S. mitis* strain NCTC 12261<sup>T</sup> as follows, 93.72%, 98.18%, 96.23%, 97.59%, respectively. All phylogenetic analyses confirmed the affiliation of strain Marseille-Q6994 to the Mitis group. Phylogenetic inferences based on 16S rRNA, *rpoB*, *sodA*, and *gyrA* genes showed that strain Marseille-Q6994 clustered with *S. mitis*. However, in the *groEL*-based tree, strain Marseille-Q6994 formed a distinct taxon, well separated from *S. mitis* and the other related type strains. Only 16S rRNA and *groEL*-based phylogenetic trees are shown in Fig. 1.

### Phenotypic characteristics and comparison

Strain Marseille-Q6994 colonies were observed after 24 h of incubation in both aerobic and microaerophilic atmospheres

**Table 1** *Streptococcus*-type strains used in this study

Species	Type strain	Size (bp)	G + C (%)	Accession numbers
<i>Streptococcus chosunense</i>	ChDC B353	1,902,053	40.10	RBCK00000000.1
<i>Streptococcus downii</i>	CECT 9732	1,922,750	41.10	MZNX00000000.1
<i>Streptococcus gwangjuense</i>	ChDC B345	1,972,481	40.20	NZ_CP032621.1
<i>Streptococcus mitis</i>	NCTC 12,261	1,879,851	40.50	UHFS00000000.1
<i>Streptococcus oralis</i> subsp. <i>dentisani</i>	7747	1,884,389	40.80	CAUK00000000.1
<i>Streptococcus pneumoniae</i>	NCTC7465	2,110,968	39.70	LN831051.1
<i>Streptococcus pseudopneumoniae</i>	CCUG 49,455	2,172,620	39.80	MWSM00000000.1
<i>Streptococcus agalactiae</i>	NCTC 8181	2,448,053	35.70	UAVB01000001.1



**Fig. 1** Phylogenetic analysis showing the position of strain Marseille-Q6994 compared to closely related *Streptococcus* genus based on the 16S rRNA gene (a) and *groEL* gene (b) sequences. Sequences were aligned by MUSCLE. Phylogenetic inferences were obtained using the maximum likelihood method within the Mega software. Values at

the nodes are bootstrap values obtained using 1000 replicates. *Halobacillus halophilus* DSM 2266<sup>T</sup> served as an outgroup of the genus (Accession number: HE717023.1). The scale bars indicate 2% and 5% sequence divergences for 16S rRNA and *groEL*, respectively



at 28 °C and 37 °C. Very small colonies appeared at 28 °C in anaerobic atmosphere. In the tested range pH range, growth occurred at pH 7. Regarding NaCl tolerance, our strain showed no salt tolerance to the tested NaCl concentration ranges. In optimal growth conditions on blood-enriched Columbia agar (37 °C, aerobic atmosphere, PH7, no NaCl), colonies appeared to be small (0.6 mm), white, with regular edges, and surrounded by an alpha hemolysis halo. SEM micrographs revealed a coccus-shaped bacteria with septa, with an average size of 649 nm in diameter and 694 nm in length (Fig. 1S). Phenotypic characteristics of our isolate compared with those of the closest bacterial species are shown in Table 2. According to FAME analysis, the major fatty acids of strain Marseille-Q6994 were even-chained saturated structures (Table 1S). Minor amounts of unsaturated and odd-chained saturated fatty acids were detected.

The most abundant fatty acid was the hexadecenoic acid (palmitic acid) as previously described (Drucker et al. 1974; Labbé et al. 1985). Strain Marseille-Q6994 differed from *S. mitis* and *S. pseudopneumoniae* by the positive assimilation of starch and its inability to assimilate ribose sugar. A positive alkaline phosphatase activity differentiated it from *S. mitis*. In addition, positive activity to alpha-galactosidase and a negative activity to beta-galactosidase differentiated it from *S. pseudopneumoniae*, in addition to the positive assimilation of mannose.

### Genome characteristics and comparison

The strain Marseille-Q6994 genome was 3,766,235-bp long with G + C content of 40.1%. It contained 4031 genes. Among them, 3927 were protein-coding genes, and 104

**Table 2** Phenotypic characteristics of strain Marseille-Q6994 compared to other members of the ‘Mitis group’ (Lim et al. 2019; Martínez-Lamas et al. 2020; Liu et al. 2021)

Properties	Q6994	SD	SO	SC	SG	SP	SPS	SM
Source	Bronchoalveolar lavage	Dental plaque	Carie-free tooth	Maxillary cyst	Pericoronitis lesion	NA	Human sputum	NA
Gram stain	+	+	+	+	+	+	+	+
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Hemolysis	α	α	α	α	α	α	α	α
Temperature	28–37 °C	37 °C	37 °C	30–37 °C	37–40 °C	37 °C	37 °C	37 °C
Oxygen tolerance	Microaerophilic	Aerobic	Microaerophilic	Facultative anaerobic	Facultative anaerobic	Microaerophilic	Microaerophilic	Facultative anaerobic
Incubation	24H	24H	24-48H	48H	48H	24-48H	24-48H	24-48H
Salt tolerance	–	NA	0.79%	0.50%	0.50%	NA	NA	NA
Optimum pH	7	7	6–7.5	7–7.5	7–7.5	NA	NA	NA
Alkaline Phosphatase	+	+	+	NA	NA	–	+	–
Esculin	–	–	–	–	–	v	–	–
Arginine	–	–	NA	–	–	+	–	v
α-galactosidase	+	+	+	–	–	+	–	+
β-Galactosidase	–	+	–	+	+	+	+	–
B-glucosidase	–	NA	–	–	–	–	v	–
Ribose	–	NA	–	–	–	–	v	v
Mannose	+	+	+	NA	NA	NA	–	+
Mannitol	–	NA	–	–	–	v	v	–
Sucrose	+	+	+	NA	NA	+	v	+
Raffinose	+	+	+	–	–	+	–	v
Glycogen	V	–	NA	NA	NA	NA	NA	–
Starch	+	–	–	+	–	v	–	–
Lactose	+	+	+	+	+	+	+	+

SC *S. chosunense* strain chDC B353<sup>T</sup>, SD *S. downii* strain CECT 9732<sup>T</sup>, SG *S. gwangjuense* strain chDC B345<sup>T</sup>, SM *S. mitis* strain NCTC 12261<sup>T</sup>, SO *S. oralis* subsp. *dentisani* strain 7747<sup>T</sup>, SP *S. pneumoniae* strain NCTC 7465<sup>T</sup>, SPS *S. pseudopneumoniae* strain 49455<sup>T</sup>, Q6994 strain Marseille-Q6994

+ positive, – negative, v variable, NA not detected

All compared *Streptococcus*-type strains were non-motile, non-endospore forming and exhibited negative catalase reaction

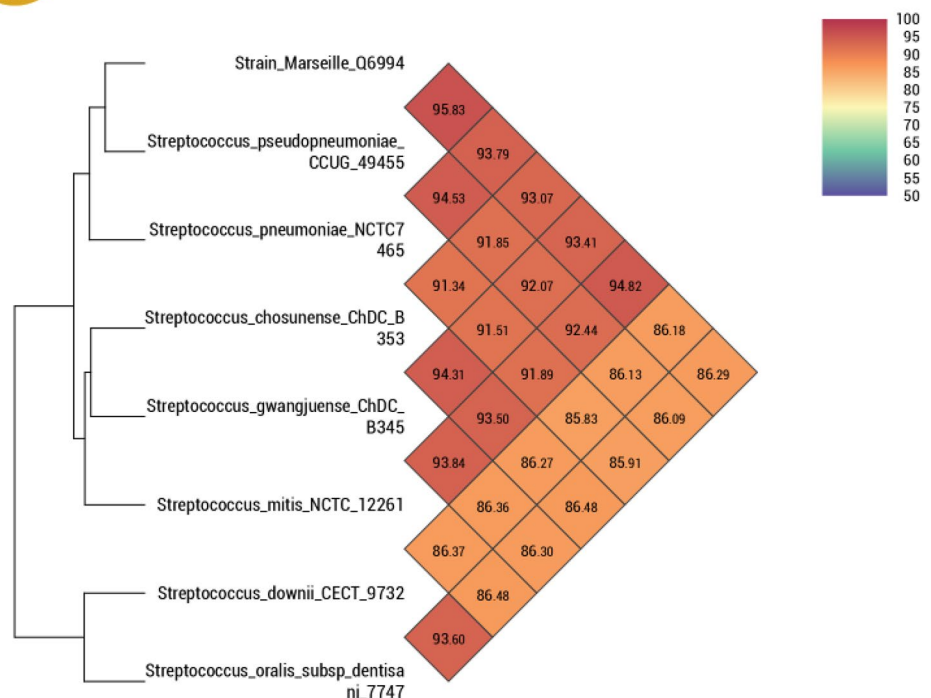
were RNA (84 were tRNAs, 1 was an mRNA, 6 were 16S rRNAs, 7 were 23S rRNAs, and 6 were 5S rRNAs). Its genome size (3.7 Mb) was larger than those of the closest species which ranged from 1.8 Mb to 2.1 Mb (Table 1). Using Orthologous ANI tool, the strain exhibited 86.18% of similarity with *S. downii* strain CECT 9732T and 95.83% with *S. pseudopneumoniae* strain CCUG 49455T (Fig. 2). Taken into consideration that ANI can be not sufficient to circumscribe a novel species in the Mitis group as previously described (Jensen et al. 2016), digital DDH was in support of the decision to propose strain

Marseille-Q6994 as a novel species. dDDH values ranged from 31.8% with *S. downii* strain CECT 9732T to 64.1% (Table 3). These values were lower than the 70% threshold recommended to delineate a novel bacterial species (Goris et al. 2007). Genome-based phylogenetic analysis revealed the closest relationship of the studied strain with *S. pseudopneumoniae* strain CCUG 49455 T as confirmed by OrthoANI and DDH values (Fig. 3). The pangenome analysis demonstrated that strain Marseille-Q6994 was clearly distinct from other compared *Streptococcus* species (Fig. 4).

**Fig. 2** Heatmap generated with OrthoANI values using the OAT software version 0.93.1 between Marseille-Q6994 and related *Streptococcus* species



Heatmap generated with OrthoANI values calculated from the OAT software. Please cite Lee et al. 2015.

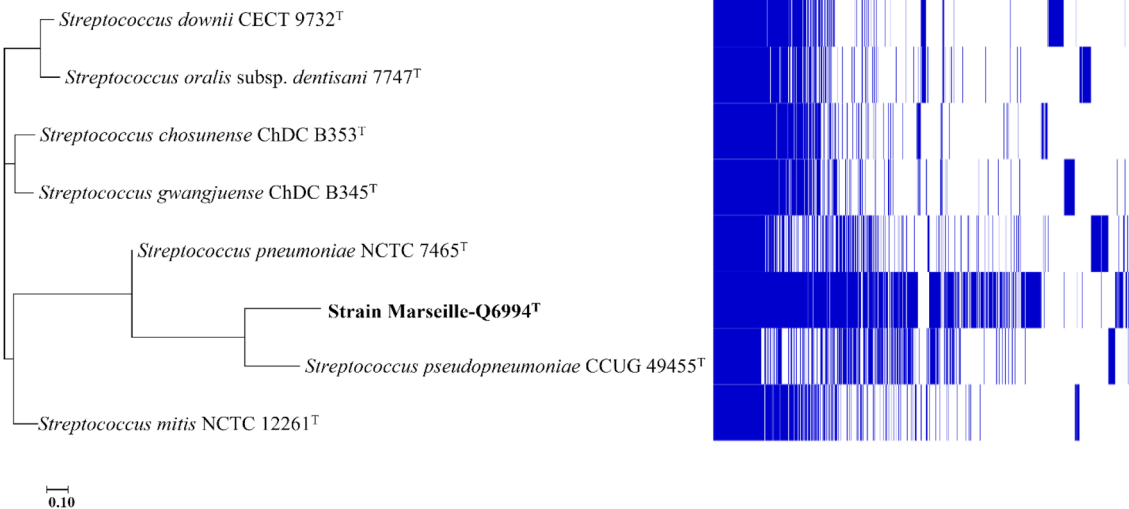
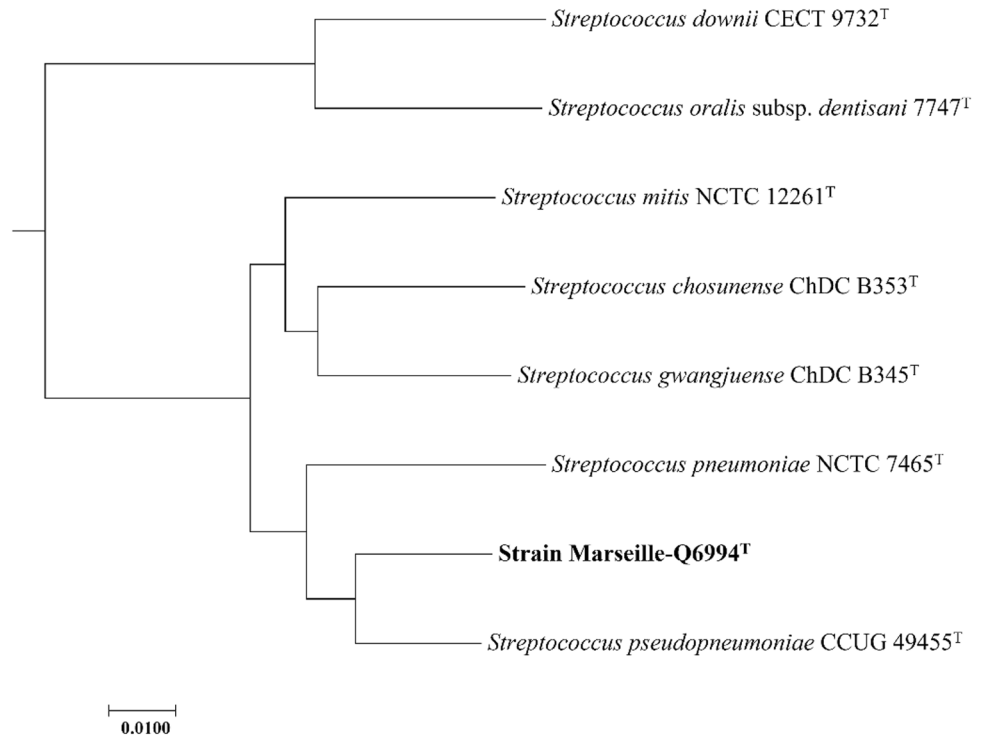


**Table 3** Digital DNA–DNA hybridization values (%) between strain Marseille-Q6994 and related *Streptococci* using formula 2 of the GGDC web server (Estimation was based on identities /HSP length)

	Q6994	SC	SD	SG	SM	SO	SP	SPS
Q6994	100	51.4	31.8	52.4	57.7	32.2	53.9	64.1
SC		100	31.2	56.3	53.2	31.1	45.2	46.4
SD			100	31.6	31.5	52.2	30.9	31.5
SG				100	54.1	31.3	46.1	47.6
SM					100	31.7	46.7	48.3
SO						100	31.2	31.4
SP							100	58.9
SPS								100

SC *S. chosunense* strain chDC B353<sup>T</sup>, SD *S. downii* strain CECT 9732<sup>T</sup>, SG *S. gwangjuense* strain chDC B345<sup>T</sup>, SM *S. mitis* strain NCTC 12261<sup>T</sup>, SO *S. oralis* subsp. *dentisani* strain 7747<sup>T</sup>, SP *S. pneumoniae* strain NCTC 7465<sup>T</sup>, SPS *S. pseudopneumoniae* strain 49455<sup>T</sup>, Q6994 strain Marseille-Q6994

**Fig. 3** Genome-based tree inferred with FastME version 2.1.6.1 (Lefort et al. 2015). Distances were calculated using the Genome Blast Distance Phylogeny (GBDP) approach. The tree was rooted with midpoint. The scale bar represents a 1% nucleotide divergence



**Fig. 4** Pangenome-based tree showing the position of Marseille-Q6994 among related *Streptococcus* species. Pangenomic analysis was carried out using the Roary software. The scale represents a 10% nucleotide sequence divergence

## Conclusion

According to our findings, strain Marseille-Q6994 which exhibits several unique characteristics among *Streptococcus* species may be classified within a new species for which we propose the name *Streptococcus bouchesdurhonensis* sp. nov.

## Description of *Streptococcus bouchesdurhonensis* sp. nov.

*Streptococcus bouchesdurhonensis* (bou.ches.du.rho.nen'sis. N.L. masc. adj., *bouchesdurhonensis*, pertaining to Bouches-du-Rhône, the name of the French territory where strain Marseille-Q6994 was isolated).

Growth occurs at 37 °C, after 24 h of incubation under aerobic conditions on 5% sheep blood-enriched Columbia agar. Cells are aerobic and anaerobic facultative. Growth pH is 7. No salt tolerance is observed. Colonies are small and white and show alpha hemolysis on sheep blood-agar. Mean length of bacterial cell is 694 nm, and the mean width is 649 nm. Using an API 50CH strip, positive reactions were obtained for D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, D-maltose, D-lactose, D-saccharose, D-raffinose, starch and glycogen. Positive activities were observed for esterase (C-4), leucine arylamidase, valine arylamidase,  $\alpha$ -chymotrypsin, phosphatase acid, naphthol phosphohydrolase,  $\alpha$ -galactosidase and  $\alpha$ -glucosidase using API Zym strip. The API 20 STREP strip showed positive results with 6-bromo-2-naphthyl- $\alpha$ -D-galactopyranoside, L-leucine- $\beta$ -naphthylamide, D-lactose (bovine origin) and D-raffinose. *Streptococcus bouchesdurhonensis* sp. nov. caused pneumonia in the index patient. The major fatty acids are the hexadecanoic (47.4%) and tetradecanoic acids (28.3%). The genome size is 3,766,235-bp long with mol% G + C content of 40.1%.

Our strain was isolated from a bronchoalveolar lavage of an hospitalized 72-year-old patient and was deposited in the Collection de Souches de l'Unité des Rickettsies, CSUR—Marseille as strain Marseille-Q6994 and in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen—Braunschweig) as DSMZ = 113892.

### Accession numbers

16S rRNA and genome of strain Marseille-Q6994 were deposited in Genbank under accession numbers OX216967 and CALYYQ010000000, respectively.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00203-022-03348-0>.

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**Author contributions** MZ drafted the manuscript and analyzed the data. RMW carried out the technical characterization of strain Marseille-Q6994. AC submitted the genome sequence to Genbank. ZZ and FF conceived the study. P-EF and LH revised the manuscript and supervised the project.

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**Data availability** The 16S rRNA gene sequence and genome sequence from *Streptococcus bouchesdurhonensis* strain Marseille-Q6994T were, respectively, deposited in NCBI

under the following accession numbers: OX216967 and CALYYQ010000000.

### Declarations

**Conflict of interest** The authors declare that they have no conflicts of interest.

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## **Article 11**

**Taxonogenomics of *Culturomica massiliensis* gen. nov.,  
sp. nov., and *Emergencia timonensis* gen. nov., sp. nov.  
new bacteria isolated from human stool microbiota**

Afaf Hamame, **Reham Magdy Wasfy**, Cheikh Ibrahima Lo, Florence Fenollar,  
Didier Raoult, Pierre-Edouard Fournier and Linda Houhamdi

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## OPEN Taxonogenomics of *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov. new bacteria isolated from human stool microbiota

Afaf Hamame<sup>1,2</sup>, Reham Magdy Wasfy<sup>1,2</sup>, Cheikh Ibrahima Lo<sup>2,3</sup>, Florence Fenollar<sup>2,3</sup>, Didier Raoult<sup>1,2</sup>, Pierre-Edouard Fournier<sup>2,3</sup> & Linda Houhamdi<sup>2,3</sup>✉

Two new bacterial strains, Marseille-P2698<sup>T</sup> (CSUR P2698 = DSM 103,121) and Marseille-P2260<sup>T</sup> (CSUR P2260 = DSM 101,844 = SN18), were isolated from human stools by the culturomic method. We used the taxonogenomic approach to fully describe these two new bacterial strains. The Marseille-P2698<sup>T</sup> strain was a Gram-negative, motile, non-spore-forming, rod-shaped bacterium. The Marseille-P2260<sup>T</sup> strain was a Gram-positive, motile, spore-forming rod-shaped bacterium. Major fatty acids found in Marseille-P2698<sup>T</sup> were C<sub>15:0 iso</sub> (63%), C<sub>15:0 anteiso</sub> (11%), and C<sub>17:0 3-OH iso</sub> (8%). Those found in Marseille-P2260<sup>T</sup> strain were C<sub>16:00</sub> (39%), C<sub>18:1n9</sub> (16%) and C<sub>18:1n7</sub> (14%). Strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> had 16S rRNA gene sequence similarities of 91.50% with *Odoribacter laneus*<sup>T</sup>, and of 90.98% and 95.07% with *Odoribacter splanchnicus*<sup>T</sup> and *Eubacterium sulci*<sup>T</sup>, respectively. The exhibited digital DNA-DNA Hybridization values lower than 20.7%, and Orthologous Average Nucleotide Identity values lower than 73% compared to their closest related bacterial species *O. splanchnicus*<sup>T</sup> and *E. sulci*<sup>T</sup> respectively. Phenotypic, biochemical, phylogenetic, and genomic results obtained by comparative analyses provided sufficient evidence that both of the two studied strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> are two new bacterial species and new bacterial genera for which the names *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov. were proposed, respectively.

The human gut microbiota is considered currently as one of the most active research fields in microbiology<sup>1</sup>. In fact, this microflora harbours a huge biodiversity of bacteria of which a large part is still unknown<sup>2</sup>. Researchers used a variety of strategies to speed up and simplify the description of new bacterial species by optimizing their in vitro growth conditions<sup>3-5</sup>. Culturomics is one among these strategies, which relies on a diversification of culture conditions that allowed the identification of several new bacterial species isolated from the human gastrointestinal tract<sup>3-5</sup>. Since 2012, it allowed the isolation of over 1000 different human-associated bacterial species, including several hundreds of new species<sup>3-6</sup>. This method highlighted the need to adopt taxonomic approaches to clinical microbiology by including the use of modern and reproducible tools, such as high throughput genomic and proteomic analyses.

In November 30, 2015, two putative new bacterial species, *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov., were isolated from patient's stools, and partially described<sup>7,8</sup>. The genomic sequencing of new described bacterial species constitutes currently a necessary step for performing their comparative taxogenomic descriptions with their closest related known species. In fact, several recent publications have used genomic descriptions to characterize the new species by comparison to their closest relatives strains<sup>9-11</sup>. The aim of our current study was to complete the phenotypic, taxonomic and genomic characterization proposal of new genera and new species of *Culturomica massiliensis* gen. nov., sp. nov., strain

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Marseille-P2698<sup>T</sup>, and *Emergencia timonensis* gen. nov., sp. nov., strain Marseille-P2260<sup>T</sup> and formally expose the creation of both species.

## Materials and methods

**Sample collection and ethics approval.** In November 30, 2015, stool samples were collected from hospitalized patients in the Timone Hospital (Marseille, France) as a part of a study of human microbiota diversity. Patients provided signed informed consent<sup>7,8</sup>. The study protocol was approved by the ethics committee of the institut de recherche fédératif 48, under agreement number 09-022. In addition, all methods were performed in accordance with the relevant guidelines and regulations. Each sample was then cultured according to the culturomics method previously established in our laboratory<sup>3,5</sup>. Various types of bacterial colonies were isolated on 5% of sheep blood-enriched Columbia agar (bioMérieux®, Marcy l’Etoile, France). Bacterial colonies were then screened for identification by Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) instrument (Bruker Daltonics®, Bremen, Germany) as previously reported<sup>12</sup>. Both two strains studied herein had a MALDI-TOF score lower than 2.0, which did not allow their correct identification. Their spectra were then added to the local MALDI-TOF MS database (<https://www.mediterranee-infection.com/urms-data-base>).

**16S rRNA gene sequencing and identification.** The 16S rRNA sequences from both strains were directly extracted from their whole genomes sequences and then, compared by Basic Local Alignment Search Tool nucleotide (BLASTn) to the non-redundant (nr) databases<sup>13</sup>. The obtained sequence similarity percentages allowed identification of the closest species to each strain, and to predict if it was new species (<98.65% of similarity). Then, the phylogenetic tree was constructed based on these 16S rRNA gene sequences in comparison to the closest related species of each studied strain. Designated species sequences were downloaded from nr<sup>14</sup>, and aligned with ClustalW. Phylogenetic trees were constructed using MEGA 11 version 11.0.10 with the maximum likelihood method and 1000 bootstrap replications<sup>15</sup>.

**Phenotypic and biochemical characterizations.** Optimal culture conditions were determined by testing various incubation temperatures (25, 28, 37, 42, and 50 °C), atmospheres (aerobic, anaerobic and micro-aerophilic), NaCl concentrations (5, 5.5, 7.5, 10, 15, and 20% of NaCl) and pH levels (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5). The morphology and motility were observed using a new-generation scanning electron microscope (Hitachi High-71 Technologies Corporation, Tokyo, Japan).

Furthermore, three semi-quantitative standardized micro-methods of Analytical Profile Index (API®, bioMérieux®) tests: API® 20A, API® 50 CH, and API® ZYM were used, according to the manufacturer’s instructions<sup>16</sup>, in order to study carbohydrate metabolism and enzymatic activities.

Fatty acid methyl ester (FAME) analysis was explored by Gas Chromatography/Mass Spectrometry, as previously reported<sup>17,18</sup>. FAMES were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500—SQ 8 S, Perkin Elmer®, Courtaboeuf, France). Obtained spectra were compared with those contained in the repertory databases using MS Search 2.0 operated with the Standard Reference Database 1A (National Institute of Standards and Technology-NIST, Gaithersburg, USA), and FAMES mass spectral database (Wiley, Chichester, UK).

**Whole genomic sequencing and bioinformatic analyses.** First, bacterial DNA was extracted using the EZ1 DNeasy Blood Tissue Kit (Qiagen® GmbH, Hilden, Germany) in line with the manufacturer’s protocol<sup>19</sup>. Whole-genome sequencing was performed using an Illumina® MiSeq sequencer (Illumina®, San Diego, CA, USA)<sup>20</sup>. Then, sequenced genomes were assembled using SPAdes 3.5.0 software<sup>21</sup>, which reduces short indels and the huge number of mismatches. Raw reads in contigs less than 700-bp-long were removed. Finally, the quality of the sequenced genome was checked using BLAST against the nr/nt database. This method allowed us to better explore the relationship between a submitted assembly of our new species to the International Nucleotide Sequence Database Collaboration (INSDC), i.e., DDBJ, ENA, or GenBank, and the assembly represented in the NCBI reference sequence (RefSeq) project. The global statistics section reported general statistics information including Gaps between scaffolds, number of scaffolds, number of contigs, total sequence length, and total ungapped length. Furthermore, taxonomic data were checked according to the best-matching-type strain with the declared new species repertory in NCBI<sup>22</sup>.

During annotation, genomic parameters were evaluated including transfer-messenger RNAs (tmRNAs) and transfer RNAs (tRNAs) using ARAGORN version 1.2 and ribosomal RNAs (rRNAs) using Barrnap version 0.9<sup>23,24</sup>. Generated file (.faa) was used for BLAST-P analyses against the Clusters of Orthologous Genes (COGs) database, and used for CRISPR-Cas identification<sup>25</sup>. Resistance genes were screened using ResFinder<sup>26</sup>. Other bioinformatic tools were also used such as AntiSMASH to search polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS)<sup>27</sup>. Circular maps of the two genomes were generated using CGView (Circular Genome Viewer) software. This Java application converts XML or tab-delimited input into a Vector Graphics format<sup>28</sup>.

Besides, phylogenetic trees of interest were generated with the FastME 2.1.6.1 software to highlight the position of each new bacterial strain among its closest relatives<sup>29</sup>. Digital DNA-DNA Hybridization (dDDH) values were calculated to check the difference between the genomes using the following website (<https://ggdc.dsmz.de>). Critical limit was set at 70% below which a prokaryotic species may be considered as new<sup>30</sup>. Orthologous average nucleotide identity (OrthoANI) version 0.93.1 was also used to calculate genomic similarities between studied species and their related taxa.



**Ethics approval.** The study was approved by the ethics committee of the Institut de Recherche Fédératif 48 under Authorization number 09-022 with the consent of the patients.

## Results

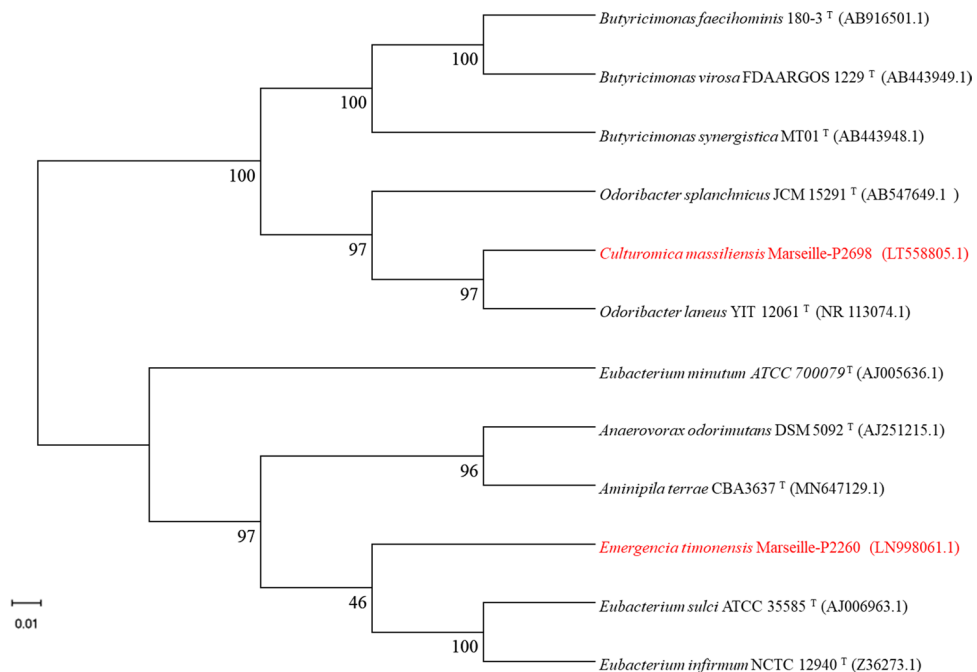
**Strain identification and phylogenetic analyses.** The species names *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov., had been previously proposed for the new species mainly as representative strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup>, respectively<sup>7,8</sup>. As these previous descriptions were not exhaustive, we revisited the work by including phylogenetic, morphological, and genomic data.

Strain Marseille-P2698<sup>T</sup> had a 16S rRNA gene sequence similarity and a query coverage of 91.5% and 99% respectively with *Odoribacter laneus* strain YIT 12061<sup>T</sup> (Fig. 1). Strain Marseille-P2260<sup>T</sup> exhibited 16S rRNA gene similarity and a query coverage of 92.72%, and 100% respectively, with *Eubacterium sulci* strain ATCC 35585<sup>T</sup> (Fig. 1).

**Phenotypic and biochemical characterizations.** Growth of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> occurred on 5% sheep blood-enriched Columbia agar (bioMérieux®), after 48 h of incubation at 37 °C in a strict anaerobic atmosphere. Optimal growth was obtained at pH 7. However, strain Marseille-P2260<sup>T</sup> did not tolerate NaCl, whereas strain Marseille-P2698<sup>T</sup> could grow with a NaCl concentration of 0.5%.

Strain Marseille-P2698<sup>T</sup> is a Gram-negative rod, strictly anaerobic, motile, and non-spore-forming with a size of 1.5–3 µm in length and 0.3 to 0.4 µm in diameter. It exhibits a positive catalase, but no oxidase activity. The colonies are circular, beige and from 0.7 to 1.2 mm in diameter.

Strain Marseille-P2260<sup>T</sup> is a Gram-positive, strictly anaerobic rod, ranging in length from 1 to 1.5 µm, and in diameter from 0.5 to 1 µm. It has no catalase or oxidase activity. Colonies of strain Marseille-P2260<sup>T</sup> are translucent with a diameter of 0.5 to 1 mm. The remaining cell characteristics of both strains compared to their closest relatives are summarized in Table 1. Using API® 50CH strips (bioMérieux®), positive reactions were obtained for both studied strains for glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, D-melezitose, gentiobiose, D-tagatose. Using API® ZYM strips (bioMérieux®), positive activities were observed for esterase lipase (C8), leucine arylamidase, phosphatase acid, and naphthol-as-bi-phosphohydrolase for both studied strains. In contrast, phosphatase alkaline, esterase (C4), α-chymotrypsin, β-galactosidase, α-glucosidase, β-glucosidase, and N-acetyl-β-glucosaminidase were only positive for strain Marseille-P2260<sup>T</sup>. Using API® 20A strips, positive results for the two strains were obtained for D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, esculin ferric citrate, glycerol, D-cellobiose,



**Figure 1.** Phylogenetic tree with the position of new species (strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup>) among closely related species. The following phylogenetic tree was performed from the comparison of 16S rRNA sequences. The accession numbers of 16S rRNA gene are mentioned in parentheses. Bootstrap appears at the nodes. MUSCLE software was used to align sequences. The tree was designed with the MEGA-X software. The used methodology is the Maximum Likelihood method and Kimura 2-parameter model.

Properties	<i>Culturomica massiliensis</i> gen. nov., sp. nov. Marseille-P2698 <sup>T</sup>	<i>Odoribacter laneus</i> YIT 12061 <sup>T</sup>	<i>Odoribacter splanchnicus</i> JCM 15291 <sup>T</sup>	<i>Butyricimonas synergistica</i> MT01 <sup>T</sup>	<i>Emergencia timonensis</i> gen. nov., sp. nov. Marseille-P2260 <sup>T</sup>	<i>Eubacterium sulci</i> ATCC 35585 <sup>T</sup>	<i>Eubacterium infirmum</i> NCTC 12940 <sup>T</sup>
Gram stain	–	–	–	–	+	–	+
Cell shape	Rod	Rod	Fusiform	Rod	Rod Bacilli	Rod	Rod
Motility	+	NA	–	–	+	–	–
Cell diameter(μm)	0.7–1.2	NA	NA	1.0	0.5–1.0	1.0	1.0
Length (μm)	0.3–0.4 × 1.5–3	9.2 × 1.15	0.7 × 1.0–5.0	1.5	1 to 1.5	0.5 × 1–2	0.5 × 1–2
Endospore	–	–	–	–	–	–	–
Optimum growth temperature	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C
Oxygen tolerance	Strict anaerobic	Anaerobic	Anaerobic	Anaerobic	Strict anaerobic	Anaerobic	Strict anaerobic
Incubation	4 days	4 days	2–3 days	2 days	3 days	3 days	7 days
Salt tolerance	0.5%	NA	NA	NA	–	NA	NA
Optimum pH	7	NA	NA	6–7.5	7	NA	NA
Acid phosphatase	+	+	NA	NA	+	NA	NA
Catalase	+	NA	–	–	–	NA	–
Oxidase	–	NA	NA	–	–	NA	NA
Indole	–	+	+	+	–	–	–
Urease	–	–	–	–	–	–	–
βGalactosidase	–	+	–	–	+	–	NA
Ribose	+	NA	NA	NA	+	–	–
Mannose	+	–	+	–	+	–	NA
Mannitol	+	–	NA	–	+	–	–
Sucrose	+	–	–	–	+	–	–
Glucose	+	–	+	+	+	NA	–
Fructose	+	NA	+	NA	+	NA	NA
Maltose	+	–	NA	–	+	–	–
Sorbitol	+	–	NA	–	+	–	NA
Starch	+	NA	NA	NA	–	NA	NA
Lactose	+	–	–	–	+	–	–
Source	Human faeces in diabetic patient	Human faeces	Human Abdominal abscess	Mouse feces	Human faeces	Human gingival sulcus	human periodontal pockets

**Table 1.** Phenotypic characteristics of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> compared with closely related species. + positive result. – negative result. NA not available data.

D-mannose, D-melezitose, D-sorbitol, L-rhamnose, and D-trehalose. However, strain Marseille-P2698<sup>T</sup> was positive to hydrolysis of gelatin, unlike strain Marseille-P2260<sup>T</sup> which was negative.

The most abundant fatty acids of strain Marseille-P2698<sup>T</sup> were 13-methyl-tetradecanoic acid (63%), 12-methyl-tetradecanoic acid (11%), and 3-hydroxy-15-methyl-hexadecanoic acid (8%). Several other branched structures, mainly iso, were also detected. Specific 3-hydroxy structures were detected, mainly branched as well. For strain Marseille-P2260<sup>T</sup>, the major fatty acid was hexadecanoic acid (39%) (Table 2).

**Genomic properties and analyses.** The whole genome of strain Marseille-P2698<sup>T</sup> was composed of 14 contigs, for a total size of 4,410,591 bp, with a G+C content of 43 mol% (Fig. 2). This genome contained 3679 genes, of which 3487 were protein-coding genes. In addition, 59 RNA sequences were also identified and distributed as follows: 8 rRNAs (three 16S, two 23S, and three 5S), 51 tRNAs, and 1 tmRNA.

Genome from strain Marseille-P2260<sup>T</sup> was composed of 9 contigs, with a size of 4,661,482 bp, and a 45.8 mol% G + C content (Fig. 2). Genome annotation identified 4380 genes, of which 4288 were protein-coding genes. There were 56 RNA sequences including 5 rRNAs (one 16S, one 23S, and three 5S), 51 tRNAs, and 1 tmRNA.

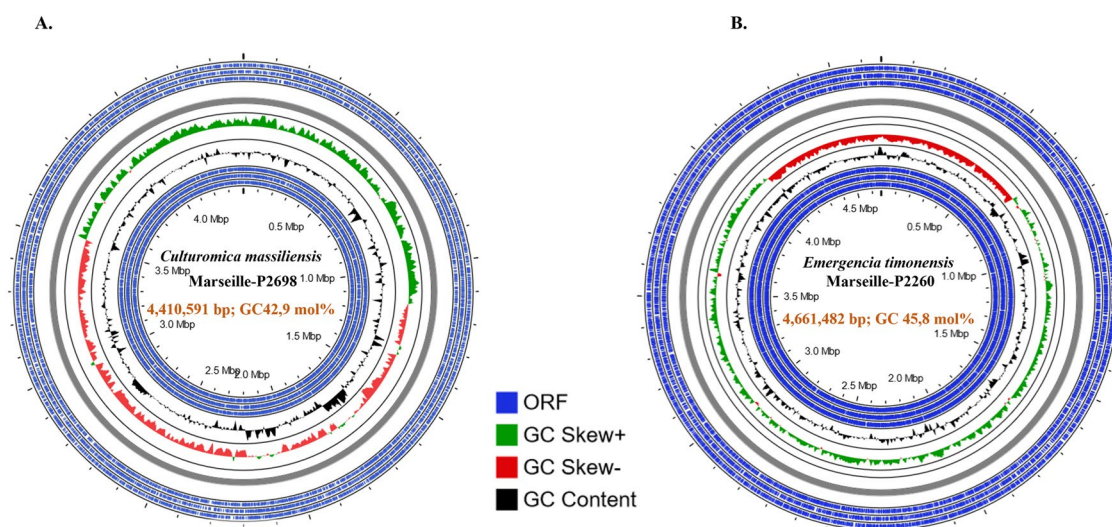
*Odoribacter laneus*<sup>T</sup>, *O. splanchnicus*<sup>T</sup>, *Butyricimonas synergistica*<sup>T</sup>, *B. faecihominis*<sup>T</sup> and *B. virosa*<sup>T</sup> exhibited genome sizes ranging from 3.77 to 4.81 Mbp. The closest bacteria of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> are presented in Table 3. *Eubacterium sulci*<sup>T</sup>, *E. infirmum*<sup>T</sup>, *A. terrae*<sup>T</sup>, *E. minutum*<sup>T</sup>, and *Aminipila odorimutans*<sup>T</sup> exhibited genome sizes ranging from 1.73 to 4.66 Mbp.

Strain Marseille-P2698<sup>T</sup> shared dDDH values of 21.6% with *Odoribacter laneus*<sup>T</sup>, 20.6% with *O. splanchnicus*<sup>T</sup>, 30.6% with *Butyricimonas faecihominis*<sup>T</sup>, 21.8% with *B. virosa*<sup>T</sup>, and 18.9% with *B. synergistica*<sup>T</sup>. Strain Marseille-P2260<sup>T</sup> exhibited dDDH values of 20.1% with *Eubacterium sulci*<sup>T</sup>, 21.1% with *E. infirmum*<sup>T</sup>, 23.1% with *A. terrae*<sup>T</sup>, 24.4% with *E. minutum*, and 21.1% with *Aminipila odorimutans* (Table 4).

The phylogenetic relationships of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> with relative strains, based on whole-genome sequencing, is represented in Fig. 3.

	Fatty acids	Name	Mean relative % (a)
	15:0 iso	13-methyl-tetradecanoic acid	63.4 ± 1.8
	15:0 anteiso	12-methyl-tetradecanoic acid	10.9 ± 0.2
	17:0 3-OH iso	3-hydroxy-15-methyl-Hexadecanoic acid	8.0 ± 0.7
	16:00	Hexadecanoic acid	7.8 ± 0.3
	16:0 3-OH	3-hydroxy-Hexadecanoic acid	3.1 ± 0.3
	5:0 iso	3-methyl-Butanoic acid	1.2 ± 0.0
<i>Culturomica</i>	18:2n6	9,12-Octadecadienoic acid	1.1 ± 0.1
<i>massiliensis</i>	18:1n9	9-Octadecenoic acid	1.0 ± 0.1
Strain	15:00	Pentadecanoic acid	TR
Marseille-	14:00	Tetradecanoic acid	TR
P2698 <sup>T</sup>	18:00	Octadecanoic acid	TR
	15:0 3-OH iso	3-hydroxy-13-methyl-Tetradecanoic acid	TR
	16:0 iso	14-methyl-Pentadecanoic acid	TR
	18:1n7	11-Octadecenoic acid	TR
	17:0 iso	15-methyl-Hexadecanoic acid	TR
	14:0 iso	12-methyl-Tridecanoic acid	TR
	16:0 3-OH iso	3-hydroxy-14-methyl-Pentadecanoic acid	TR
	17:00	Heptadecanoic acid	TR
	15:00	3-OH anteiso 3-hydroxy-12-methyl-Tetradecanoic acid	TR
	16:00	Hexadecanoic acid	39.2 ± 2.4
	18:1n9	9-Octadecenoic acid	16.5 ± 1.3
	18:1n7	11-Octadecenoic acid	14.5 ± 4.8
<i>Emergencia</i>	14:00	Tetradecanoic acid	11.9 ± 0.6
<i>timonensis</i>	18:00	Octadecanoic acid	9.5 ± 1.4
Strain	18:1n3	15-Octadecenoic acid	5.6 ± 0.9
Marseille-	18:2n6	9,12-Octadecadienoic acid	1.1 ± 0.3
P2260 <sup>T</sup>	15:00	Pentadecanoic acid	TR
	15:0 anteiso	12-methyl-tetradecanoic acid	TR
	12:00	Dodecanoic acid	TR

**Table 2.** Cellular fatty acids composition of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup>. a: Mean peak area percentage. TR trace amount.



**Figure 2.** Circular genome map of strains Marseille-P2698<sup>T</sup> (left) and Marseille-P2260<sup>T</sup> (right) generated by the CGView software. From outside to the center: blue rings demonstrate the ORFs (Open Reading Frames) on both forward and reverse strands, green and red rings represent both positive and negative GC skew respectively, and black ring represents the GC content plot.

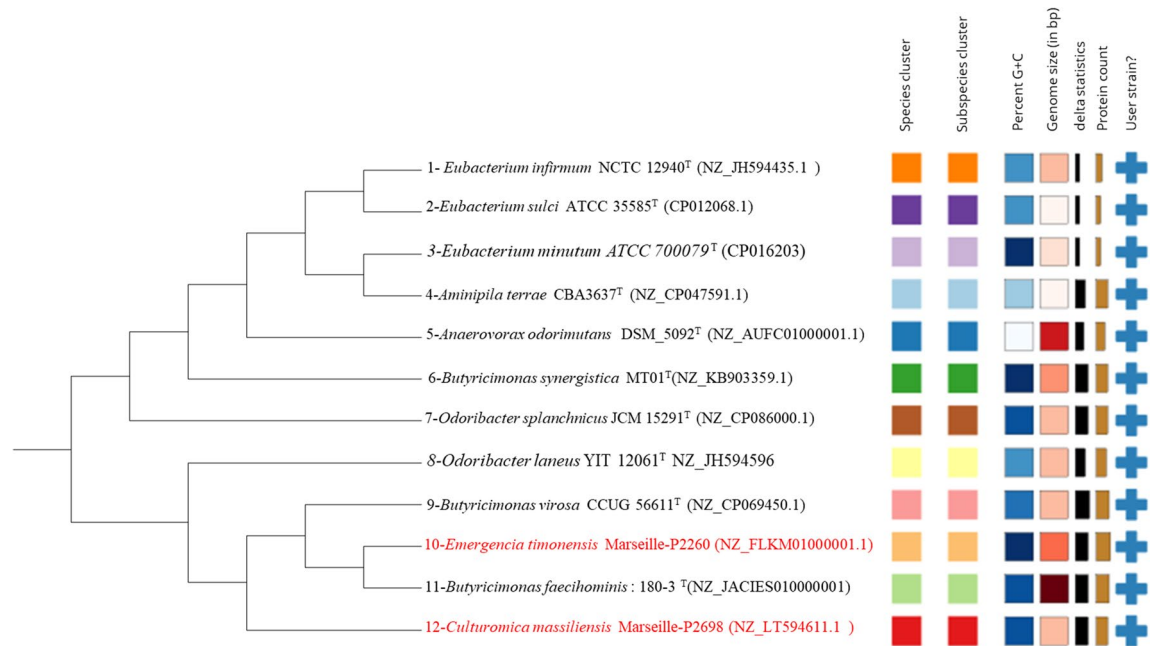
	Organisms	INSDC/RefSeq	Size (M bp)	G + C %	Protein-coding genes	rRNA	tRNA	tmRNA	genes	Genes assigned to COGs	Contigs	Bases	CDS
	<b>Strain Marseille-P2698<sup>T</sup></b>	FLSN01000000	4.41	43	3487	8	50	1	3679	2113	11	4410591	3620
<i>Culturomica</i>	<i>Odoribacter laneus</i> YIT 12,061 <sup>T</sup>	NZ_JH594596	3.77	40.6	2993	10	53	3	3100	2995	42	3,794,909	3095
<i>massiliensis</i>	<i>Odoribacter splanchnicus</i> JCM 15291 <sup>T</sup>	CP086000.1	4.39	43.4	3545	12	59	1	3757	2106	1	4,393,364	3685
strain	<i>Butyricimonas synergistica</i> MT01 <sup>T</sup>	NR_041690.1	4.77	43.8	3741	8	54	1	3922	2113	16	4,770,838	3859
Marseille-P2698 <sup>T</sup>	<i>Butyricimonas faecihominis</i> 180-3 <sup>T</sup>	NR_126194.1	4.79	42.9	3832	4	57	1	4017	2156	29	4,793,705	3955
	<i>Butyricimonas virosa</i> CCUG 56611 <sup>T</sup>	CP069450.1	4.81	42.4	3837	15	57	1	4032	2169	1	4,813,143	3959
	<b>Strain Marseille-P2260<sup>T</sup></b>	FLKM01000000	4.66	45.8	4288	5	50	1	4380	2895	9	4,661,482	4324
<i>Emergencia</i>	<i>Eubacterium sulci</i> ATCC 35585 <sup>T</sup>	CP012068.1	1.73	39.9	3062	6	40	1	1646	1235	1	1,739,380	1599
<i>timonensis</i>	<i>Eubacterium infirmum</i> NCTC 12940 <sup>T</sup>	NZ_JH594435.1	1.91	39.9	3510	4	43	1	1781	1305	13	1,910,927	1733
strain	<i>Aminipila terrae</i> CBA3637 <sup>T</sup>	CP047591.1	3.51	37	3102	18	65	1	3581	2346	1	3,512,404	3497
Marseille-P2260 <sup>T</sup>	<i>Eubacterium minutum</i> ATCC 700,079 <sup>T</sup>	CP016203	1.9	45.79	1454	7	43	1	1620	1035	124	1,903,428	1246
	<i>Anaerovorax odorimutans</i> DSM_5092 <sup>T</sup>	NZ_AUFC01000001.1	3.26	31.5	2894	4	42	1	2944	1976	54	3,263,520	2897

**Table 3.** Summary of comparative genomes and characteristics for strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup>.

	CM	OL	OS	BS	BF	BV	ET	EM	AT	AO	ES	EI
CM	100%	21.6% (± 4.7%)	20.6 (± 4.7%)	18.9 (± 4.5%)	30.6 (± 4.9%)	21.8 (± 4.7%)	17.8 (± 4.4%)	16 (± 4.4%)	17.1 (± 4.4%)	21.5 (± 4.7%)	18.3 (± 4.5%)	18.2 (± 4.5%)
OL		100%	13.3 (± 6.5%)	18.6 (± 4.66%)	18.5 (± 4.5%)	19.9 (± 4.6%)	18.1 (± 4.5%)	17.7 (± 4.5%)	18.1 (± 4.5%)	21 (± 4.7%)	18.2 (± 4.6%)	18.1 (± 4%)
OS			100%	23.7 (± 4.8%)	19.2 (± 4.6%)	23 (± 4.8%)	19.3 (± 4.5%)	18.5 (± 4.5%)	19.1 (± 4.6%)	21.5 (± 4.7%)	19.1 (± 4.6%)	17.3 (± 4.5%)
BS				100%	21 (± 4.7%)	20.9 (± 4.7%)	19.6 (± 4.6%)	20.9 (± 4.7%)	20.6 (± 4.6%)	22.9 (± 4.8%)	20.9 (± 4.7%)	18.1 (± 4.5%)
BF					100%	42.2 (± 5.1%)	43.4 (± 5.1%)	3.7 (± 2%)	3.7 (± 2%)	30.4 (± 4.9%)	3.7 (± 2%)	3.7 (± 2%)
BV						100%	18 (± 4.5%)	17.7 (± 4.5%)	17.9 (± 4.5%)	21 (± 4.7%)	18.3 (± 4.5%)	18.3 (± 4.6%)
ET							100%	24.4 (± 4.8%)	23.1 (± 4.8%)	21.1 (± 4.7%)	20.1 (± 4.6%)	21.1 (± 4.6%)
EM								100%	29.5 (± 4.9%)	31.2 (± 4.9%)	25.4 (± 4.8%)	24.9 (± 4.8%)
AT									100%	23 (± 4.8%)	28.8 (± 4.9%)	24.4 (± 4.8%)
AO										100%	19.8 (± 4.6%)	23.4 (± 4.8%)
ES											100%	28.9 (± 4.9%)
EI												100%

**Table 4.** Comparative digital DNA-DNA Hybridization (dDDH) values (%) between studied bacterial genomes.

CM. *Culturomica massiliensis* gen. nov., sp. nov., Marseille-P2698<sup>T</sup>. OL. *Odoribacter laneus*<sup>T</sup>. OS. *Odoribacter splanchnicus*<sup>T</sup>. BS. *Butyricimonas synergistica*<sup>T</sup>. BF. *Butyricimonas faecihominis*<sup>T</sup>. BV. *Butyricimonas virosa*<sup>T</sup>. ET. *Emergencia timonensis* gen. nov., sp. nov., Marseille-P2260<sup>T</sup>. EM. *Eubacterium minutum*<sup>T</sup>. AT. *Aminipila terrae*<sup>T</sup>. AO. *Anaerovorax odorimutans*<sup>T</sup>. ES. *Eubacterium sulci*<sup>T</sup>. EI. *Eubacterium infirmum*<sup>T</sup>.

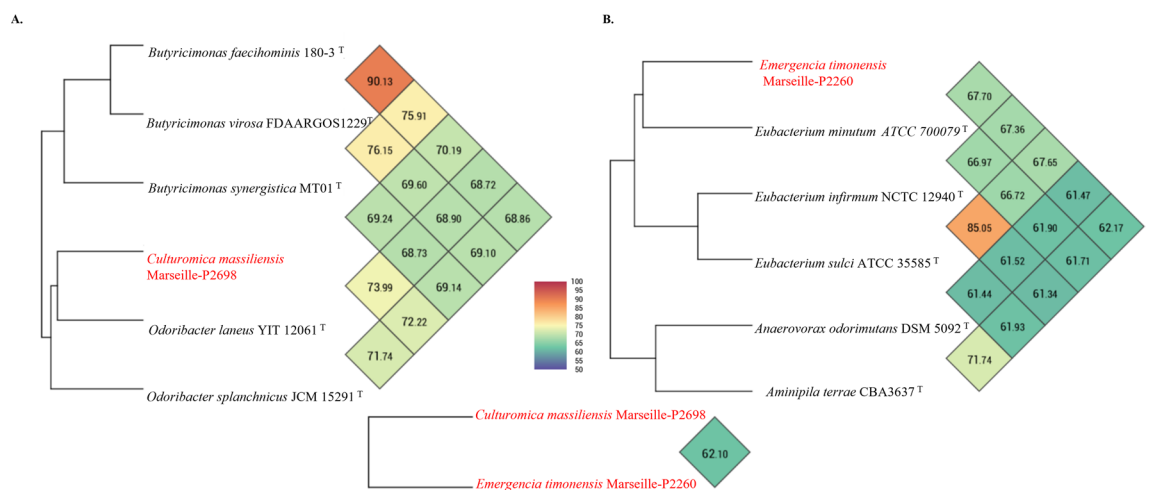


**Figure 3.** Whole-Genome sequence-based phylogenetic tree of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> using TYGS design tree with FastME 2.1.4 software based on Genome BLAST Distance Phylogeny (GBDP) parameters. Distances were calculated from the genome sequences, and branch lengths were calculated by GBDP distance formula d5.

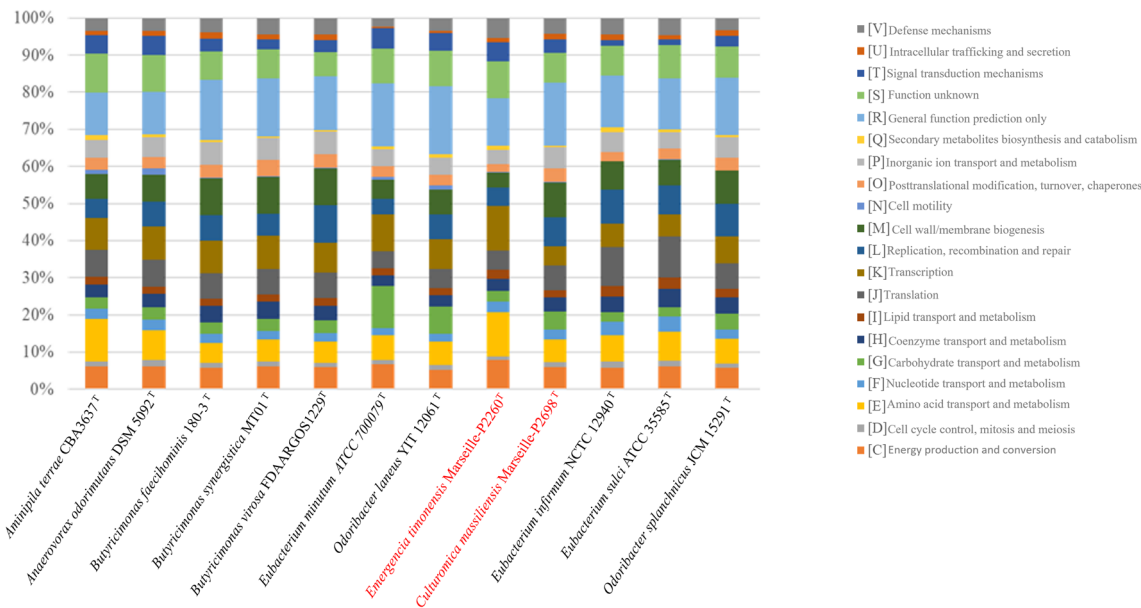
The obtained dDDH values were lower than the 70% threshold used for delineating prokaryotic species<sup>4</sup>. In addition, strain Marseille-P2698<sup>T</sup> exhibited OrthoANI values of 73.99% with *O. laneus*<sup>T</sup>, 72.22% with *O. splanchnicus*<sup>T</sup>, and 69.24% with *B. synergistica*<sup>T</sup>. Strain Marseille-P2260<sup>T</sup> had an OrthoANI value of 67.65% with *E. sulci*<sup>T</sup> and 67.36% with *E. infirmum*<sup>T</sup>. These values were lower than 95%, also suggesting that strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> belonged to distinct species (Fig. 4).

Genes encoding proteins are divided into several categories according to their functions and others have unknown functions. COGs of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> have different functional distributions from their closest species (Fig. 5).

**Antibiotic resistance genes and defense mechanisms.** Using the ResFinder software, antibiotic resistance genes (ARG) *erm* (F) and *tet* (Q) were detected within the genome of strain Marseille-P2698<sup>T</sup>, with identity percentages of 100% and 99.8%, respectively. For strain Marseille-P2260<sup>T</sup>, ARG included *erm* (B), *tet* (M), and *tet* (O), with identity percentage ranging from 99.7% to 100% (Table 5).



**Figure 4.** Heatmap and phylogenetic trees showing the average nucleotide identity based on calculated orthology (OrthoANI) of Marseille-P2698<sup>T</sup> (A) and Marseille-P2260<sup>T</sup> (B) compared to their closest related bacterial species.



**Figure 5.** Distribution of functional classes of predicted genes expressing group of proteins that clusters according to functions.

	Resistance gene	Identity	Alignment Length/Gene Length	Position in contig	Phenotype
	<b>Macrolide</b>				
	erm(F)	100	801/801	1,826,565.0.1827365	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
	<b>Lincosamide</b>				
Culturomica	erm(F)	100	801/801	1,826,565.0.1827365	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
massiliensis	<b>Streptogramin b</b>				
Marseille-P2698 <sup>T</sup>	erm(F)	100	801/801	1,826,565.0.1827365	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
	<b>Tetracycline</b>				
	tet(Q)	99.8	1926/1926	1,055,038.0.1056963	doxycycline,tetracycline,minocycline
	<b>Macrolide</b>				
	erm(B)	100	738/738	1,359,368.0.1360105	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
Emergencia	<b>Lincosamide</b>				
timonensis	erm(B)	99.86	738/738	1,359,368.0.1360105	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
Marseille-P2260 <sup>T</sup>	<b>Streptogramin b</b>				
	erm(B)	99.9	738/738	1,359,368.0.1360105	erythromycin,lincomycin,clindamycin,quinupristin,pristinamycin ia,virginiamycin s
	<b>Tetracycline</b>				
	tet(M)	99.84	1920/1920	1,222,665.0.1224584	doxycycline,tetracycline,minocycline
	tet(O)	99.79	1920/1920	2,008,154.0.2010073	doxycycline,tetracycline,minocycline

**Table 5.** Antibiotic resistance genes detected in the genomes strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> using ResFinder software.

The genomes of strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup> contained CRISPR-Cas modules (Table 6). Strain Marseille-P2698<sup>T</sup> contains one defense mechanism composed of polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) enzymes. NRPS-PKS was previously demonstrated to have a role in the biosynthesis of pharmaceutically-important natural products<sup>31</sup>. Using ANTI SMASH software, NRPS-PKS-like genes cluster had been detected in genome of strain Marseille-P2698<sup>T</sup>, and β-lactone; containing potent medicinal properties<sup>32, 33</sup>, had been detected in strain Marseille-P2698<sup>T</sup> genome. In addition, according to our results in ToxFinder-1.0 software detecting toxins<sup>34</sup>, our two strains did not possess toxin genes.

Bacteria	Element	CRISPR Id /gene name	Start	End	Spacer /Gene	Repeat consensus /cas genes	Orientation
	CRISPR		112	199	1	ATGGGACTCTTTTGTGAAATA	
	Cas cluster	CAS	71,544	73,337	1	Cas3_0_I	+
	Cas cluster	CAS-TypeIB	613,607	622,650	7	Cas6_0_I-III, Cas7_3_IB, Cas5_1_IB, Cas3_0_I,	+
		Cas6_0_I-III				Cas4_0_I-II, Cas1_0_I-II-III, Cas2_0_I-II-III-V	
<i>Culturomica</i>	CRISPR	Cas7_3_IB	622,870	625,330	37	CTTTAATTGAACCTAAGGTAGAATTGAAAC	+
<i>massiliensis</i>	CRISPR	Cas5_1_IB	992,376	993,764	21	GTTTCAATACTACTTAGTCTATTAAAAG	+
Marseille-P2698 <sup>T</sup>	CRISPR	Cas3_0_I	2,048,339	2,049,405	15	GTCTCAATGCCGTGATTACAGCTATTCTCTAAATC	+
	Cas cluster	Cas4_0_I-II	2,049,852	2,057,279	6	Cas2_0_I-II-III-V, Cas1_0_I-II-III, Cmr6_0_IIIb,	+
						Cmr4_0_IIIb, Cmr3_0_IIIb, Cas10_1_IIIb	
	CRISPR	Cas1_0_I-II-III	3,461,867	3,462,226	5	ATTTCAATTCTACTCCAGTTCTATTAAAAT	+
	CRISPR	Cas2_0_I-II-III-V	3,750,732	3,750,812	1	AATTCTCTTTAGCGTTGGTTTGTG	+
	CRISPR		882	1086	2	GGCATATAATTATCAATACAGTATTTCCCTATTTT	
	CRISPR		300,401	300,525	1	TTGCGGCAACGCCGTGAGATTTTGAATCATTATA	+
	CRISPR		522,369	522,503	1	TTGCAGCAACGCTGTCAATATCGTTGTTCATTAT	+
						ACCATGA	
	CRISPR		1,380,564	1,381,183	9	GTCCGATCCTCATGGATGCGTGGATTGAAAT	-
	CRISPR	CAS-TypeIC	1,429,920	1,430,223	4	GTCTTGCTCCGCATGGAGCAAGTGGATTGAAAT	+
	Cas cluster		1,453,194	1,461,082	7	Cas3_0_I, Cas5_0_IC, Cas8c_0_IC, Cas7_0_IC, Cas4_0_I-	
						II, Cas1_0_IC, Cas2_0_I-II-III-V	
<i>Emergencia</i>	CRISPR		1,461,248	1,463,328	30	GTCCCACTCCGCATGGAGTGCCTGGATTGAAAT	+
<i>timonensis</i>	CRISPR		1,608,002	1,608,146	1	AAAGCATAAGGACTTTCATCAATTTGTTTCAAAAT	-
						TGTGACAAGTCCATTA	
Marseille-P2260 <sup>T</sup>	CRISPR		1,944,682	1,944,814	1	GGTATAATGAATCCATAACCAACGACGTTGTCGCA	+
						AGGTAG	
	CRISPR		225,209	225,286	1	ATCCAAGCCAATTCTTATCGAGGCT	-
	CRISPR		530,585	530,712	1	TATTTTCAGCGGAATGCACATGGTATAATAACGCA	-
	CRISPR		853,819	853,957	1	TGGTATAATGGTTCACGATCTTACGGCGTTGCCGA	+
						AGTGTAGGAACCA	
	CRISPR		1,673,013	1,673,146	1	CATTATACGACAAAACGCGCCAAATCAGAGATTG	+
						GGCGTT	
	CRISPR		1,798,696	1,798,841	1	TCCTTATGCTTAAACGCTAAATCTGTGATTAGC	-
						TGCGTTTTGTCGTATAA	
	CRISPR		1,910,685	1,910,831	1	AATGAACGCTTCCTTGTGTCAGCAATCTGATTAAGAT	-
						TGTGCGTTTATTATACCA	
	CRISPR		1,927,335	1,927,459	1	CCGTTTCATCTTTCGGCGTTGCCGCAAGGTAGGAA	+
	CRISPR		2,025,969	2,026,111	1	TGTTCTGCTAACTGACTCCATATCCAGCCCTCTGT	+
						GTTACGCAGATT	

**Table 6.** Identification of CRISPR-Cas type and subtype genes in user-submitted sequence for strains Marseille-P2698<sup>T</sup> and Marseille-P2260<sup>T</sup>.

## Conclusion

Culturomics combined with Taxogenomics allowed the isolation and the full characterization of two new bacterial species isolated from human intestine. Primitive phylogenetic comparisons were previously performed<sup>7,8</sup>, while a thorough phylogenetic and genomic analyses were performed in our study. The results of phenotypic, biochemical, phylogenetic, and genomic analyses obtained for both studied strains proved that they belong to new species. Thus, we propose the creation of two new bacterial genera and new species, *Culturomica massiliensis* gen. nov., sp. nov., and *Emergencia timonensis* gen. nov., sp. nov..

**Description of *Culturomica* gen. nov.** *Culturomica* (*Cul.tu.ro.mi'ca*. N.L. fem, *culturomica*, referring to a new method of diversified bacterial culture). Cells are anaerobic, Gram-negative, motile and rod-shaped. The type species is *Culturomica massiliensis* gen. nov., sp. nov.. It was isolated from human feces.

**Description of *Culturomica massiliensis* gen. nov., sp. nov.** *Culturomica massiliensis* (*mas.si.li.en'sis*. L. fem. adj. *massiliensis*, form “*Massilia*”, the Latin name of Marseille, France, where the type strain was isolated). Bacterial cells are Gram-negative and rod-shaped. They are motile and non-spore-forming, with lengths ranging from 1.5 to 3.0 µm, and diameters from 0.3 to 0.4 µm. The optimal growth conditions are 37 °C, a strict anaerobic atmosphere, and neutral pH. On 5% sheep blood-enriched Columbia agar, colonies appear small, circular, beige to white, and measure between 0.7 and 1.2 mm in diameter. They ferment D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-glucose, glycerol, D-lactose, D-saccharose, D-trehalose, D-melezitose,

Amidon, gentiobiose, D-tagatose, potassium gluconate and potassium 5-ketogluconate. Enzymatic activities for phosphatase al-kaline, esterase, esterase lipase, leucine arylamidase,  $\alpha$ -chymotrypsin, phosphatase acid, naphthol-as-bi-phosphohydrolase,  $\beta$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, and N-acetyl- $\beta$ -glucosaminidase are present. The major cell wall fatty acids are C<sub>15:0 iso</sub> (63%), C<sub>15:0 anteiso</sub> (11%), and C<sub>17:0 3-OH iso</sub> (8%). The genome size from strain Marseille-P2698<sup>T</sup> is 4.41 Mbp-long with a G+C content of 43 mol%.

The type strain Marseille-P2698<sup>T</sup> (CSUR P2698 = DSM 103,121) was isolated from a stool specimen of a 66-year-old man with diabetes mellitus.

The accession numbers for the genomic and 16S rRNA gene sequences of strain Marseille-P2698<sup>T</sup> are deposited in the GenBank database under references FLSN00000000 and LT558805, respectively.

**Description of *Emergencia* gen. nov.** *Emergencia timonensis* (*e.mer.gencia* N.L. fem. n., *Emergencia*, for emergence, in reference to the discovery of emerging human bacteria).

**Description of *Emergencia timonensis* gen. nov., sp. nov.** *Emergencia timonensis* (*ti.mo.nen'sis*. L. fem. adj., *timonensis*, from Timone, the name of a university hospital in Marseille, France, where the type strain was isolated).

Bacterial cells are Gram-positive rod-shaped and bacilli. They are motile, non-spore-forming, with lengths ranging from 1.0 to 1.5  $\mu$ m, and a diameter of 0.5 to 1.0  $\mu$ m. The optimal growth conditions are 37 °C, a strict anaerobic atmosphere, and pH.7. On 5% sheep, blood-enriched Columbia agar, colonies of strain Marseille-P2260<sup>T</sup> appear small, translucent, and measure from 0.7 to 1.2 mm in diameter. Cells ferment D-glucose, D-mannitol, D-lactose, D-saccharose, D-maltose, salicin, esculin ferric citrate, glycerol, D-cellobiose, D-mannose, D-melezitose, D-sorbitol, L-rhamnose, D-trehalose, glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, Methyl- $\alpha$ -glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, gentiobiose, D-turanose, D-tagalose, L-fucose, and potassium gluconate. In addition, enzymatic activities such as esterase lipase, leucine arylamidase, acid phosphatase, and naphthol phosphohydrolase are present. The major cell wall fatty acids are C<sub>16:00</sub> (39%), C<sub>18:1n9</sub> (16%), and C<sub>18:1n7</sub> (14%).

The genome size from strain Marseille-P2260<sup>T</sup> is 4.66 Mbp with a G+C content of 45.8 mol%.

The type strain Marseille-P2260<sup>T</sup> (CSUR P2260 = DSM 101,844 = SN18) was isolated from a stool sample the feces of a healthy patient with an unremarkable medical history.

## Data availability

The accession numbers for the genomic and 16S rRNA gene sequences are deposited in the GenBank database under references: FLSN00000000 and LT558805 respectively for Marseille-P2698<sup>T</sup> strain, and FLKM00000000 and LN998061 respectively for Marseille-P2260<sup>T</sup> strain.

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Conceptualization and methodology: D.R., P.E.F., F.F., L.H.; Software and analysis: A.H.; Validation: L.H., P.E.F., C.I.L.; Investigation: A.H., R.M.W.; Writing-original draft preparation: A.H., R.M.W., P.E.F., L.H.; Supervision, L.H., P.E.F.; Project administration, P.E.F., D.R.; Funding acquisition, D.R. All authors have read and agreed to the published version of the manuscript.

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## Competing interests

The authors declare no competing interests.

## Additional information

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# CONCLUSIONS AND PERSPECTIVES

Hepatitis B is one of the most common liver diseases worldwide, and studies describing HBV-associated dysbiosis lack consensus on a signature microbiota profile in most disease stages. Therefore, our systematic review provided a comprehensive overview of the most recent research on HBV-associated dysbiosis investigating microbiome, mycobiome, and virome studies. We used traditional databases and AI tools to retrieve all the available studies. We did not consider using other AI-assisted technology or tools to retrieve the key results of the articles as they could produce incorrect or incomplete outputs in many situations compared to our manual analysis. Indicating that the use of technology with human oversight and control is required and vital.

Our analysis showed that the results of the included studies were heterogeneous in some cases, particularly in patient subgroups. This could be due to different factors influencing gut microbiota, such as genetics, age, and diet, which were not adjusted in many cases.

Eventually, reproducible results of a signature microbiota profile in most HBV-related diseases were obtained. An increase in *Pseudomonadota*, *Bacilli*, *Prevotella*, *Streptococcus*, *Veillonella* and a decrease in *Clostridia*, *Lachnospiraceae*, and *Roseburria* characterizes this signature. High abundance of *Saccharomyces cerevisiae*, and *Candida tropicalis* was a constant finding. These results should be considered when prescribing *Saccharomyces* as probiotic fungi, especially in patients with HBV infection.

Notably, dysbiosis was observed in all groups except for HBV carriers, who seemed similar to the control group. This indicated the role of microbiota in the pathogenesis of HBV infection.

Our culturomics study in HBV-infected patients showed enrichment in potentially pathogenic *Enterocloster* and *Clostridium* species. Future research should consider more diverse samples and focus on elucidating the mechanisms by which *E. bolteae* might contribute to liver inflammation and HBV disease progression. Unlike earlier research, our study found an increase in *Clostridium* species. This could be related to various factors, including sample size, the study populations, and various diagnostic techniques (culturomics with different culture conditions), while previous studies utilized solely the metagenomic approach.

Moreover, the present results suggest that endogenous alcohol production by gut microbiota might participate in HBV-related liver disease, as recently described in NASH patients [88]. Notably, microbial culturomics allowed us to obtain live bacterial species that could contribute to the pathophysiology of the disease.

Additionally, members of uncultivated CPR were reported for the first time in HBV-associated dysbiosis, such as *Candidatus Saccharibacteria*, and *Atribacter* phyla. These phyla were found to be negatively correlated with prothrombin time in HBV-infected patients. As a result, further study into establishing culture methods for CPR is recommended to isolate for further research into HBV-associated disorders.

Although studies on bacterial overgrowth and dysbiosis are increasing, no specific intestinal bacterial pattern is identified to have an etiological role in the development of ALD or its related complications, such as HCC. The highly abundant *Thomasclavelia*

*ramosa* bacterial species isolated from patients with ALD-associated HCC indicated its potential role in mediating carcinoma. Therefore, experimental studies and further research are needed to investigate the causality effect. Indeed, the fact that alcohol consumption causes intestinal bacterial dysbiosis provides an opportunity for the treatment and/or prevention of ALD by targeting intestinal microbiota.

Probiotics and FMT have proved to be promising gut microbiota-targeted therapies. However, more extensive FMT randomized clinical trials are needed to confirm the practicability in clinical settings. Hence, synthetic probiotics and selective microbiota transplantation may make these therapies more precise and bring fewer side effects.

Finally, our findings shed light on the possible significance of certain bacterial species in the pathophysiology of HBV and ALD, paving the way for more studies into microbiome-targeted therapy alternatives.

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