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# The role of dietary sugars in intestinal homeostasis and microbiota composition

Xufei Zhang

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# Sorbonne Université

Ecole doctorale 394: Physiologie, Physiopathologie et Thérapeutique

*INRA Jouy-en-Josas, Institut MICALIS, Equipe AMIPEM*

## **The role of dietary sugars in intestinal homeostasis and microbiota composition**

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Thèse de doctorat

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## ABBREVIATIONS

<b>ABCB1</b>	ATP-binding cassette B1
<b>AICAR</b>	5-aminoimidazole-4-carboxamide 1 $\beta$ -D-ribose nucleoside
<b>AJs</b>	Adherent junctions
<b>AMP</b>	Adenosine monophosphate
<b>AMPK</b>	AMP-activated protein kinase
<b>ARC</b>	Arcuate nucleus
<b>Arx</b>	Aristaless related homeobox
<b>Atoh1/Math1</b>	Atonal homologue 1
<b>BBM</b>	Brush border membrane
<b>CaSR</b>	Ca <sup>2+</sup> sensing receptor
<b>CCK</b>	Cholecystokinin
<b>CCK1R</b>	Cholecystokinin receptor 1
<b>CCK2R</b>	Cholecystokinin receptor 2
<b>CNS</b>	Central nervous system
<b>EECs</b>	Enteroendocrine cells
<b>FFAR2</b>	Free fatty acid receptor 2
<b>FFAR3</b>	Free fatty acid receptor 3
<b>FFAR4</b>	Free fatty acid receptor 4
<b>ENS</b>	Enteric nervous system
<b>EGCs</b>	Enteric glial cells
<b>FXR</b>	Farnesoid X receptor
<b>FOXA1/2</b>	The winged helix factors 1/2
<b>FITC</b>	Fluorescein isothiocyanate
<b>GCG</b>	Pre-proglucagon
<b>GFAP</b>	Glial fibrillary acidic protein
<b>GF</b>	Germ-free
<b>GFP</b>	Green fluorescent protein
<b>GI</b>	Gastrointestinal
<b>GIP</b>	Glucose-dependent insulinotropic peptide
<b>GLP-1/GLP-2</b>	Glucagon-like peptide-1 or 2

<b>GLUT2</b>	Glucose transporter 2
<b>GLUT5</b>	Glucose transporter 5
<b>GLUT5<sup>-/-</sup></b>	GLUT5 knockout
<b>GPCR</b>	G-protein coupled receptor
<b>GPR40</b>	G-protein coupled receptor 40
<b>GPR41</b>	G-protein coupled receptor 41
<b>GPR120</b>	G-protein coupled receptor 120
<b>GPR93</b>	G-protein-coupled receptor 93
<b>HDL</b>	High-density lipoproteins
<b>HES1</b>	Hairy/enhancer fo split
<b>HFCS</b>	High fructose corn syrup
<b>HFD</b>	High-fat diet
<b>HRP</b>	Horseradish peroxidase
<b>IBD</b>	Inflammatory bowel disease
<b>IBS</b>	Irritable bowel disease
<b>IECs</b>	Intestinal epithelium cells
<b>IFN<math>\gamma</math></b>	Interferon gamma
<b>IL13</b>	Interleukin 13
<b>IL1<math>\beta</math></b>	Interleukin 1 beta
<b>ILDR1</b>	Immunoglobulin-like domain containing receptor 1
<b>LPS</b>	Lipopolysaccharides
<b>JAMs</b>	Junctional adhesion molecules
<b>KHK</b>	Ketohexokinase or fructokinase
<b>KHK<sup>-/-</sup></b>	KHK knockout
<b>Klf4</b>	Kruppel-like factor 4
<b>M</b>	Microfold
<b>MAGUK</b>	Membrane Associated Guanylate Kinase Homologs
<b>MetaHit</b>	Metagenomics of human intestinal tract
<b>MLC</b>	Myosin light chain
<b>MLCK</b>	Myosin light chain kinase
<b>NAFLD</b>	Non-alcoholic fatty liver disease
<b>NEFAs</b>	Non-esterified fatty acids
<b>NeuroD1</b>	Neurogenic differentiation 1

<b>Ngn3</b>	Neurogenin 3
<b>NHE3</b>	Na <sup>+</sup> /H <sup>+</sup> exchanger
<b>NMDA</b>	NR2B-containing N-methyl-d-aspartate
<b>NTS</b>	Neurotensin
<b>NTSRs</b>	Neurotensin receptors
<b>Oxo-FAs</b>	Oxo-fatty acids
<b>Pax4</b>	Paired box 4
<b>Pax6</b>	Paired box 6
<b>PEG</b>	Polyethylene glycol
<b>POMC</b>	Proopiomelanocortin
<b>Pou2f3</b>	Pou domain, class2, transcription factor 3
<b>PYY</b>	Peptide YY
<b>RYGB</b>	Roux-en-Y gastric bypass
<b>SBS</b>	Short bowel syndrome
<b>SCFAs</b>	Short-chain fatty acids
<b>SCT</b>	Secretin
<b>SGLT1</b>	Sodium glucose cotransport protein
<b>Sox9</b>	Sry-box 9
<b>SREBP1</b>	Sterol response element binding protein
<b>T2R14</b>	Taste 2 receptor member 14
<b>T1R2/3</b>	Taste receptor type 2 or type 3
<b>T2D</b>	Type 2 diabetes
<b>TEER</b>	Transepithelial electrical resistance
<b>TGR5</b>	G protein-coupled bile acid receptor
<b>TJs</b>	Tight junctions
<b>TLR4</b>	Toll-like receptor 4
<b>TNF<math>\alpha</math></b>	Tumor necrosis factor alpha
<b>TRPA1</b>	Transient receptor potential ankyrin 1
<b>UC</b>	Ulcerative colitis
<b>VLDL</b>	Very-low-density lipoproteins
<b>WT</b>	Wild type
<b>ZO</b>	Zonula Occludens
<b>5-HT</b>	5-hydroxy-tryptamine/serotonin



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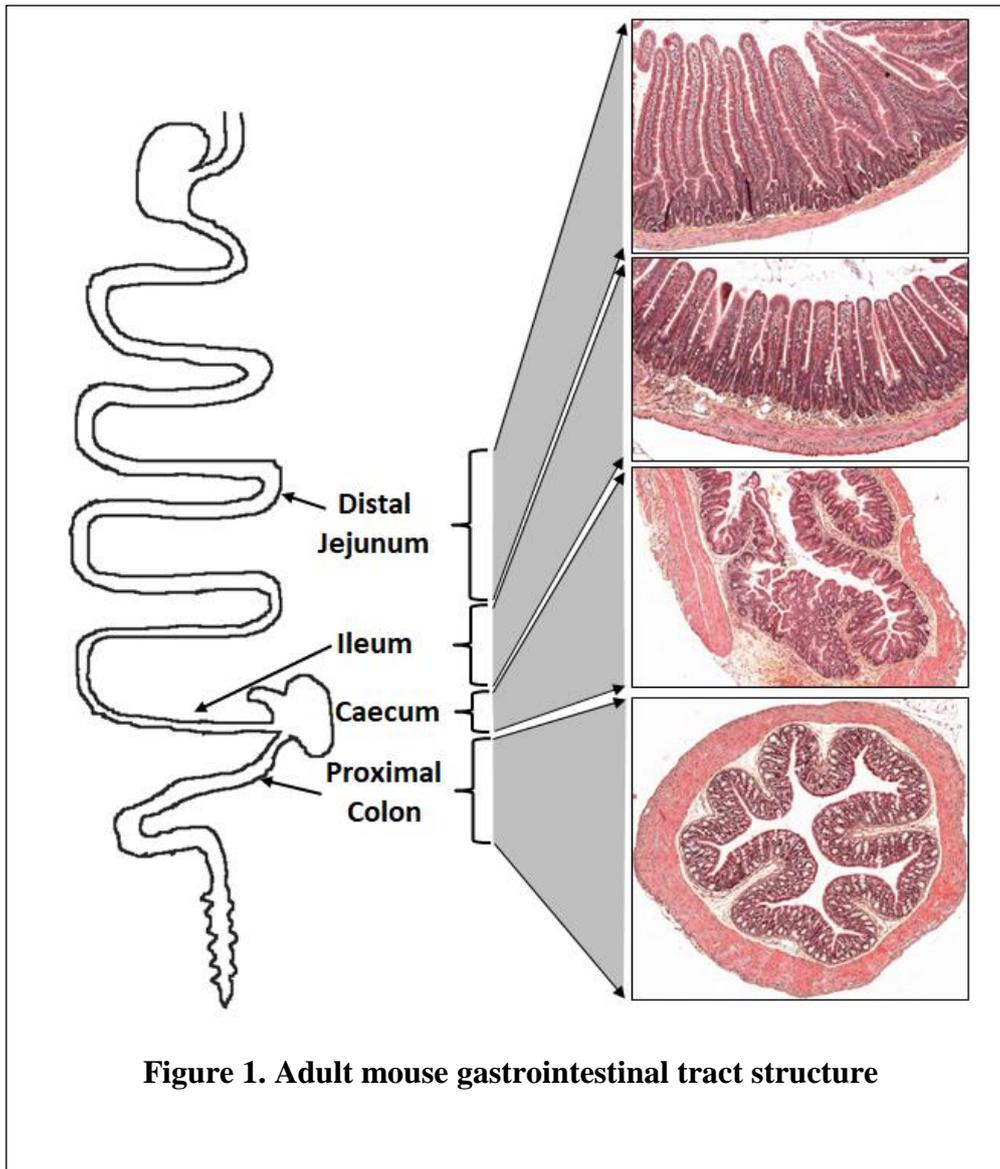
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# **INTRODUCTION**



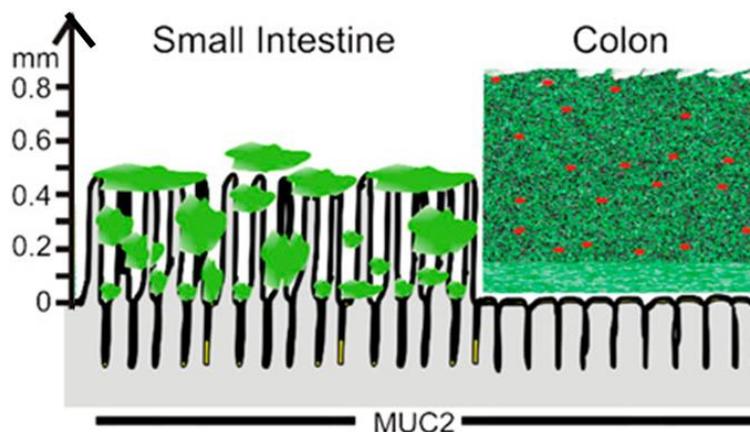
## **I-Structures and functions of the gastrointestinal tract**

The gastrointestinal (GI) tract goes from the mouth to the anus. It is responsible for food digestion, nutrient absorption, gut-hormone secretion, and immune functions. It is also the first barrier for the protection of the internal milieu from the non-regulated passage of compounds and microorganisms originating from the luminal environment. This long tract can be subdivided into several specific regions that differ in terms of structure and function (**Figure 1**).

### **I-1 Structures of the intestinal epithelium**

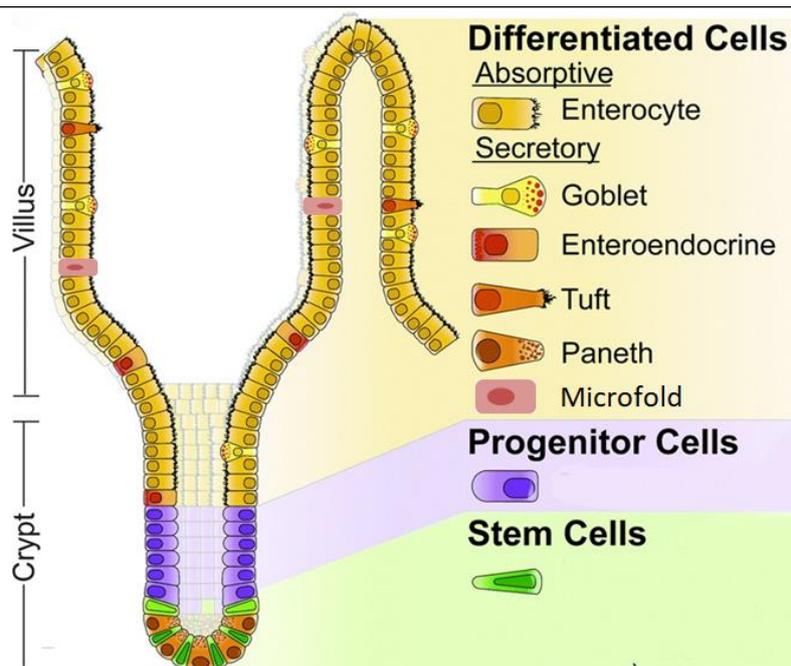
The upper GI tract is composed of the oral cavity, pharynx, esophagus, and stomach. After the pylorus, the GI tract consists of the small and large intestines, of which the small intestine (proximal intestine) is divided into the duodenum, jejunum, and ileum and the large intestine (distal intestine) into the caecum, colon, and rectum (Greenwood-Van Meerveld et al., 2017). Along most of the GI tract, the structure of the intestinal wall is composed of four layers, including, from the lumen outwards: the mucosa, submucosa, muscularis propria, and serosa (Aibe et al., 1986). Despite being composed of the same tissue layers from the mouth to the anus, the mucosal layer exhibits different features depending on the GI tract region. In the stomach, the mucosa folds into rugae (Krag, 1966). In the small intestine, the mucosa consists of crypt invaginations and circular folds, villi, and microvilli, which increase the absorptive surface area of the small intestine by more than 600-fold (Ferguson et al., 1978). In contrast, the colonic mucosa consists of simple flat epithelium with successive crypt invaginations (Ross, 1921, Baker et al., 2014). The caecum is less well-described, likely because it is prominent in mostly herbivorous species. It is located at the transition between the small and large intestine. Its structure is close to that observed in the colon with no prominent villi. However, the surface is less flat and crypt invaginations less deep.

The mucus layers also differ depending on the intestinal segment. Mucus is secreted continuously by goblet cells localized at the villi. The mucus in the colon is composed of an inner and outer mucus layer. The outer layer is loose (~4 times thicker than the inner layer), whereas the inner layer is firm and impermeable. In the colon, the inner layer



**Figure 2. Schematic organization of the mucus layer in the gut of rats**  
(Adapted from (Johansson et al., 2011))

In the small intestine, the mucus is composed of only one irregular layer. In contrast, the inner layer of the colon provides protection against the luminal bacteria, whereas the outer layer harbors the commensal bacteria represented by the red dots.



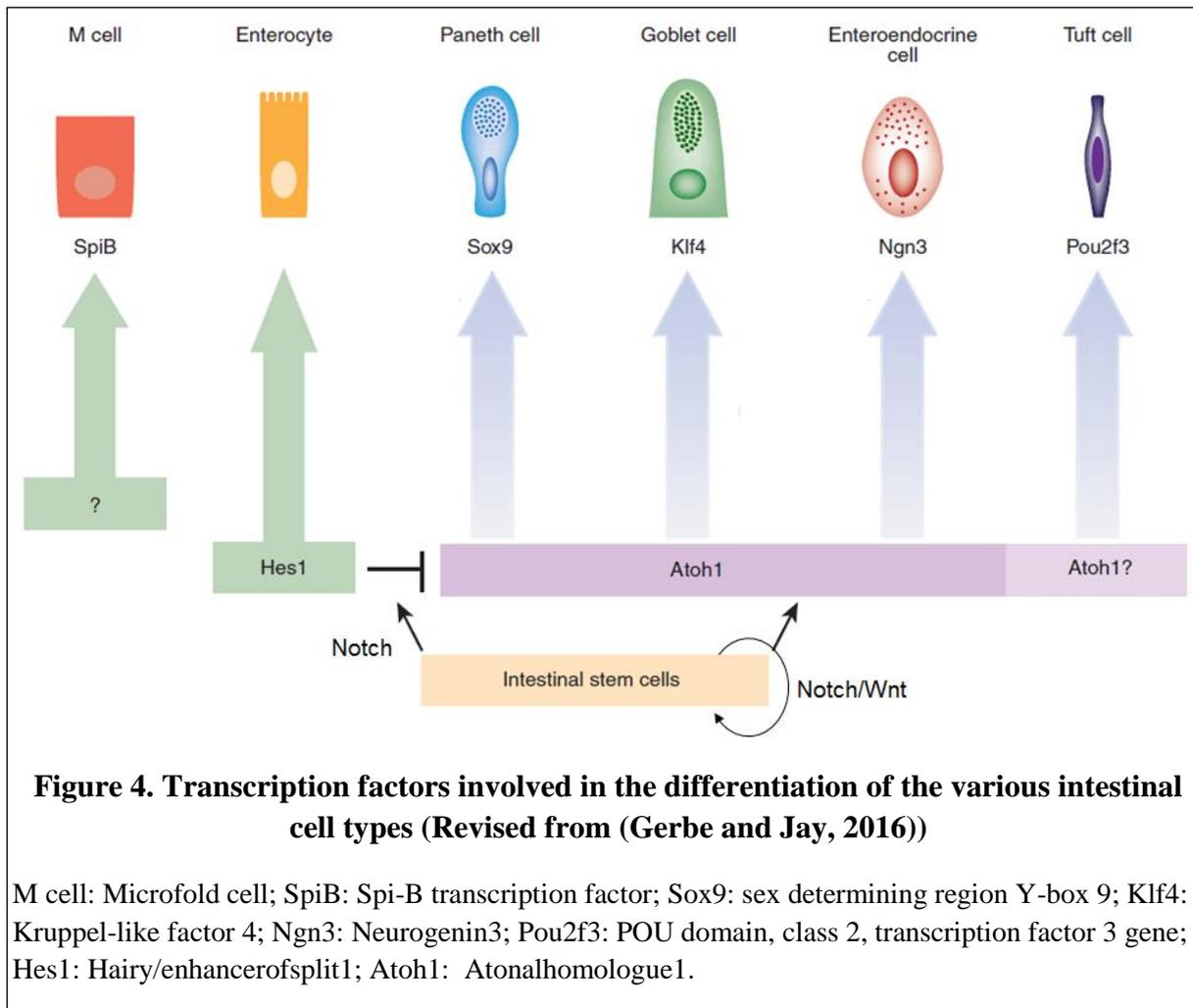
**Figure 3. Schematic representation of the crypt-villus unit in the small intestine**  
(Adapted from (Carulli et al., 2014))

The stem and progenitor cells are localized in the crypts. They differentiate into a number of cell types indicated in the right side of the figure as they are displaced from the crypt to the villi.

provides protection against the luminal bacteria, whereas the outer layer harbors the commensal bacteria (Johansson et al., 2008). In contrast, the mucus in the small intestine is composed of only one irregular layer (Johansson et al., 2011) (**Figure 2**). However, early descriptions of the mucus layer also described a thin firm layer in the small intestine (Atuma et al., 2001). Mucus consists of water (98%), mucins (~2%, which are high molecular weight (~500 kDa) glycoproteins) (Andrianifahanana et al., 2006), and other proteins, including immunoglobulins (Johansson et al., 2008).

### **I-2 Cell populations of the intestinal epithelium**

The intestinal epithelium is composed of six types of differentiated epithelial cells (**Figure 3**): absorptive enterocytes, secretory Paneth cells, goblet cells, tuft cells, microfold (M) cells, and enteroendocrine cells (EECs). Enterocytes are the most abundant cells of the intestinal epithelium and are involved in absorption. In the small intestine, they absorb the nutrients degraded by digestive processes and are involved in the reabsorption of water and electrolytes, as well as the products of microbiota metabolism in the colon. Paneth cells are localized at the base of crypts in the small intestine, where they surround the stem cells (Sato et al., 2011). They secrete antimicrobial peptides, as well as growth factors for the defense against bacteria and signaling molecules for maintenance of the stem-cells (Ayabe et al., 2004, Sato et al., 2011). Goblet cells secrete the mucins that constitute the mucus layer. Their density increases from the duodenum to the colon (4 to 16% of the total epithelium respectively) parallel to the increase in the number of bacteria (Deplancke and Gaskins, 2001). Tuft cells represent less than 0.4% of the total epithelial cells and are involved in chemoreception and regulating type 2 immune responses associated with allergic conditions (Gerbe et al., 2012, Gerbe and Jay, 2016). M cells play a key role in mucosal immunity by transporting antigens from the lumen to cells of the immune system (Gebert et al., 1996). EECs are the hormone-producing cells of the intestine. They represent 1% of the intestinal epithelial cells and are expressed all along the GI tract and can be found in the crypts as well as in the villi. They have a broad spectrum of actions which are described in § III.



Overall, the epithelial cells of the intestine constitute a monolayer of polarized cells of which the basolateral side faces the *lamina propria* and the lateral surface is tightly linked with the neighboring cells through various junctions which will be described in § IV.

### I-3 Intestinal epithelium renewal

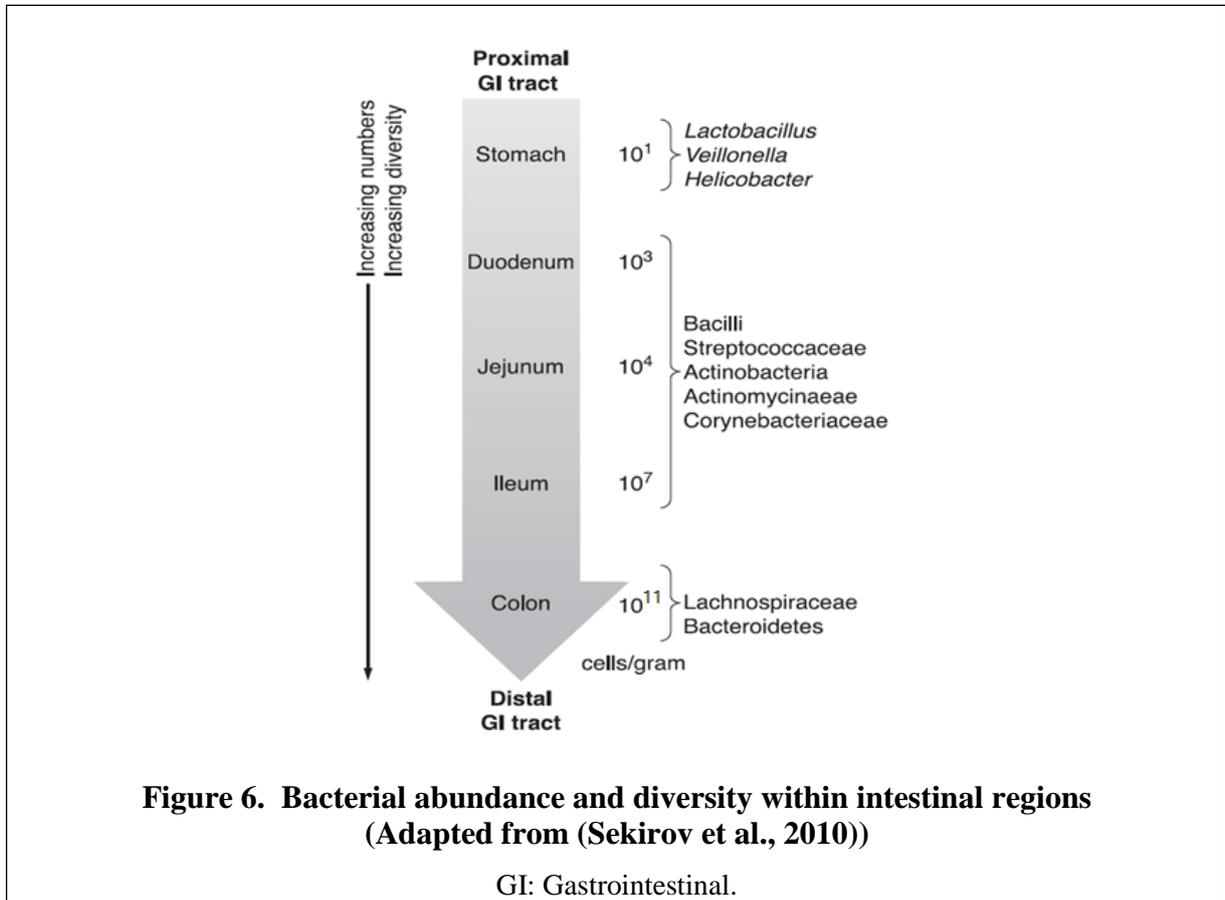
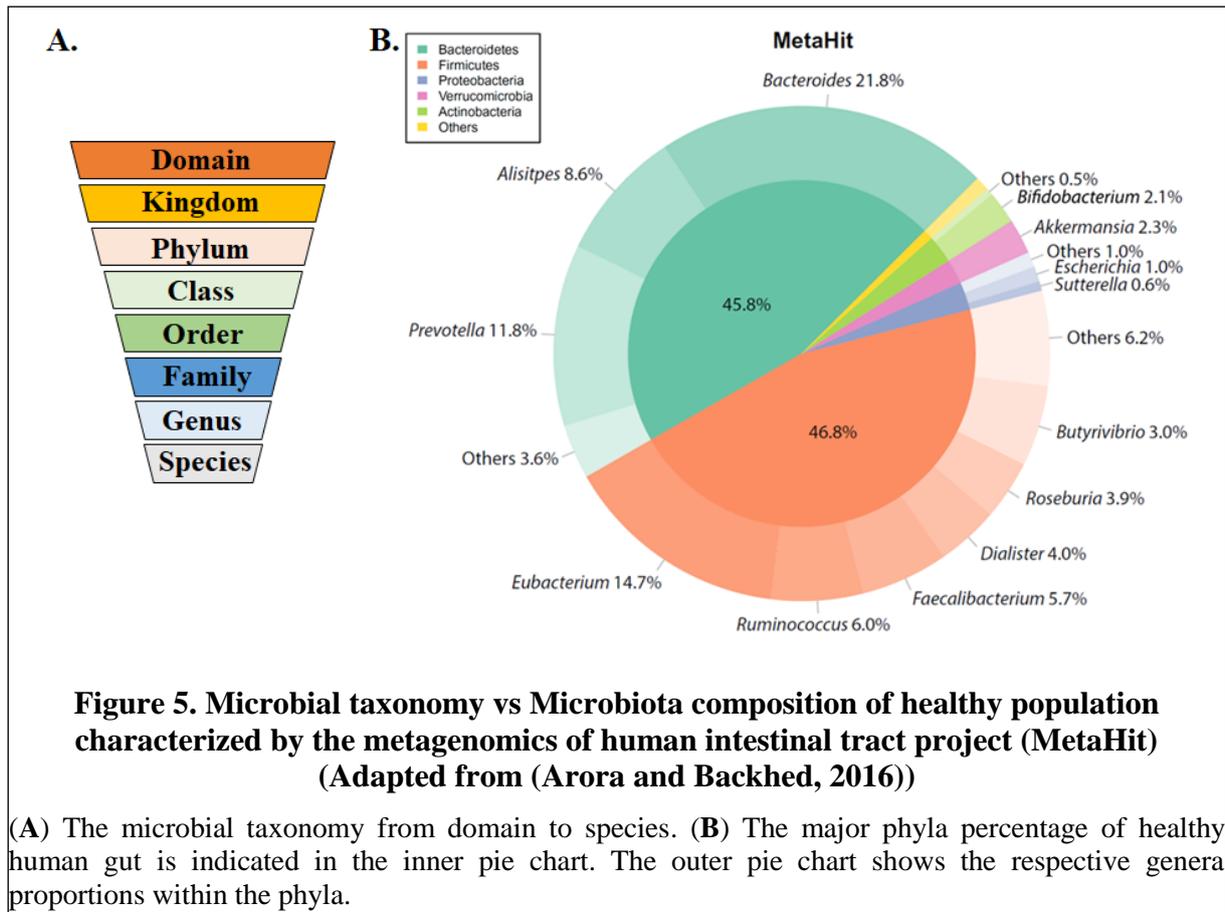
The intestinal epithelium is in a state of constant renewal because of the proliferative and differentiation properties of the stem cells, which are found at the bottom of the crypts. It can be renewed within two to three days in rats and four to five days in humans (Moore and Lemischka, 2006). However, there are differences between the cell types and GI regions. For example, the life span of EECs is up to four months in the stomach, up to 60 days in the ileum and colon, and between six and 10 days in the duodenum (Lehy and Willems, 1976, Tsubouchi and Leblond, 1979, Thompson et al., 1990). The coordinated action of various transcription factors controls the specification and maintenance of the various intestinal epithelial cell types (Sato and Clevers, 2013) (**Figure 4**). First, the Wnt and Notch pathways allow maintenance of the undifferentiated state of the stem cells. In the lower part of the crypts, the stem cells undergo a rapid series of divisions that generate progenitors which migrate to the middle region of the crypt. Under the control of the Notch pathway, the progenitor cells specialize into either secretory precursors (which give rise to goblet, enteroendocrine, tuft, and Paneth cells) or absorptive precursors (which give rise to enterocytes) (Vooijs et al., 2011). This mechanism is mediated by hairy/enhancer of split (HES1), a major transcription factor for which the expression is initiated by the Notch pathway (Fre et al., 2005, Bar et al., 2008). Activation of the Notch/HES1 pathway promotes differentiation of the absorptive lineage and inhibits differentiation of the secretory lineage via the repression of the transcription factor Atonal homologue 1 (Atoh1 also named Math1). The Math1<sup>+</sup>-lineage differentiates into EECs under the control of Neurogenin 3 (Ngn3). Then, other transcription factors downstream of Ngn3, including neurogenic differentiation 1 (NeuroD1), the paired box 4 and 6 (PAX4 and PAX6), the winged helix factors 1/2 (FOXA 1/2), and Aristaless related homeobox (Arx) are responsible for the differentiation of various EEC subtypes (described in § III) (Schonhoff et al., 2004). The Sry-box 9 (Sox9) factor (Bastide et al., 2007) is required for Paneth cell differentiation and Kruppel-like factor 4 (Klf4) (Katz et al., 2002) for that of goblet cells. Pou domain, class 2, transcription factor 3 (Pou2f3) is the first transcription factor to be identified for tuft cell differentiation (Gerbe et al., 2016).

This extraordinary capacity for renewal confers remarkable plasticity of the intestinal epithelium in response to changes in the luminal environment (nutrients, microbiota metabolites, drugs).

## II-Gut microbiota

### II-1 General overview of the gut microbiota composition

The gut microbiota is composed of a large and diverse community of  $\sim 10^{14}$  microorganisms that reside in the GI tract (Gill et al., 2006, Qin et al., 2010). Within this community the bacteria are the most abundant, but viruses, archaea, yeasts and fungi are also present (Gevers et al., 2012). Classical microbiology techniques allow the detection and characterisation of only 30% of the total bacteria present in the gut (Rajilic-Stojanovic and de Vos, 2014). Recently, the development of techniques based on the 16S RNA sequencing have allowed the identification of new bacteria and permit a better characterisation of the gut ecosystem species (Fraher et al., 2012). Although more than 1000 bacteria species have been identified within the whole intestinal microbiome, about 160 species are dominant and largely shared among individuals (Qin et al., 2010). Studies demonstrated that 18 bacterial species were present in most of the individuals and that 57 species were shared by 90% of specimens (Qin et al., 2010). Two major phyla of bacteria, Firmicutes and Bacteroidetes, are predominated in both human and mice intestine followed by Actinobacteria, Verrucomicrobia and Proteobacteria (Ley et al., 2005) (**Figure 5**). The dominant genera found in adult healthy human are *Bacteroides*, *Eubacterium*, *Ruminococcus*, *Clostridium* and *Bifidobacterium* (Ley et al., 2005, Qin et al., 2010). By comparing gut microbiota composition of large cohorts of human patients originating from different countries, the ecological “enterotype” concept emerged. According to this concept, human population could be divided into three major groups, or enterotypes: the type 1 is defined by dominance of *Bacteroides*, the type 2 by *Prevotella* and the type 3 by *Ruminococcus* (Arumugam et al., 2011, Wu et al., 2011, Costea et al., 2018). Despite this general profile, gut microbiota exhibits spatial differences at the genus level and beyond. The composition and density of the microorganisms vary throughout the digestive tract, due to variation of the pH, the redox potential, the substrates available and adhesion sites (e.g. mucus layer) (O'Hara and Shanahan, 2006). Thus, the bacterial density is about  $10^1$ - $10^3$  colony-forming unit/ml of luminal content in the duodenum,  $10^4$ - $10^7$  in the jejunum and reaches  $10^{10}$ - $10^{12}$  in the colon (**Figure 6**) (Sekirov et al., 2010, Sender et al., 2016). This increase in bacterial density also parallels an increase in density of anaerobic bacteria and a decrease in density of aerobic bacteria. For instance, predominant genus found in the stomach and the proximal small intestine is *Streptococcus* while Firmicutes and Bacteroidetes are the



main phyla inhabiting the colon. In addition, the composition of the bacterial communities also varies between the lumen and the mucosal surface of the intestine. For example, the mucosal surface is enriched in *Akkermansia muciniphila* and other bacterial groups able to metabolize the mucins as energy source (Swidsinski et al., 2005, Beaumont et al., 2017).

## **II-2 The functions of the gut microbiota**

The recent development of high throughput DNA sequencing allowed the sequencing and identification of the genes of bacteria present in the digestive ecosystem (metagenome). It was thus established that each microbiota encodes for 500 000 to 600 000 genes such as about 10 million genes are encoded in total by the human microbiota which is 150 times present in the human genome. The gut ecosystem performs various functions essential for host physiology and the maintenance of the host health. The cloning of large DNA fragments permitted the identification of the species responsible for specific physiological functions (Manichanh et al., 2006). These technical advances increased our knowledge of the microbiome and uncover its numerous functions in healthy conditions and its relationship with the development of pathologies. In healthy conditions, the gut microbiome is involved in carbohydrate and protein fermentations, the synthesis of vitamins (B12, K), the synthesis of short chain fatty acids (SCFAs), the secondary bile acids metabolism, as well as the synthesis of numerous components whose nature and functions remain unidentified (Gill et al., 2006, Rowland et al., 2018). The gut microbiota is also involved in the maturation of the immune system, the protection against pathogens and the maintenance of gut barrier integrity (Gordon and Bruckner-Kardoss, 1961, Banasaz et al., 2002).

## **II-3 The stability and dietary-dependent alterations of the microbiota composition**

The gut is colonized by the bacteria at the time of birth either by the microorganisms originating from the maternal vagina (*e.g. Lactobacillus and Prevotella*) or from the skin (*e.g. Streptococcus, Corynebacterium and Propionibacterium*) depending on the type of delivery, vaginal or caesarian, respectively. During the first years of life, the type of diet (breast milk or formula) (Donovan et al., 2012, Iozzo and Sanguinetti, 2018) together with exposure to antibiotics (Cox and Blaser, 2015) are the main factors influencing the evolution of the microbiota composition. The 3-4 years old children microbiota composition is already

stabilized and diversified and shares 40 to 60% similarity with the adult ecosystem. In healthy individuals, the predominant phyla and genera show relative stability during the course of the life (Greenhalgh et al., 2016). However as in infancy, diet is one of the main factors influencing the composition, diversity and richness of adulthood microbiota. First, dietary habits largely contribute to the establishment of enterotypes, with *Bacteroides* enterotype being mainly associated with animal protein, amino acid and fat consumption, *Prevotella* enterotype being related to high intake of carbohydrates while *Ruminococcus* enterotype was not clearly defined towards any diet (Wu et al., 2011). However, this association of the enterotype with the dietary pattern remains controversial. Indeed, another study associated *Bacteroides* enterotype with sugars fermentation and an elevated ability to synthesize the vitamins B7, B2 and C (Arumugam et al., 2011). In the same study, *Prevotella* and *Ruminococcus* enterotypes appear composed of a majority of species able to degrade the mucins and *Prevotella* enterotype also showed an enrichment in genes encoding for synthesis of the vitamins B1 and B9.

Among the various food constituents, the fibers are the most effective macronutrients able to increase the richness and diversity of the gut microbiota. Diets enriched in fiber would specifically increase the abundance of bacteria belonging to the Firmicutes phylum able to metabolize polysaccharides (e.g. *Ruminococcus bromii*, *Roseburia* and *Eubacterium*). In contrast, high-fat diets (HFD) decrease the overall gut microbiota diversity and alter the microbiome composition by decreasing the relative abundance of Bacteroidetes and increasing abundance of Firmicute and Proteobacteria (Hildebrandt et al., 2009, Do et al., 2018). At the family level, HFD decrease *Ruminococcaceae* and increase *Rikenellaceae* as well as colonic sulfite-reducing bacteria abundance (Daniel et al., 2014, Shen et al., 2014, Araujo et al., 2017). When fat was associated with high levels of sucrose or high level of fructose in the diet, similar modifications of the microbiota were detected (decreased Bacteroidetes/Firmicutes ratio, and decreased diversity) (Parks et al., 2013, Carmody et al., 2015, Volynets et al., 2017). When not associated with HFD, exposure of rats to moderate ranges of glucose/fructose-sweetened beverages reduced the abundance of *Prevotella* and *Lachnospiraceae incertae sedis* and increased *Bacteroides*, *Alistipes*, *Lactobacillus*, *Clostridium sensu stricto*, *Bifidobacteriaceae*, and *Parasutterella* (Noble et al., 2017). High fructose diet also results in the increase in *Enterobacteriaceae* in rats (Jena et al., 2014). More recent findings also showed that high glucose or high fructose (65%) diets significantly reduced the microbiota diversity similarly to a HFD poor in sugar (20% carbohydrates) when

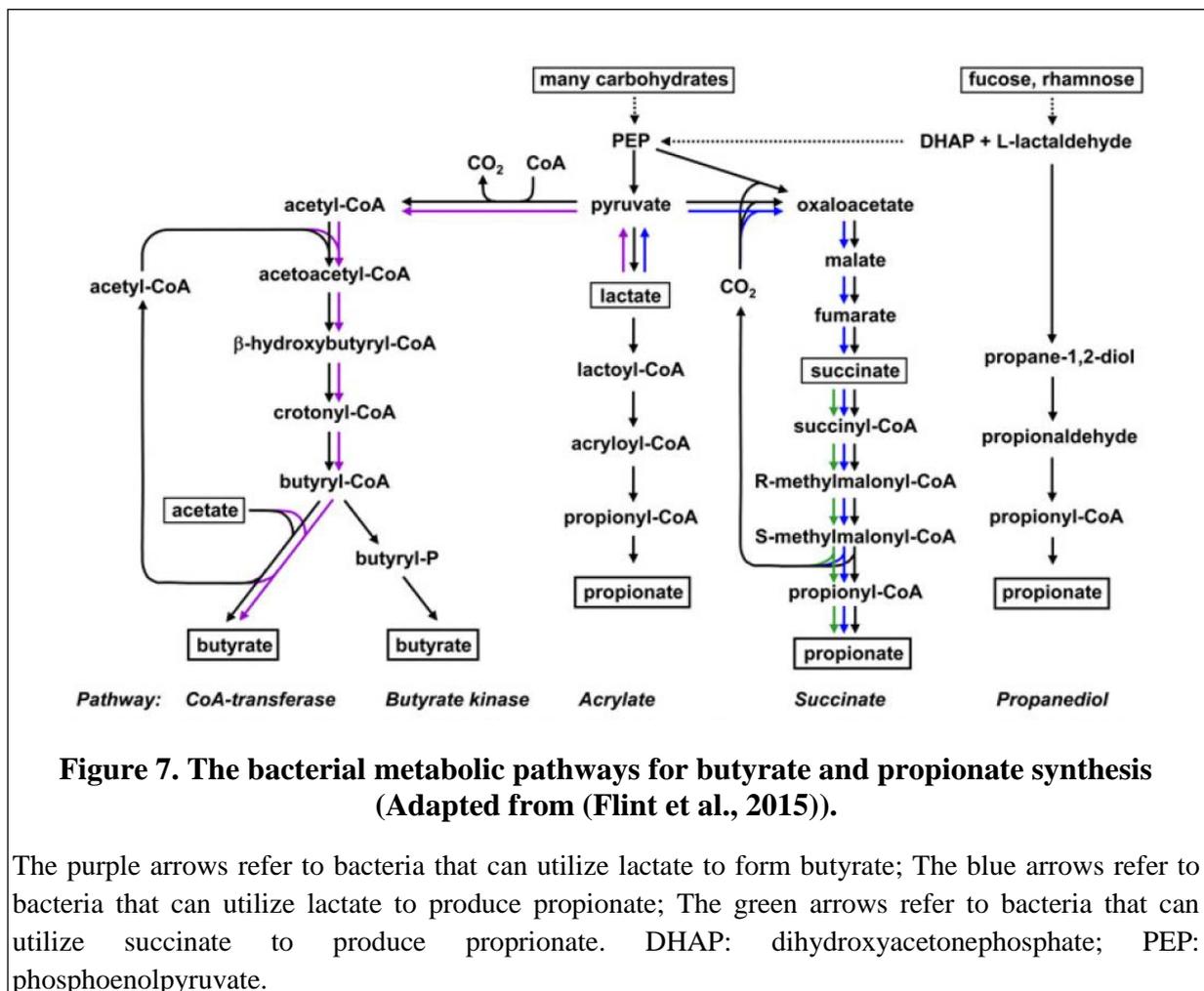
compared to control diet (Do et al., 2018). 65% glucose or fructose diets were also associated with an increase in Verrucomicrobia phylum when compared to the HFD poor in sugar or to the normal diet (Do et al., 2018). In the same study, when compared to glucose, fructose diet induced a lower decrease in the Bacteroidetes/Firmicutes ratio and a higher increase in the Deferribacteres phylum. Interestingly, non-caloric artificial sweeteners such as saccharin also modify gut microbiota composition by increasing the relative abundance of *Bacteroides* and reducing *Lactobacillus reuteri* (Suez et al., 2014). Importantly, those modifications are responsible for the development of glucose intolerance observed in response to non-caloric sweeteners intake (Suez et al., 2014).

## II-4 The microbial metabolites and components

Changes in microbiota composition regulate the host physiology via the metabolites they produce such as SCFAs, bile acids, tryptophan metabolites, or other microbial components such as lipopolysaccharide (LPS).

### II-4.1 Short-chain fatty acids

SCFAs are the main products of the fermentation of the non-digestible polysaccharides (e.g. fibers) by the microorganisms of the distal gut (Miller and Wolin, 1996, Flint et al., 2015). There are three major SCFAs: acetate, butyrate, and propionate (Cummings et al., 1987, Flint et al., 2015). Classical biochemistry techniques (Flint et al., 2015), phylogenetic analysis targeting specific bacterial functional genes of SCFA synthesis pathways (Louis et al., 2010, Reichardt et al., 2014) and metagenomic approaches (Vital et al., 2014, Vital et al., 2017) have clarified the pathways and some of the bacteria species involved in the SCFAs production (**Figure 7**). The majority of butyrate- and propionate-producing bacteria belong to families within the *Firmicutes*. Among the dominant bacterial species of the human microbiota, species belonging to the Lachnospiraceae family such as *Roseburia inulinivorans*, *Ruminococcus obeum* or *Coprococcus catus* are propionate producers while in the same family *Roseburia intestinalis* and *Clostridium symbiosum* are butyrate producers (Reichardt et al., 2014). In addition, species from the Negativicutes family as well as *Bacteroides* species from the Bacteroidetes phylum are also major propionate producers (Reichardt et al., 2014). *Roseburia intestinalis*, *Eubacterium hallii* as well as *Faecalibacterium prausnitzii* are part of the major butyrate producers (Louis et al., 2010). Acetate fermentation ability is also widely



present in gut bacteria (Morrison and Preston, 2016). In addition to polysaccharides, the lactate can also contribute to the production of acetate, butyrate and propionate through a process implying cross-feeding between bacteria such as *Bifidobacterium adolescentis* and *Roseburia spp.* (Bourriaud et al., 2005, Belenguer et al., 2006). In the caecum, the interconversion between acetate and butyrate or between butyrate and propionate are also possible (den Besten et al., 2013). The SCFAs produced by the microbiota play a major role in the energy regulation of the host. Propionate and acetate are mainly transported into the portal blood while butyrate is mostly used as energy source by the colonocytes. Propionate can be used as a precursor for glucose synthesis by the peripheral organs while acetate and butyrate participate in the synthesis of cholesterol and fatty acid (den Besten et al., 2013). The utilization of butyrate by the colonocytes supports their proliferation and participates in the maintenance of the intestinal barrier integrity (Donohoe et al., 2012, Wang et al., 2012). Numerous signaling effects of SCFAs such as the regulation of glucose homeostasis, appetite and gut peptide secretion are mostly mediated via the free fatty acid receptors 2/3 (FFAR2/3 also named G protein-coupled receptors 43 and 41 (GPR43 and GPR41)) (Ulven, 2012, Morrison and Preston, 2016). SCFAs produced by the microbiota can also suppress insulin-mediated adipose accumulation through the activation of adipocyte GPR43 (Kimura et al., 2013, Morrison and Preston, 2016).

#### ***II-4.2 Bile acids***

Bile acids are amphipathic, saturated steroid acids (Hofmann et al., 2010). Primary bile acids (chenodeoxycholic acid and cholic acid in humans) are synthesized from the cholesterol oxidation in the hepatocytes and then conjugated with the amino acids, taurine or glycine, before to be released into the bile in the small intestine (Gerard, 2013, Ridlon et al., 2014). While most of the bile acids are reabsorbed by the small intestine via a process named the enterohepatic circulation, a fraction of them reaches the colon where they are metabolized into secondary bile acids by the bacteria. In humans, the main secondary bile acids are the lithocholic and deoxycholic acids. However, more than 20 types had been identified (Midtvedt, 1974). The microbial metabolism of bile acids involves several metabolic conversions including (1) microbial deconjugation which is carried out by bacteria expressing bile salt hydrolase such as *Bacteroides* and *Bifidobacterium*; (2) microbial 7-dehydroxylation by bacteria such as *Clostridium* and *Eubacterium*; (3) microbial oxidation and epimerization which are catalyzed by bacteria expressing hydroxysteroid dehydrogenases such as

*Clostridium* and *Eggerthella* (Jonsson et al., 1995, Gerard, 2013, Wahlstrom et al., 2016). The bile acids can also reshape the microbial community by increasing the bile acid-metabolizing bacteria and decreasing the bile sensitive bacteria (Wahlstrom et al., 2016). Functionally, bile acids activate the farnesoid X receptor (FXR) or G protein-coupled bile acid receptor (TGR5) signaling pathways (Makishima et al., 1999). FXR activation had been shown to protect against the development of hypercholesterolemia under chow diet condition, but to promote metabolic diseases such as obesity on high-fat diet (Lambert et al., 2003, Thomas et al., 2008, Parséus et al., 2017). The signaling pathway mediated by TGR5 is activated mainly by secondary bile acids and is involved in the control of glucose homeostasis through the increase in GLP-1 release by the intestinal enteroendocrine cells (Thomas et al., 2009).

#### ***II-4.3 Tryptophan metabolites***

Tryptophan is an essential non-polar aromatic amino acid that needs to be obtained from diet. In numerous gram-positive and gram-negative bacteria as well as various pathogens, tryptophan is converted into indole by the tryptophanase (Lee and Lee, 2010). Other bacteria unable to synthesize indole are however able to modify or degrade indole to produce indole derivatives such as indole-propionic acid, 5-hydroxy-indoleacetic acid or indole-acetic acid (Danaceau et al., 2003). Therefore, when compared to conventionally raised mice, germ-free mice display a major increase in their plasma concentrations of tryptophan and a decrease in indolepropionic acid (Wikoff et al., 2009). On one hand, indole is sensed by the bacteria; thus it and its derivatives play a central role in the inter bacteria communication and participate in the stability of the intestinal ecosystem (Mueller et al., 2009, Wood, 2009). On the other hand, indole is also a signaling molecule detected by the eukaryotic cells. For instance, the indole and some of its derivatives can activate the aryl-hydrocarbon receptor (AhR) in either immune cells (Hubbard, 2015) or intestinal epithelial cells (Lamas, et al., 2016) therefore regulating the immune response, intestinal barrier integrity and gut endocrine function (Chimerel et al., 2014, Hubbard et al., 2015, Lamas, et al., 2016, Wlodarska et al., 2017). The tryptophan is also a precursor for the synthesis of the serotonin by the enterochromaffin cells. Decrease in serotonin synthesis and plasmatic levels in the germ-free mice suggests that the gut microbiota likely participates in the regulation of the intestinal serotonin production (Yano et al., 2015). Despite a lack of clear mechanisms, two bacterial metabolites mentioned above, the SCFAs and the deoxycholic acid, are likely involved in this regulation (Reigstad et al., 2015, Yano et al., 2015). The serotonin acting via several serotonin receptors plays a major

role in the regulation of the GI motility and secretion (Mawe and Hoffman, 2013, Li et al. 2011). In addition to tryptophan, other aromatic amino acids such as phenylalanine and tyrosine can also be metabolized by the intestinal bacteria and produce bioactive compounds able to regulate a wide range of host physiological functions (Dodd et al., 2017).

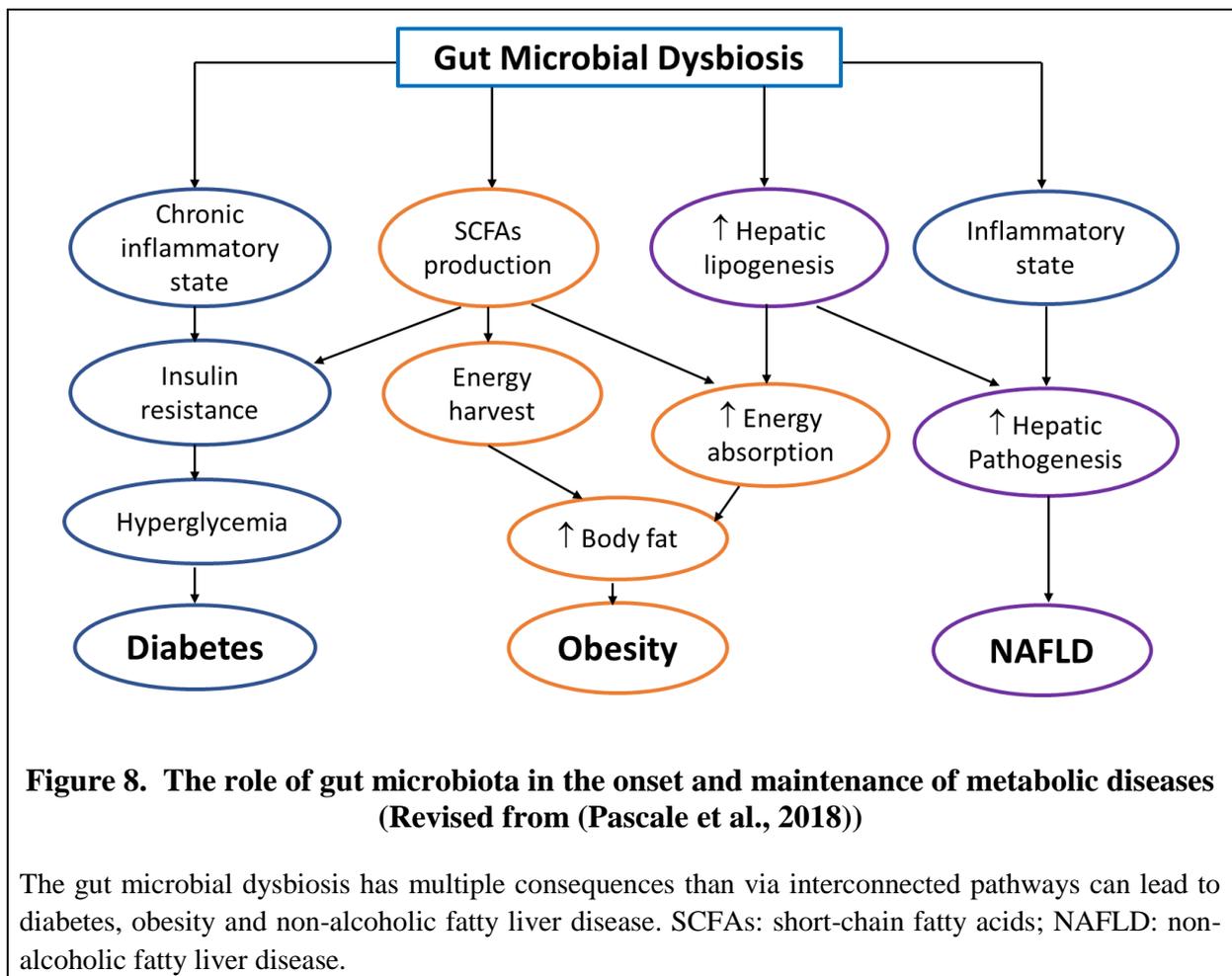
#### ***II-4.4 Lipopolysaccharides***

Lipopolysaccharides (LPS), also known as endotoxins, are composed of a hydrophobic domain (lipid A), a distal O-polysaccharide chain, and a non-repeating R-core oligosaccharide (Rietschel et al., 1994, Caroff and Karibian, 2003). They are the major components of the outer membrane of the gram-negative bacteria (Galloway and Raetz, 1990, Raetz and Whitfield, 2002). The dietary intake, particularly high-fat diet, results in the increased abundance of LPS-containing bacteria associated with a higher plasmatic level of LPS. However, the mechanisms involved in the translocation of luminal LPS into the blood remain unclear and may involve intestinal barrier dysfunction and an as yet unidentified transcellular pathway (Cani et al., 2007, Guerville and Boudry, 2016). The increase in plasmatic LPS, also named endotoxemia, is largely suspected to trigger the low-grade inflammation characteristic of metabolic diseases and to promote the insulin resistance through the activation of Toll-like receptor 4 (TLR4) and its co-receptors (Shi et al., 2006, Cani et al., 2007, Saad et al., 2016).

The above list of bacterial products represents only a subset of known potential regulators originating from bacteria. Clearly, many more remain to be discovered. Furthermore, the list of known host physiological functions regulated by the microbiota will expand significantly in the near future.

#### **II-5 The role of gut microbiota in metabolic diseases**

Humans normally exist in a remarkably harmonious relationship with its intestinal ecosystem and alteration in the bacterial profile is at the origin of several chronic diseases (**Figure 8**). Human and rodent studies demonstrated that obesity is associated with profound changes in microbiota (Cani, 2013, Everard and Cani, 2013, Nieuwdorp et al., 2014). Obese individuals are characterized by a lower microbial gene richness (low gene count) (Le Chatelier et al., 2013), a lower ratio Bacteroidetes/Firmicutes and an increased relative abundance of Actinobacteria (Ley et al., 2006, Turnbaugh et al., 2009) when compared to the non-obese counterparts. However, this association between obesity and low ratio



Bacteroidetes/Firmicutes was not always confirmed later in humans (Duncan et al., 2008, Schwartz et al., 2010). The induction of a metabolic syndrome phenotype through fecal transplants in mice confirmed the important role of the microbiota in this disease (Ridaura et al., 2013). In addition to obesity, the gut microbiota dysbiosis also contributes to the development of diabetes (Qin et al., 2012), non-alcoholic fatty liver disease (NAFLD) (Le Roy et al., 2013) as well as intestinal disorders such as inflammatory bowel diseases (IBD) (Bamola et al., 2017) and mental diseases including Alzheimer's (Hu et al., 2016) and depression (Kelly et al., 2016). Studies focusing on the mechanisms involved in the development of hepatic steatosis induced by fructose suggested that endotoxin, originating from microbiota, plays also a major role in the induction of hepatic lipogenesis by fructose (Bergheim et al., 2008, Spruss and Bergheim, 2009, Wagnerberger et al., 2013, Ritze et al., 2014, Sellmann et al., 2015). Antibiotic treatment prevented also the alterations of glucose homeostasis, the increase in tissue oxidative stress as well as the elevation of circulating levels of pro-inflammatory cytokines, LPS and non-esterified fatty acids induced by fructose (Di Luccia et al., 2015, Crescenzo et al., 2017). Similarly, probiotics such as *Lactobacillus kefir* reduced the adipose tissue inflammation, plasmatic hypertriglyceridemia and the hepatic increase in triglyceride content triggered by high fructose diet (Zubiria et al., 2017). Although those studies do not determine whether fructose is a substrate of certain intestinal bacteria, they highlight the potential role of gut microbiota in metabolic disorders induced by fructose.

**Table 1. The old dogma of localization and secretion of the enteroendocrine cells in the gastrointestinal tract**

Cell type	Hormone secretion	Localization				
		Stomach	Duodenum	Jejunum	Ileum	Colon
EC cells	Serotonin/5-HT	+	+++	+	+	+
D cells	SST	+	+	+	+	+
ECL cells	Histamine	+	-	-	-	-
G cells	Gastrin	++	+	-	-	-
X/A cells	Ghrelin	++	+	+	+	-
I cells	CCK	-	+++	+	-	-
K cells	GIP	-	+++	(+)	-	-
M cells	Motilin	-	+	+	(+)	-
S cells	SCT	-	+	+	-	-
N cells	NTS	-	-	-	+	-
L cells	GLP-1, GLP-2	-	(+)	+	++	+++
	PYY	-	-	(+)	+	+++
	INSL5	-	(+)	-	-	+++

5-HT: 5-hydroxy-tryptamine; SST: Somatostatin; CCK: Cholecystokinin; GIP: Glucose-dependent insulinotropic peptide; SCT: Secretin; NTS: Neurotensin; GLP-1/2: glucagon-like peptides 1 and 2; PYY: peptide YY; INSL5: insulin-like peptide 5. Reviewed in (Gribble and Reimann, 2016).

### III- Enteroendocrine functions of the gastrointestinal tract

#### III-1 The classification of enteroendocrine cells: from the old to a new dogma

EECs are a population of cells found from the stomach to the distal colon (Dobbins and Austin, 2009). Despite representing only approximately 1% of the intestinal epithelium, EECs constitute the main endocrine system of the entire body (Janssen and Depoortere, 2013). Approximately 30 enterohormones are produced by 15 different subpopulations of EECs which were long defined by the main enterohormones they produced and their localization along the GI tract (Rehfeld, 1998, Brubaker, 2012) (**Table 1**). Some enterohormones are released throughout the entire intestine, such as serotonin/5-hydroxy-tryptamine (5-HT) (Ormsbee and Fondacaro, 1985, Gribble and Reimann, 2016), whereas others are released by EECs in specific regions of the gut. For example, K, I, M, N, and S-cells, which secrete Glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CCK), motilin, neurotensin (NTS), and secretin (SCT), respectively, are mainly found in the small intestine (Buchan et al., 1978, Kitabgi and Freychet, 1978, Lopez et al., 1995, Itoh, 1997, Parker et al., 2009). L-cells, which secrete glucagon-like peptide-1 and 2 (GLP-1 and GLP-2) and the enterohormones peptide YY (PYY), are distributed from the distal small intestine to the colon (Drucker and Nauck, 2006, Chimere et al., 2014, Latorre et al., 2016b). However, the distribution of the main enterohormones (PYY, GLP-1, GIP and NTS) differs among species (Wewer Albrechtsen et al., 2016).

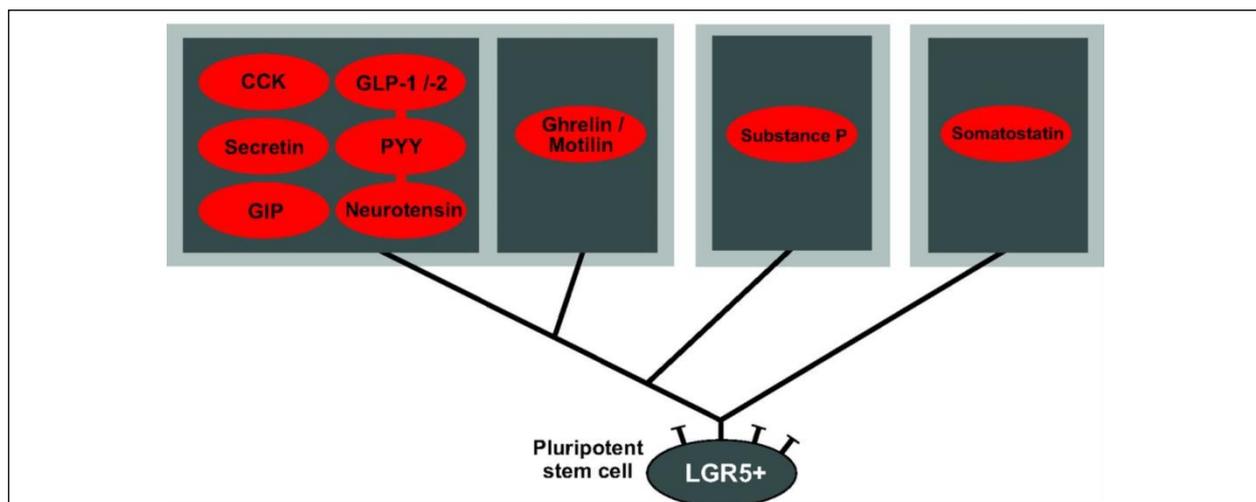
However, the dogma of one cell producing one enterohormone has been largely challenged during the past decade. The use of double or triple immunohistochemistry stains (Egerod et al., 2012, Grunddal et al., 2016), together with that of transgenic mice expressing fluorescent proteins under the control of specific hormone promoters (Aiken et al., 1994, Lopez et al., 1995, Mortensen et al., 2003, Egerod et al., 2012, Habib et al., 2012, Bohorquez et al., 2014, Sykaras et al., 2014, Grunddal et al., 2016) has uncovered the ability of most gut enterohormones to be co-expressed and secreted by the same EEC (Engelstoft et al., 2013) (**Table 2**). Numerous reporter systems have been developed in *in vivo* mouse models and have been reviewed by Engelstoft et al. (Engelstoft et al., 2013). Here, we will focus on the results provided by reporter mice in which CCK-positive (CCK-GFP mice (Liou et al., 2011a)), Gcg-positive (GLU-Venus mice (Reimann et al., 2008)), and GIP-positive (GIP-Venus mice

**Table 2. Enteroendocrine cells and gut peptides colocalization along the gastrointestinal tract**

Intestinal sections	Enteroendocrine cell types	Major produced peptide	Colocalized peptides	Species	Detection methods	References
Duodenum	K cells	GIP	GLP-1	Human, mouse	Immunofluorescence, laser capture microdissection	(Mortensen et al., 2003, Theodorakis et al., 2006, Engelstoft et al., 2013)
			CCK, GLP-1, SST	Mouse	GIP-Venus reporter mice, FACS	(Habib et al., 2012, Gribble and Reimann, 2016)
	I cells	CCK	Ghrl, NTS, GIP, SCT, PYY, GLP-1	Mouse	CCK-eGFP reporter mice, FACS, Immunohistochemistry	(Egerod et al., 2012, Engelstoft et al., 2013, Sykaras et al., 2014, Gribble and Reimann, 2016, Fothergill et al., 2017)
			GIP, SCT	Human	Immunohistochemistry	(Egerod et al., 2012)
	L cells	GLP-1	GIP, PYY	Rat Mouse Pig	Venus reporter transgenic mice, FACS, Immunohistochemistry	(Mortensen et al., 2003, Reimann et al., 2008)
			CCK, GIP	Mouse	GLU-Venus reporter mice, FACS	(Habib et al., 2012, Gribble and Reimann, 2016)
			CCK, GIP, NTS	Rat	Immunohistochemistry	(Svendsen et al., 2015)
		PYY	GIP, GLP-1	Pig	Immunofluorescence	(Cho et al., 2015)
			NTS	Rat	Immunohistochemistry	(Svendsen et al., 2015)
		S cells	SCT	GIP	Human	Immunohistochemistry
CCK, GCG, PYY, SST	Mouse			Immunohistochemistry	(Rindi et al., 1999, Habib et al., 2012)	
Jejunum	K cells	GIP	GLP-1	Mouse	Immunofluorescence	(Cho et al., 2015)
	I cells	CCK	SCT, NTS, GIP, Ghrl, GCG, PYY	Mouse	CCK-eGFP reporter mice, FACS, immunostaining	(Egerod et al., 2012)
			NTS, GLP-1	Human	Immunohistochemistry	(Egerod et al., 2012)
	L cells	GLP-1	PYY, GIP	Pig	Immunofluorescence	(Cho et al., 2015)
S cells	SCT	NTS	Human	Immunohistochemistry	(Egerod et al., 2012)	

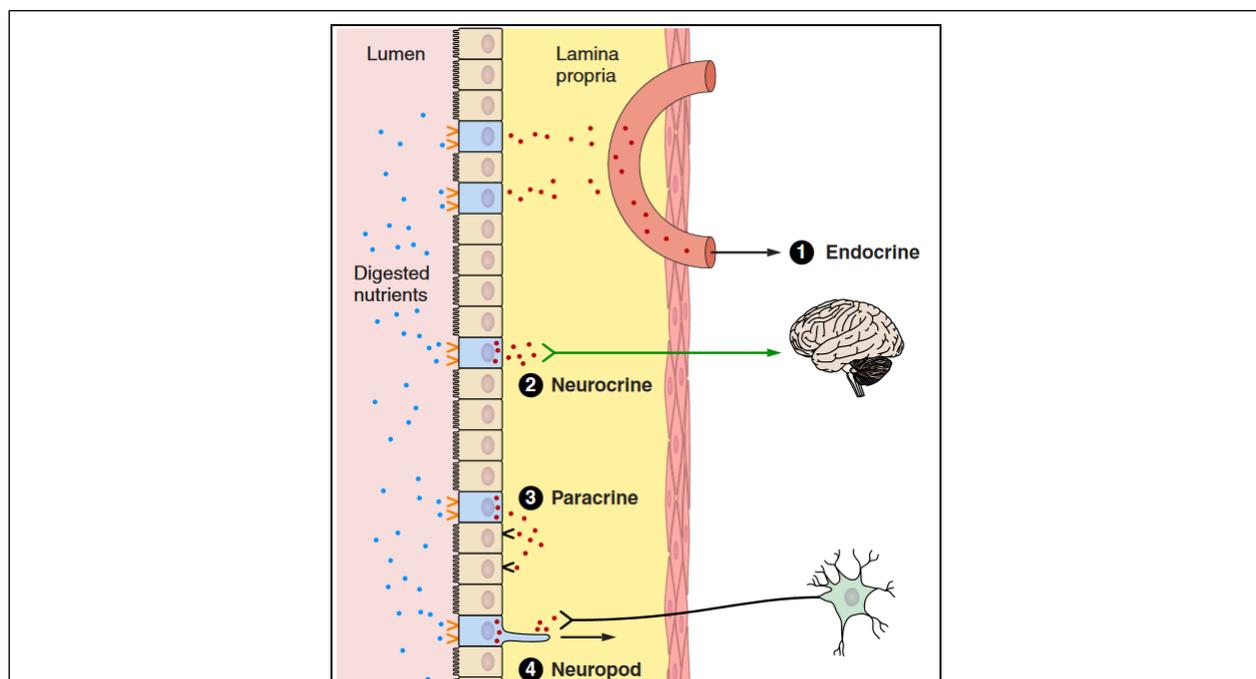
Intestinal sections	Enteroendocrine cell types	Major produced peptide	Colocalized peptides	Species	Detection methods	References
Ileum	K cells	GIP	GLP-1, PYY	Mouse, Pig	Immunofluorescence	(Cho et al., 2015)
	I cells	CCK	Sct, Nts, Gip, Gcg, Pyy	Mouse	CCK-eGFP reporter mice, FACS, qPCR	(Egerod et al., 2012)
			5-HT	Mouse	Immunohistochemistry	(Fakhry et al., 2017)
	L cells	GLP-1	PYY	Mouse, Pig	Immunofluorescence	(Cho et al., 2015)
			PYY, NTS	Mouse	GPR41-RFP reporter mice, FACS, laser capture microdissection	(Grunddal et al., 2016)
			CCK, GIP	Mouse	GLU-Venus reporter mice, FACS	(Habib et al., 2012)
	N cells	NTS	GLP-1, PYY	Mouse	GPR41-RFP reporter mice, FACS, laser capture microdissection	(Grunddal et al., 2016)
S cells	SCT	GLP-1	Human	Immunohistochemistry	(Egerod et al., 2012)	
Caecum	I cells?	CCK	5-HT	Mouse	Immunohistochemistry	(Fakhry et al., 2017)
Colon	L cells	GLP-1	PYY	Mouse	GLU-Venus reporter mice, FACS, Immunofluorescence	(Habib et al., 2012, Cho et al., 2015)
		PYY	CCK	Mouse	Immunohistochemistry	(Fakhry et al., 2017)

GIP: Glucose-dependent insulinotropic peptide; GLP-1: Glucagon-like peptides 1; CCK: Cholecystokinin; SST: Somatostatin; Ghrl: Ghrelin; NTS: Neurotensin; SCT: Secretin; PYY: peptide YY; 5-HT: 5-hydroxy-tryptamine ; GFP : Green fluorescent protein ; FACS : Fluorescence-activated cell sorting ; GPR41 : G protein-coupled receptors 41.



**Figure 9. Schematic overview of proposed main enteroendocrine cell lineages of the small intestine (Adapted from (Egerod et al., 2012))**

Enteroendocrine cells (EECs) expressing somatostatin represent a distinct lineage from EECs expressing the rest of the GI peptides. The non-somatostatin cells can be subdivided into three subtypes: 1) the cells having the potential to express CCK, GLP-1/2, secretin, PYY, GPI and neurotensin; 2) the cells able to express ghrelin and motilin and 3) the enterochromafin cells expressing substance P. GI: Gastrointestinal; CCK: Cholecystokinin; GLP-1/-2: Glucagon-like peptides 1/2; GIP: Glucose-dependent insulinotropic peptide; PYY: peptide YY; LGR5+: Leucine-rich repeat-containing G-protein coupled receptor 5.



**Figure 10. The four gut hormonal signaling pathways (Adapted from (Steinert et al., 2017))**

The four types of gut hormone signaling pathways include: (1) the classical endocrine pathway in which hormones diffuse into the blood; (2) the neurocrine pathway signaling through the afferent nerves, (3) the paracrine pathway acting on neighboring cells and (4) the neuropod pathway in which hormones such as CCK or PYY are released by EECs neuropods that end in synapse-like appositions connecting directly to the enteric neurons. CCK: Cholecystokinin; PYY: Peptide YY; EECs: Enteroendocrine cells.

(Parker et al., 2009)) EEC populations are labeled. The use of transgenic mice in which the expression of green fluorescent protein (GFP) was driven by the *Cck* promoter established that most CCK-positive cells of the duodenum express *Pyy*, *Gcg*, *Ghrelin*, and *Gip* (Egerod et al., 2012, Sykaras et al., 2014). Only the co-expression of CCK and ghrelin was not confirmed by immunohistochemistry, in agreement with previous data obtained in pigs and humans (Wierup et al., 2007). Interestingly, the population of CCK-positive cells purified from the ileum, express *Gcg*, *Pyy*, and *Nts* at equal levels and the specific ablation of GLP-1-positive cells decreased the number of CCK-positive cells by ~ 60% in the ileum (Egerod et al., 2012), strengthening the assumption of an overlap of CCK and GLP-1 production by L-cells in the distal small intestine. The study of transgenic mice expressing Venus fluorescent protein under the control of the *Gcg* promoter revealed that *Gcg*-Venus-positive cells isolated from the upper small intestine not only secrete PYY, as expected, but also GIP (Reimann et al., 2008), and display similar expression levels for *Gip*, *Cck*, *Sct*, *Nts*, and *Pyy* (Habib et al., 2012). Only SST secretion does not appear to overlap with that of PYY, GLP-1/2, CCK, SCT, NTS, and GIP in the same EECs (Egerod et al., 2012). Overall, these studies suggest that most EEC cells (except SST-positive cells) can express and secrete a broad range of enterohormones (**Figure 9**). A comparison of the transcription factor expression profiles by microarray of K- and L-cells isolated from GIP-Venus and GLU-Venus mice, respectively, revealed that upper small-intestine L-cells (defined as *Gcg*-Venus-positive EECs) are more closely related to duodenal K-cells (defined as GIP-Venus-positive EECs) than colonic L-cells (Habib et al., 2012). This suggests that the regulation of specific enterohormones in the lower intestine may differ from that in the upper small intestine. Thus, the panel of enterohormones expressed or secreted in each EEC is likely regulated by the position of the EECs along the GI tract in association with nutritional or other luminal (*e.g.* microbial metabolites) factors.

### III-2 Gut hormonal signaling pathways

There are four types of pathways through which gut hormones signal to exert their regulatory functions (**Figure 10**): (1) the endocrine pathway via the blood, (2) the paracrine pathway by acting on neighboring cells, (3) the neurocrine pathway, mostly by signaling through afferent nerves (*e.g.* the vagal nerve), and (4) the neurocrine-like pathway, directly connecting glial cells to the EECs. This latter pathway was suggested recently by the discovery of EEC structures

called neuropods, which displays axon-like features (Bohorquez et al., 2014). These structures have so far been described for CCK- and PYY-positive EECs (Bohorquez et al., 2015).

### **III-3 The regulation of enterohormone secretion**

A wide range of luminal compounds, such as nutrients (per review (Furness et al., 2013), microbial metabolites (*e.g.* SCFAs or bile acids) (Christiansen et al., 2018, Kuhre et al., 2018, Lu et al., 2018), and microbial components (*e.g.* LPS or toxins) (Bogunovic et al., 2007, Lebrun et al., 2017) can stimulate the expression and secretion of gut enterohormones. We will not exhaustively review all activators for all enterohormones. We will first focus on the intestinal endocrine response to acute or chronic exposure to fructose. Then, we will examine the endocrine intestinal response to major changes in nutrient influx into the lower GI tract and discuss the role of microbial metabolites on the regulation of the enterohormones. Finally, the regulation of CCK secretion and its function will be described in more detail.

#### ***III-3.1 The impact of fructose on the secretion of gut enterohormones***

Sugars, such as fructose and glucose, as well as polysaccharides (fiber and starch), are major components of the diet and play a major role in the regulation of the expression and secretion of gut enterohormones. We will mostly center our review on the acute response of EECs to fructose relative to their response to glucose, as well as the consequences of chronic fructose intake on the intestinal endocrine response.

##### ***III-3.1.1 The intestinal endocrine response to acute fructose exposure***

GLP-1 secretion is stimulated in response to oral fructose challenge in healthy humans and type 2 diabetic patients (Ganda et al., 1979, Vozzo et al., 2002, Kuhre et al., 2014, Yau et al., 2017). Similar results have been observed for mice and rats (Kuhre et al., 2014). Fructose also stimulates GLP-1 secretion in GLUTag cells (Gribble et al., 2003, Kuhre et al., 2014). In contrast, fructose does not activate the secretion of the other incretin hormone, GIP, in neither humans, rats, or mice (Vozzo et al., 2002, Kuhre et al., 2014). However, GIP secretion is mildly stimulated in response to 10mM fructose in primary mouse cells in culture (Parker et al., 2009). Over the course of a day, fructose was shown to prolong the postprandial release of GLP-1 relative to glucose when glucose and fructose were consumed in the context of isocaloric meals (Teff et al., 2004). In the same study, the postprandial suppression of ghrelin was lower after

ingestion of a meal associated with a fructose-containing beverage than when associated with a glucose-containing beverage (Teff et al., 2004). This confirmed previous data found in rats, in which fasting ghrelin levels increased after two weeks of fructose consumption, whereas they remained unchanged after two weeks of glucose consumption (Lindqvist et al., 2008). In humans, acute fructose intake also stimulates the release of CCK and NTS to a similar extent as glucose, whereas PYY blood levels modestly increase in response to fructose but not glucose (Kuhre et al., 2014). Thus, acute exposure of EECs to fructose stimulates the secretion of most enterohormones.

### *III-3.1.2 The intestinal endocrine response to chronic fructose exposure*

Numerous studies in rodents and humans have associated the chronic intake of fructose with impairment in insulin sensitivity and glycemic homeostasis (Thorburn et al., 1989, Aeberli et al., 2013). This suggests that chronic exposure to dietary fructose may impair intestinal GLP-1 and GIP secretion in response to nutrients, as GLP-1 and GIP mediate the insulinotropic response to luminal nutrients. This hypothesis was tested, but the measured effects of long-term fructose intake have been largely variable and contradictory (Madani et al., 2015, Maekawa et al., 2017, Matikainen et al., 2017). The differences observed between studies can be explained largely by differences in the duration of exposure to fructose, the mode of administration (liquid or solid), the composition of other nutrients ingested (*e.g.* in association with a HFD or not), and the concentration of fructose used. This reflects the complexity of the intestinal endocrine response *in vivo* and may also reflect the contradictory food intake behavior and satiety response to fructose (Soenen and Westerterp-Plantenga, 2007, Lindqvist et al., 2008, Moran, 2009, Page et al., 2013, Purnell and Fair, 2013).

### *III-3.1.3 The potential sensor of fructose in enteroendocrine cells*

The mechanism by which fructose activates enterohormone secretion is less clear than for that of glucose. In the intestine, sweets exert part of their regulatory action through the sweet taste G-protein coupled receptor (GPCR) heterodimer system, taste receptor type 2 or 3 (T1R2/T1R3) (Shirazi-Beechey et al., 2011). This receptor can detect the large variety of sweet-tasting molecules (*e.g.* glucose, fructose, sucrose, aspartame, and sucralose) (Li et al., 2002). T1R2/T1R3 has been found along the intestinal tract at the apical membrane of most of EECs (Dyer et al., 2005, Jang et al., 2007, Margolskee et al., 2007). Rodent EEC cell lines, such as

STC-1 and GLUTag, also express T1R2/T1R3 (Dyer et al., 2005, Margolskee et al., 2007), as well as the human L-cell line, NCI-H716 (Jang et al., 2007). Among the various EEC types, T1R2/T1R3 is strongly expressed by GLP-1 and PYY-positive EECs (Meyer-Gerspach et al., 2014) and GIP-positive cells (Moran et al., 2010), but is poorly expressed in duodenal I-cells that secrete CCK (Daly et al., 2013). Interestingly, lactitol, a competitive inhibitor of T1R3, reduces glucose-induced GLP-1 and PYY release *in vivo* and *in vitro* (Margolskee et al., 2007, Gerspach et al., 2011), but has no effect on CCK secretion (Gerspach et al., 2011), indicating that sweet taste receptors are unlikely to be involved in the regulation of CCK secretion. The activation of GLP-1/2, GIP, and PYY in response to fructose through the T1R2/T1R3 pathway is theoretically possible since fructose also activates T1R2/T1R3 in EECs (Shirazi-Beechey et al., 2014). However, no study has investigated the role of the sweet taste receptor in mediating the effect of fructose in EECs. Another possible mechanism may involve the fructose transporters, GLUT2 and GLUT5. The GLUT5 transcript has been detected by qRT-PCR in isolated K-cells of the upper small intestine and isolated colonic L-cells (Reimann et al., 2008, Parker et al., 2009). GLUT2 is expressed at the apical and basolateral membranes of L-cells in rodents and humans (Parker et al., 2012, Schmitt, 2016).

### ***III-3.2 The impact of change in the nutrient flow on enterohormone expression***

Numerous studies have shown that changes in nutrient flow in the intestine can modify endocrine function along the GI tract through modification of the distribution or transcriptional activity of EECs. In a rodent model of Roux-en-Y gastric bypass (RYGB), the number of GLP-1-, GIP- and PYY-positive cells increased in the alimentary and common limbs (intestinal segments in which the nutrient flow increased after the surgery) but not the biliopancreatic limb (intestinal segment without nutrient flow) (Cavin et al., 2016). Similar results have also been observed in humans after RYGB (Nergård et al., Rhee et al., 2015, Cavin et al., 2016). In addition to GLP-1, GIP, and PYY, which are the most extensively studied gut enterohormones in the context of RYGB, a few studies have also investigated changes in CCK and NTS; CCK-positive cell density increased in the alimentary and common limbs after RYGB in humans (Rhee et al., 2015) and rat models (Mumphrey et al., 2013) and postprandial CCK and NTS serum levels were greater in response to a meal in RYGB patients than control subjects (Jacobsen et al., 2012, Dirksen et al., 2013). In rats, interposition of the ileum into the jejunum led to an increased in mRNA levels of the precursor of GLP-1 and the GLP-2, pre-proglucagon (*Gcg*), in the remnant ileum segments and the colon (Holst, 1997, Brubaker and Anini, 2003). In

humans, short bowel syndrome (SBS), a strong malabsorption disorder resulting from resection of the small intestine, is associated with elevated plasma levels of GLP-1, GLP-2, and PYY (Jeppesen et al., 2000, Gillard et al., 2016). SBS animal models also exhibit major endocrine adaptations in the GI tract, including elevated *Pyy* and *Gcg* transcript levels in the colon (Gillard et al., 2016).

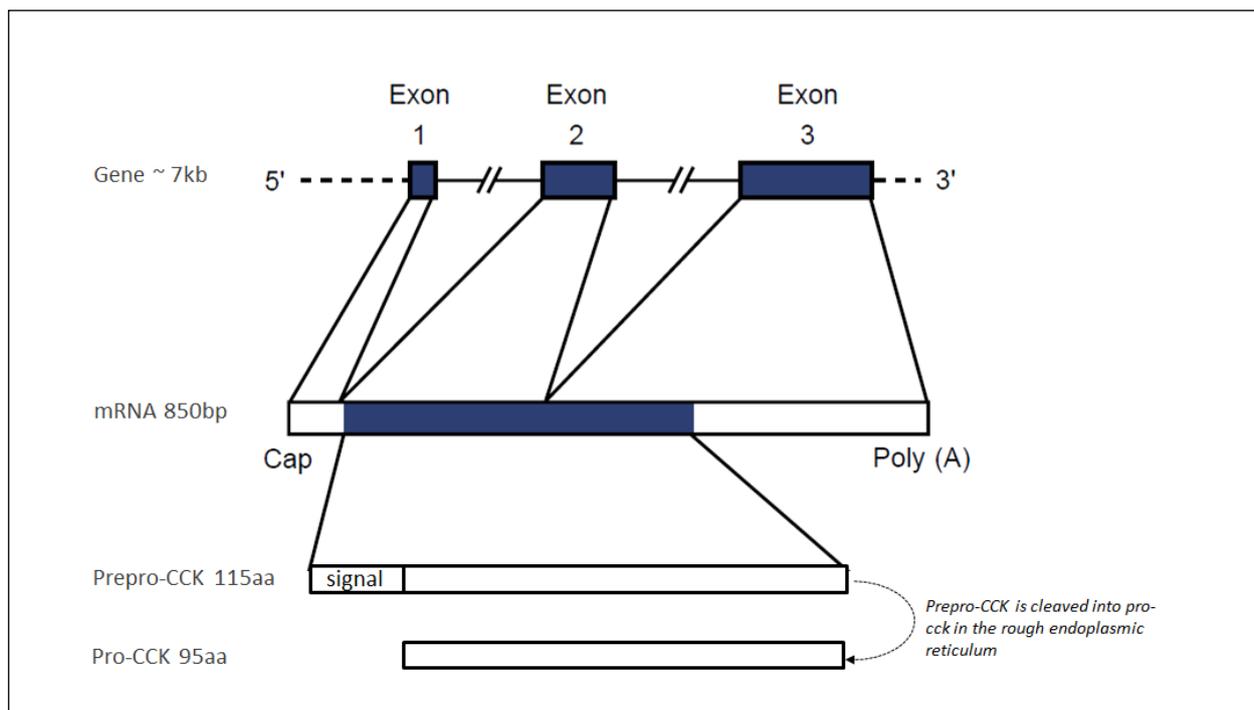
### ***III-3.3 The role of the gut microbiota and its metabolites on the regulation of enteroendocrine cells***

The transfer of SBS patient microbiota into germ-free (GF) rats increases the density of GLP-1-positive cells in the colon of recipient animals relative to that of rats colonized with conventional microbiota, emphasizing the role of the intestinal ecosystem in intestinal EECs proliferation and differentiation (Gillard et al., 2017). Numerous studies have highlighted the ability of microbiota-derived products, such as SCFAs (Zhou et al., 2008, Tolhurst et al., 2012, Psichas et al., 2015, Plovier and Cani, 2017) and bile acids (Thomas et al., 2009, Trabelsi et al., 2015, Fuchs et al., 2018), to regulate *Gcg* expression and GLP-1 or PYY secretion. Interestingly, GF rodents and rodents treated with antibiotics display higher *Gcg* expression levels, GLP-1-positive cell density in the colon, and elevated circulating levels of GLP-1 than conventionally raised mice (Wichmann et al., 2013, Arora et al., 2018). The gut microbiota also profoundly impairs the transcriptional responses of the *Gcg*-positive cells in the ileum and colon (Arora et al., 2018). However, although it is now clearly established that most of EECs secrete more than one enterohormone (Gribble and Reimann, 2016), the ability of the microbiota to specifically modulate the panel of secreted hormones in individual EECs is still unclear. Moreover, the impact of microbiota on enterohormones that have long been assumed to be expressed and secreted in the upper small intestine has not been investigated.

## **III-4 Cholecystokinin: its regulation and main function**

### ***III-4.1 Transcript structure and derived molecular forms of cholecystokinin***

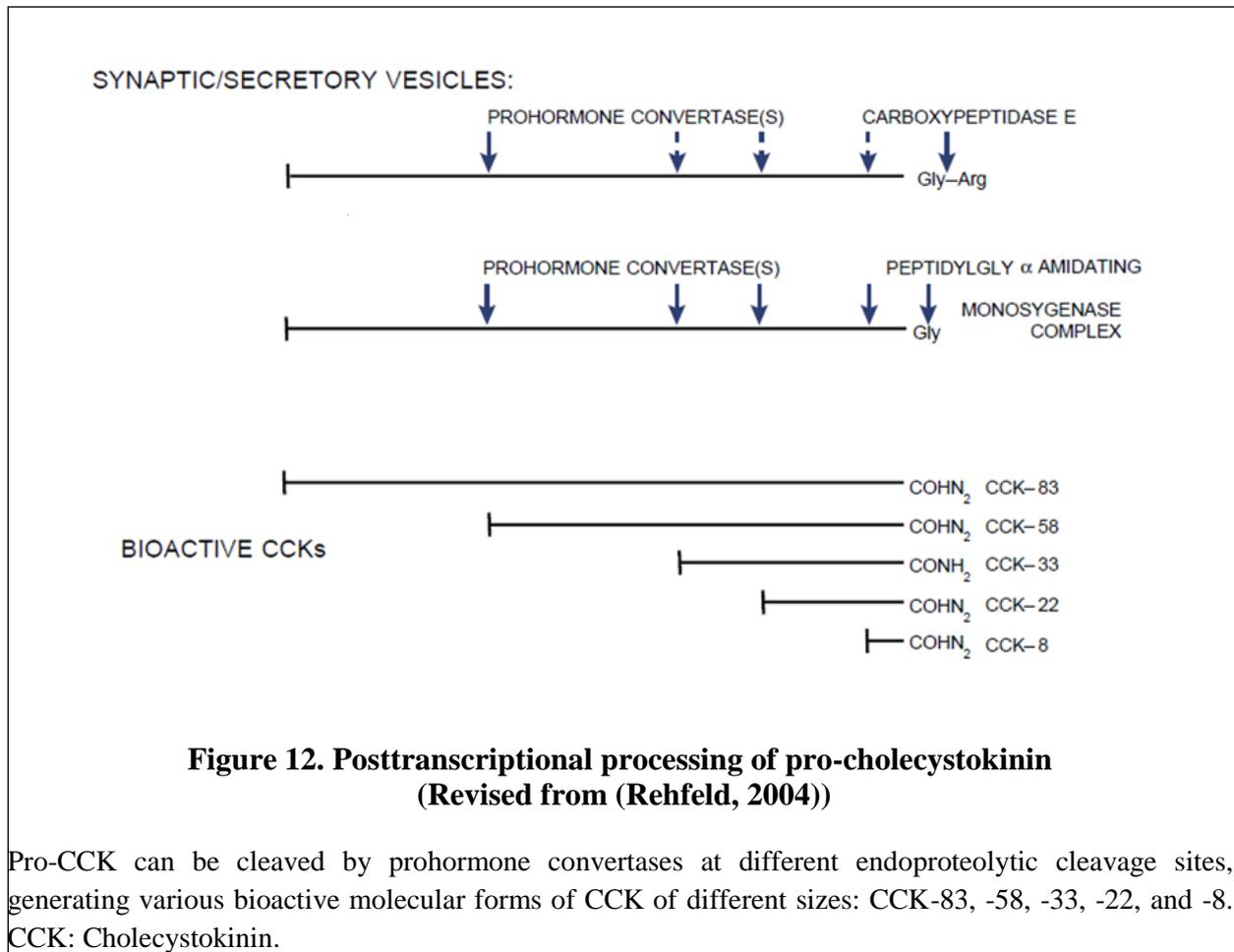
The gene encoding CCK in humans is approximately 7 kb and composed of two introns and three exons. The transcript of this gene is a mRNA of ~ 850 bp, of which 345 encodes a protein of 115 amino acids which is the precursor of CCK (prepro-CCK) (**Figure 11**)



**Figure 11. Overall structure of the cholecystokinin gene, mRNA, prepro-cholecystokinin and pro-cholecystokinin (Revised from (Rehfeld, 2004))**

CCK gene is about 7kb and has 3 exons. It is transcribed into 850 base pair (bp) mRNA with an 345bp open reading frame. The translated 115 amino acid protein is the precursor of CCK (prepro-CCK). The N-terminal 20 amino acids are removed to generate a smaller peptide of 95 amino acids (pro-CCK). CCK: Cholecystokinin.

(Deschenes et al., 1984, Gubler et al., 1984). The 20 amino acids of the N terminal region composed the signal peptide and is removed in the rough endoplasmic reticulum, generating a smaller peptide of 95 amino acids, pro-CCK. Then, pro-CCK migrates into the secretory vesicles, where multiple posttranslational modifications occur before the release of bioactive forms of CCK. Pro-CCK can be cleaved by prohormone convertases at different endoproteolytic cleavage sites, generating various bioactive molecular forms of CCK peptides of different sizes: CCK-83, -58, -39, -33, -22, and -8 (**Figure 12**) (Deschenes et al., 1984, Rehfeld et al., 2008). In addition, the posttranslational processing of CCK also requires carboxyamidation and O-sulfation of the peptides (Eysselein et al., 1982, Eng et al., 1984, Ruiz-Gayo et al., 1985, Reeve et al., 1986, Paloheimo and Rehfeld, 1994, Bundgaard et al., 1997, Rehfeld et al., 2001). Among the various molecular forms generated, the amidated CCK-8 sulfate is generally considered to be the biologically active form (Rehfeld et al., 2007). The processing of pro-CCK into bioactive CCKs is tissue specific. Thus, specific patterns of CCK peptides have been measured in specific tissues, mostly because of the tissue specificity of the prohormone convertases (Beinfeld, 2003). In the gut, EECs release various molecular forms of the hormone, such as CCK-58, -33, -22, and -8 (Rehfeld, 2004, Rehfeld et al., 2008). However, all the molecular forms of CCK are not secreted equally along the GI tract. In humans, for example, the CCK-8/CCK-33 ratio goes from 1/1 to 1/6 from the proximal- to the mid-jejunum (Maton et al., 1984). More recently, the molecular forms of CCK released by EECs of the colon were investigated and showed the presence of amidated and sulfated forms, including CCK-8 and CCK-33, suggesting that CCKs secreted in the colon are also bioactive peptides (Fakhry et al., 2017). This variety of molecular forms of CCK in the gut also translates into the plasma and is also influenced by species specificity. The predominant forms in human plasma are CCK-33 and CCK-58, whereas CCK-8 and CCK-22 are predominantly found in rodents and rabbits (Liddle et al., 1984, Rehfeld, 1994, Rehfeld et al., 2001). CCK is also largely present in the enteric and central nervous tissues (Larsson and Rehfeld, 1979). In the central nervous system (CNS), CCK is mostly present in the cortical brain (Larsson and Rehfeld, 1979), where CCK-8 is the main molecular form and ~95% is sulfated (Reeve et al., 1986, Rehfeld and Hansen, 1986, Agersnap and Rehfeld, 2015, Agersnap et al., 2016). This multiplicity of molecular forms of CCK make its accurate measurement challenging and the various properties of each molecular form are still not clearly established.

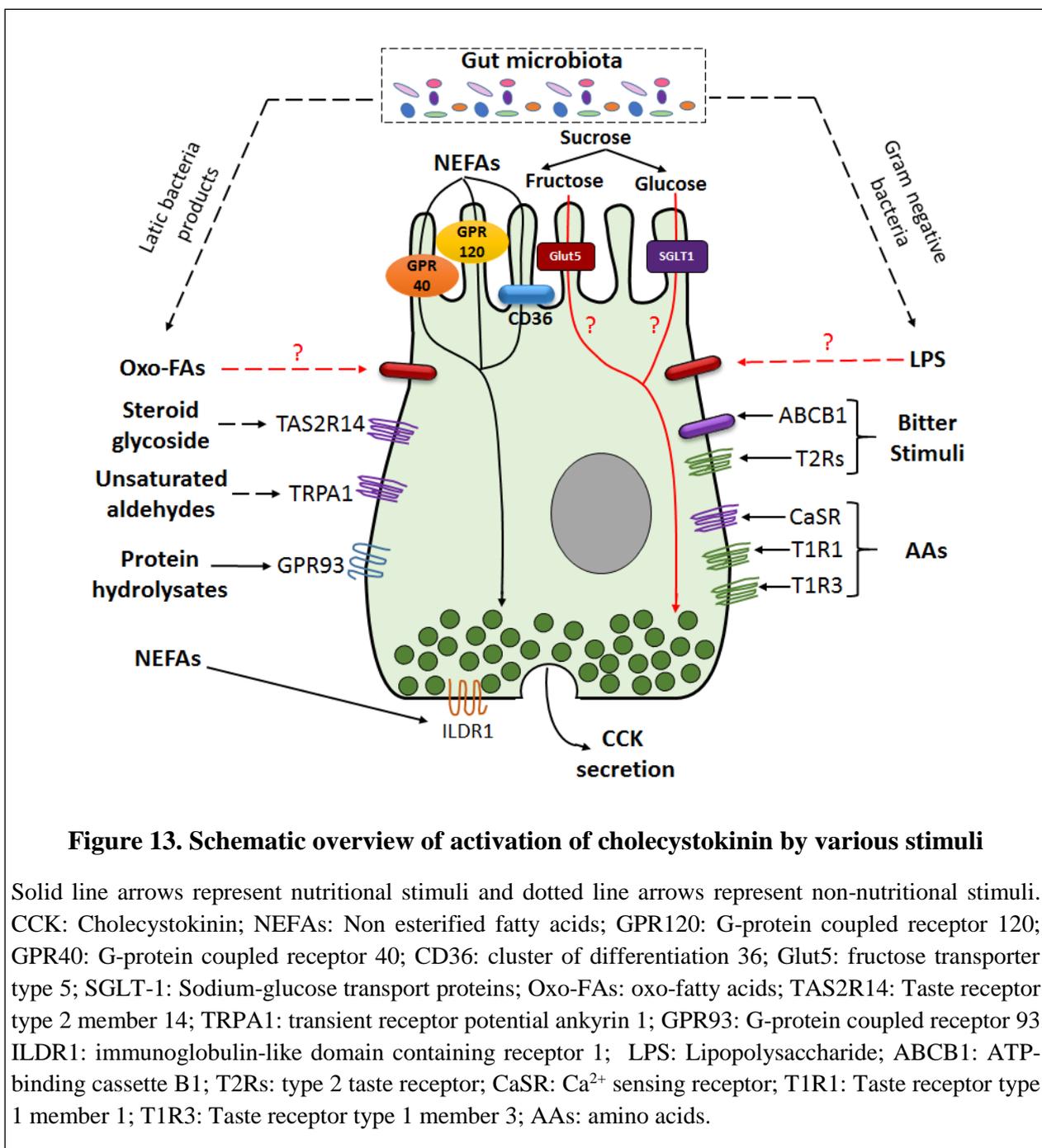


### ***III-4.2 Stimulation of cholecystinin secretion***

The regulation of CCK secretion in response to nutrient stimuli has been mostly studied in the I-cells of the small intestine. Although it is now clear that CCK can be secreted by EECs localized elsewhere in the gut, here we will mostly review the effect of nutrients on CCK expression and secretion in the proximal small intestine or EEC cell-line models. In the pre-prandial situation, the plasma CCK concentration is  $\leq 1$  pmol/L and increases to 2-5 pmol/L within 80 min following ingestion of a meal (Rehfeld, 2004). The major nutrients that stimulate CCK release are fats (Costarelli and Sanders, 2007, Sankoda et al., 2017) and protein hydrolysates or amino acids (Choi et al., 2007, Wang et al., 2011). However, evidence also suggest that sugar and bitter compounds can also regulate the release of CCK (Chen et al., 2006, Geraedts et al., 2012), as well as non-nutritional factors that originate from the gut microbiota (*e.g.* LPS) (**Figure 13**).

#### ***III-4.2.1 The fatty acids***

Non-esterified fatty acids (NEFAs), originating from the digestion of dietary triglycerides, are potent activators of CCK secretion in numerous species *in vivo* (Costarelli and Sanders, 2001, Little et al., 2007, Steinert et al., 2013) and *in vitro* in GLUtag or STC-1 cell lines (Sidhu et al., 2000, Harden et al., 2012). The length and extent of non-saturation of the acyl chains positively correlate with the potency of NEFAs to activate the release of CCK (Jonkers et al., 2000, Matzinger et al., 2000, Costarelli and Sanders, 2001, Harden et al., 2012). *In vitro* and *in vivo* (using knockout mice models) studies have demonstrated that the two long chain free fatty acid receptors, GPR40 and GP120 (also named free fatty acid receptor 4 (FFAR4) and FFAR2, respectively), and the scavenger receptor CD36 are involved in NEFA-induced release of CCK by EECs (Tanaka et al., 2008, Liou et al., 2011a, Sundaresan et al., 2013, Sankoda et al., 2017). More recently, a member of the lipoprotein remnant receptor family, immunoglobulin-like domain containing receptor 1 (ILDR1), has been shown to be expressed in CCK-positive EECs in mice and the effects of NEFA on the secretion of CCK were abolished in *Ildr1* knockout mice (Chandra et al., 2013). A mixture of high-density lipoproteins (HDL) and NEFA was also able to induce CCK release in EECs isolated from wild type (WT) but not *Ildr1* knockout mice (Chandra et al., 2013). This discovery suggests that both sides of CCK-positive cells may be involved in the stimulation of the cells by lipid molecules: lipoprotein can activate CCK



secretion from the basolateral surface via ILDR1, whereas CD36 and FFARs detect NEFAs at the apical surface of CCK-EECs.

#### *III-4.2.2 Protein hydrolysates and amino acids*

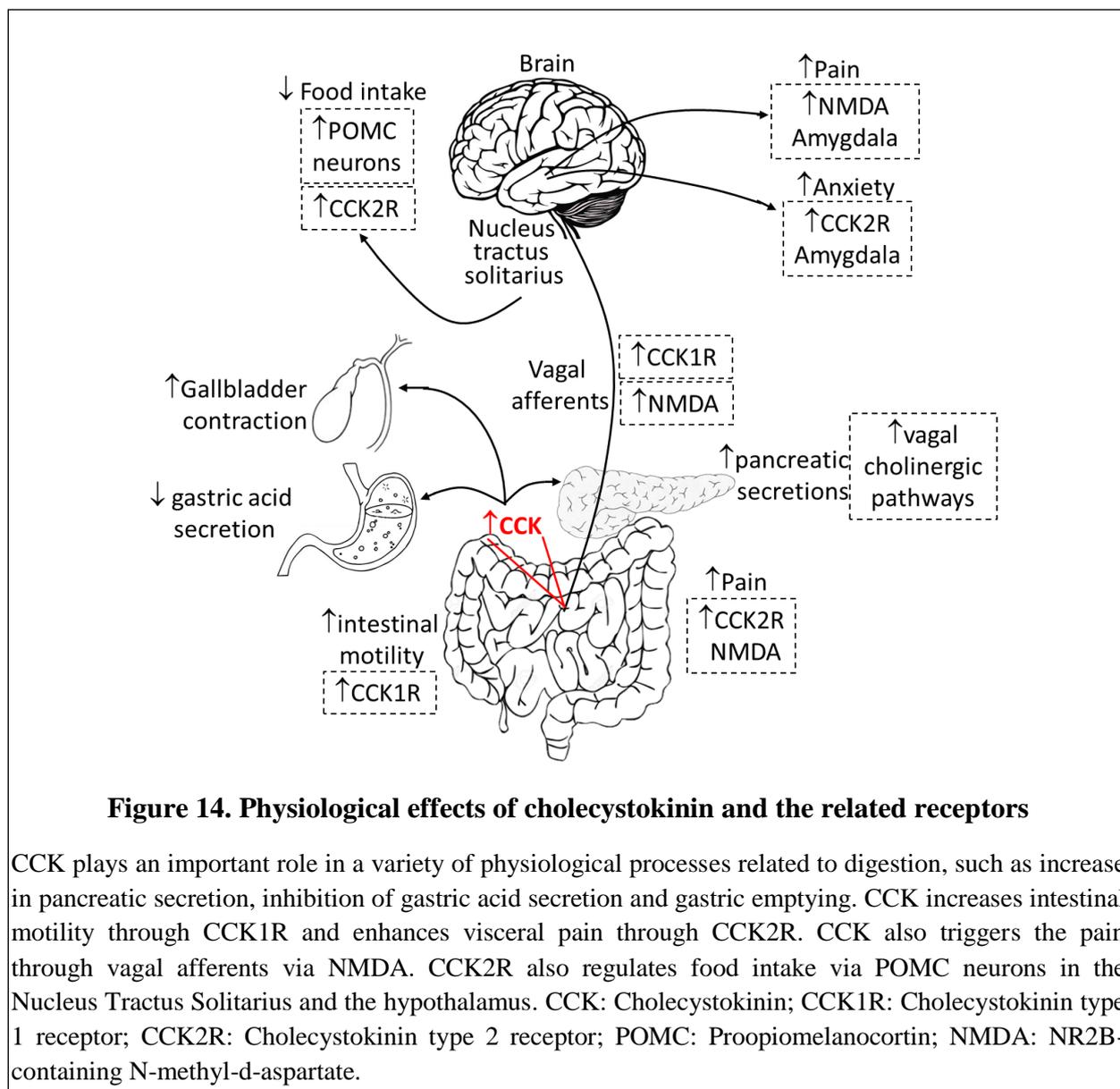
Protein hydrolysates and amino acids have been shown to stimulate CCK release through multiple types of receptors. Protein hydrolysates promote the secretion of CCK through G-protein-coupled receptor GPR93 in STC-1 cells (Choi et al., 2007). Among the amino acids that stimulate CCK release, the aromatic amino acids, L-phenylalanine and L-tryptophan, are the most effective and regulate CCK secretion via the activation of the Ca<sup>2+</sup> sensing receptor (CaSR) (Hira et al., 2008, Liou et al., 2011b, Wang et al., 2011, Shirazi-Beechey et al., 2014). The taste receptors T1R1 and T1R3 expressed in CCK-positive cells have also been shown to support the release of CCK in response to phenylalanine, leucine, and glutamate (Daly et al., 2013).

#### *III-4.2.3 Sweet and Bitter stimuli*

Although less studied than NEFAs or amino acids, the sweets including sucrose, glucose, and fructose, have been shown to stimulate CCK secretion in EEC cell lines *in vitro* and humans and rodents *in vivo* (Fried et al., 1991, Buemann et al., 2000, Geraedts et al., 2012, Kuhre et al., 2014, Meyer-Gerspach et al., 2018). However, as mentioned previously, the molecular mechanism by which glucose and fructose stimulate the secretion of CCK is yet to be clarified. Intestinal CCK is also released *in vivo* or *in vitro* in response to bitter compounds (Chen et al., 2006, Le Neve et al., 2010, Jeon et al., 2011, Andreozzi et al., 2015). Bitter taste-sensing G protein-coupled receptor family (type 2 taste receptors) expressed in EECs (Latorre et al., 2016a) regulates the bitter-induced secretion of CCK. Functional consequences of bitter-induced CCK secretion are a decrease in food intake (Schier et al., 2011) and an increase in expression of ATP-binding cassette B1 (ABCB1, an efflux transporter) in the intestinal cells. Both are likely involved in limiting the absorption of dietary-derived bitter-tasting toxins (Jeon et al., 2011).

#### *III-4.2.4 Non-nutrient stimuli*

Non-nutritional stimuli, such as gut microbiota metabolites (Bogunovic et al., 2007, Hira et al., 2018), non-nutritional compounds, (Le Neve et al., 2010, Nakajima et al., 2014) and



neuropeptide (Ramesh et al., 2016) also influence intestinal CCK secretion. Two bacterial compounds, LPS (cell wall compound of gram-negative bacteria) and specific oxo-fatty acids (oxo-FAs, metabolites produced by lactic acid bacteria) can activate the secretion of CCK *in vitro* (Bogunovic et al., 2007, Hira et al., 2018). However, their capacity to activate CCK secretion in the gut and the physiological responses associated with their ability to stimulate CCK are yet to be investigated. Non-nutritional compounds, such as unsaturated aldehydes and steroid glycoside, also stimulate CCK secretion through transient receptor potential ankyrin 1 (TRPA1) (Nakajima et al., 2014) and the bitter receptor taste 2 receptor member 14 (T2R14) (Le Neve et al., 2010), respectively. Finally, the neuropeptide nesfatin-1, which colocalizes with CCK in EECs *in vivo*, triggers CCK secretion *in vitro*, (Ramesh et al., 2016). Non-nutritional stimuli need further investigation to understand their potential physiological link to their ability to stimulate CCK release. However, they may be interesting candidates to better understand the regulation of CCK secretion and expression in the lower intestine, where luminal lipid, amino acid, and sugar concentrations are expected to be reduced subsequent to their absorption in the small intestine.

### ***III-4.3 Physiological effects of cholecystinin***

CCK plays an important role in a variety of physiological process related to digestion, such as contraction of the gallbladder (Xu et al., 2008b), increasing intestinal motility (Meyer et al., 1989), and inhibiting gastric acid secretion and gastric emptying (Liddle et al., 1986). CCK also regulates food intake and induces satiety (Lo et al., 2014, Schroeter et al., 2015). In addition to those digestion related functions, CCK also enhances visceral pain (Friedrich and Gebhart, 2000), and participates in the regulation of anxiety via its actions in the CNS (Abelson and Nesse, 1990, Desai et al., 2014). Two distinct G protein-coupled receptors mediate the effects of CCK: the CCK1 (CCK1R) and the CCK2 (CCK2R) receptors. They both bind CCK and gastrin, but with different binding affinity. The CCK1R is highly discriminating toward CCK vs. gastrin, whereas the CCK2R binds CCK and gastrin with comparable affinities (**Figure 14**) (Miller and Desai, 2016). CCK1Rs are mainly localized in peripheral organs, whereas CCK2Rs are primarily expressed in the brain and stomach (**Figure 14**).

#### ***III-4.3.1 Food intake***

The role of CCK in controlling food intake and inducing satiety has long been known (Gibbs et al., 1973). Intravenous infusions of CCK-8 or purified CCK result in a dose-dependent decrease in meal size when injected at the beginning of a meal (Gibbs et al., 1997). Behavioral studies have also shown that chronic CCK treatment reduces meal size but increases their frequency (Crawley and Beinfeld, 1983, West et al., 1987), possibly explaining why long-term CCK administration has no effect on body weight. CCK signals via the vagal nerve to the nucleus tractus solitarius by activating the CCK1R at the level of the vagal nerve and the CCK2R at the level of the postrema area in the nucleus tractus solitarius (Zarbin et al., 1981, Moran et al., 1990, Moran et al., 1998, Kopin et al., 1999, Bellissimo and Anderson, 2003, Clerc et al., 2007). From the nucleus tractus solitarius, afferent fibers project to the arcuate nucleus (ARC) of the hypothalamus, where the CCK satiety signal is integrated with leptin signals (Blevins et al., 2009), creating a complex response that regulates food intake. In addition, CCK leads to the activation of proopiomelanocortin (POMC) neurons (in the hypothalamus the stimulation of POMC neurons results in satiety) of the nucleus tractus solitarius (Appleyard et al., 2005), suggesting that, in addition to its role in transmitting afferent signals to the hypothalamus, the nucleus tractus solitarius may also participate in the regulation of food intake.

#### *III-4.3.2 Pancreatic secretion, gut motility and gallbladder contraction*

The pancreatic secretagogue and gallbladder contractile properties of CCK have long been known (Kerstens et al., 1985). Indeed, CCK can activate the secretion of most pancreatic proteases (*e.g.* amylase and trypsin) (Liddle et al., 1985, Evilevitch et al., 2004, Jaworek et al., 2010), as well as those of the intestine (Dyck et al., 1973a, Dyck et al., 1973b, Dyck et al., 1974). Stimulation of pancreatic enzyme secretion in response to CCK results from the activation of the vagal cholinergic pathways (Owyang and Logsdon, 2004). In addition to its impact on GI secretion, CCK increases the contraction of the smooth muscles of the pylorus, boosts the motility of the small intestine and colon, and inhibits gastric emptying (Per review (Dockray, 2012)). Deletion of the CCK1R decreases intestinal motility, suggesting a role for the CCK1R in this process (Berna and Jensen, 2007).

#### *III-4.3.3 Pain*

The role of CCK in mediating visceral pain has been suggested in mice, rats, and humans (Harvey and Read, 1973, Friedrich and Gebhart, 2000, Chua and Keeling, 2006, Cao et al.,

2012). Moreover, both subtypes of CCK receptor, CCK1R and CCK2R, have been found in colonic smooth muscle and enteric neurons, suggesting that they can regulate sensory and motor function in the lower intestine (Dinosa et al., 1973, Harvey and Read, 1973, Morton et al., 2002, Fornai et al., 2007, Ko et al., 2011). Specific antagonists of the CCK2R increase the antinociceptive effect of morphine in a dose-dependent manner, suggesting that the inhibition of the CCK2R may alleviate visceral pain induced by CCK-8 (Friedrich and Gebhart, 2000). Conversely, the CCK1R does not appear to be involved in this process. The nociception induced by CCK is also implicated in vagal afferent signaling and mediated through NR2B-containing N-methyl-d-aspartate (NMDA) receptors in the central amygdale (Wang et al., 2015). In irritable bowel syndrome (IBS) patients, plasma and colonic levels of CCK are elevated (Zhang et al., 2008), suggesting that abdominal pain symptoms associated with specific GI disorders may be related to mechanisms regulated by CCK.

#### *III-4.3.4 The central action of cholecystokinin on various behavior processes*

In the CNS, CCK is also involved in the regulation of a wide range of behaviors. This is explained by its colocalization with numerous neurotransmitters (dopamine, serotonin, gamma-aminobutyric acid) in neuron cell bodies and the broad localization and function of CCK receptor subtypes (Hokfelt et al., 1980, van der Kooy et al., 1981, Hendry et al., 1984, Ballaz, 2017). This central action and function of CCK is not reviewed here; only the most notable effects of CCK are briefly highlighted. CCK plays a major regulatory function in reward behavior via mesolimbic dopamine structures (Josselyn et al., 1996, Josselyn and Vaccarino, 1996). Numerous studies have described the ability of intravenous infusions of CCK to induce anxiety disorder in humans and rodents (van Megen et al., 1996), and CCK2R antagonists can reduce these effects (Bradwejn et al., 1994). These data are coherent with the high expression of CCK2R receptors in areas associated with anxiety, such as the amygdale (Mercer et al., 2000).

#### **III-5 Regulation of the other gut peptides: Peptide YY, glucagon-like peptide-1, and neurotensin**

Like CCK, GLP-1, PYY, and NTS regulate appetite and food intake in response to a meal. Their secretion is also stimulated by carbohydrate, fat, and protein intake, as well as byproducts of intestinal microbiota metabolism.

### ***III-5.1 Peptide YY***

PYY belongs to the family of protein-fold polypeptides. Its secretion is stimulated by food intake, the presence of nutrients in the GI tract, and SCFAs produced by intestinal bacteria (Pfluger et al., 2007). PYY crosses the hemato-encephalic barrier and binds to the Y2 receptor in the hypothalamus, inhibiting the expression of Neuropeptide Y neurons and thus reducing food intake (Batterham et al., 2002). In addition to its central effects, PYY regulates GI motility through the "ileal brake". The "ileal brake" is activated by the presence of nutrients in the ileum, especially lipids. It limits gastric emptying and intestinal transit, thereby increasing the efficiency of nutrients absorption (Taylor, 1993). PYY also acts in the periphery via vagal afferent neurons or neurons of the enteric nervous system (ENS) (Konturek et al., 2004, Koda et al., 2005, Glavas et al., 2008).

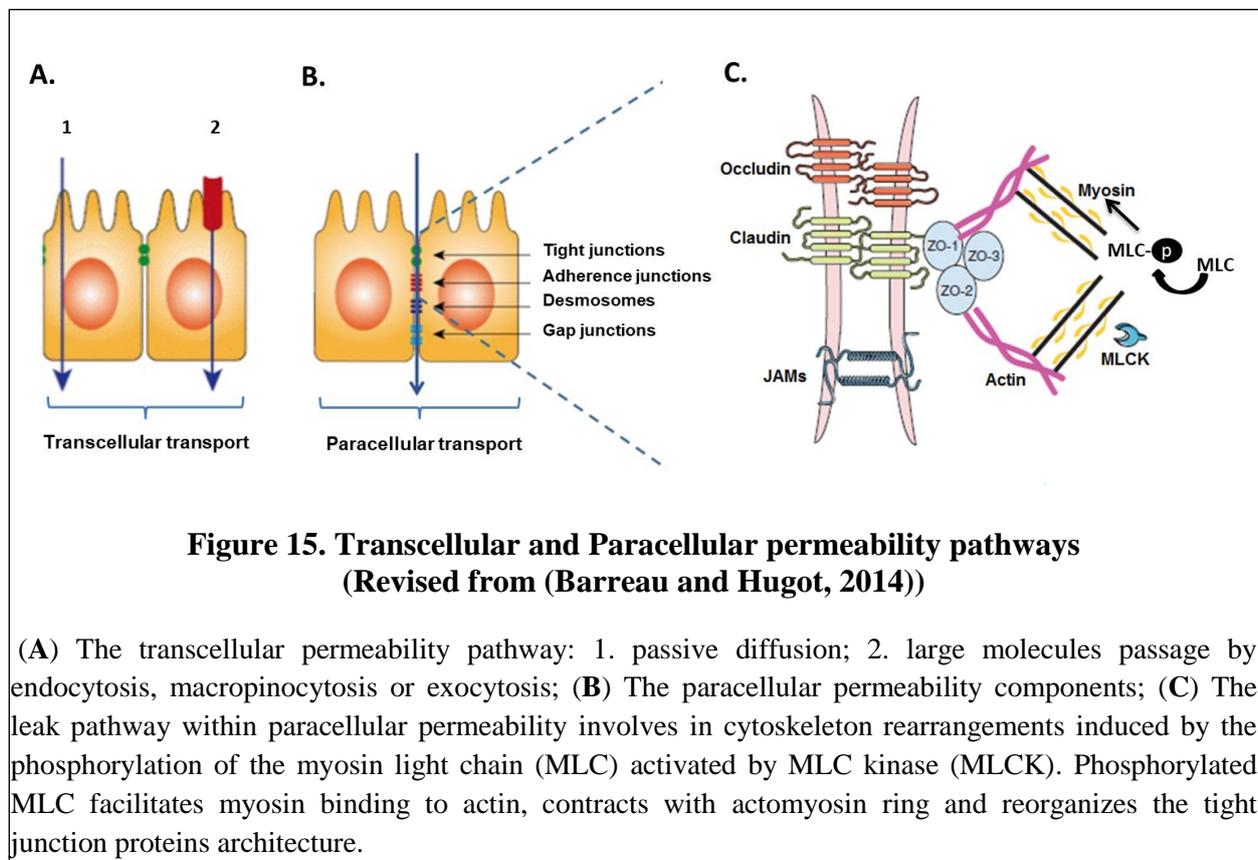
### ***III-5.2 Glucagon-like peptide-1***

The ileum and colon have been identified as the primary sites that contain most of the proglucagon expressing L-cell populations in rats, pigs, dogs, primates, and humans. However, as mentioned previously, it is now clear the proglucagon- and GLP-1-positive cells are expressed from the small intestine to the colon (Eissele et al., 1992), with an increasing density gradient from the proximal to the distal region of the gut (Furness et al., 2013). The secretion of GLP-1 is stimulated by nutrients, especially sugars and lipids, as well as by microbial compounds, such as SCFAs or bile acids. GLP-1 increases pancreatic glucose-dependent insulin secretion (the incretin effect), regulates the glucose flux, inhibits gastric emptying, and reduces appetite.

### ***III-5.3 Neurotensin***

NTS is an important peptide secreted by N cells in both the intestine and the CNS. Ten percent of NTS is detected in the brain, whereas the other 90% is found in the rest of the body with 85% in the intestine (Carraway and Leeman, 1976). In most mammals, NTS is detected in the jejuno-ileum section of intestine (Sundler et al., 1977). However, variations in intestinal distribution have been observed among species (Iwasaki et al., 1980). In the small intestine, lipids and sugars are both potent activators of NTS (Ferris et al., 1981, Drewe et al., 2008, Kuhre et al., 2015). In addition, infusion of SCFAs, such as acetate, butyrate, and propionate, has been found

to stimulate NTS secretion in the ileum and colon (Sileikiene et al., 2008). NTS exerts a wide range of physiological functions through three NTS receptors (NTSRs) (Vincent, 1995, Mazella et al., 2012). In the CNS, the satiety function of NTS has long been known in rodents (Stanley et al., 1983), but the role and mechanism of action of intestinal NTS in the regulation of food intake has only been recently clarified (Ratner et al., 2016). This action involves stimulation by both the vagal afferent system and blood circulation (Ratner et al., 2016). In addition to being co-stored (Egerod et al., 2012) and co-secreted (Grunddal et al., 2016) with PYY and GLP-1, NTS acts in synergy with these two peptides to regulate gastric emptying and glycemia in response to food intake (Grunddal et al., 2016). Intestinal NTS is also involved in gastric-acid secretion (Andersson et al., 1976), the delay of gastric emptying (Blackburn et al., 1980), the control of gallbladder contraction (Degolier et al., 2013), and the stimulation insulin secretion (Kaneto et al., 1978).



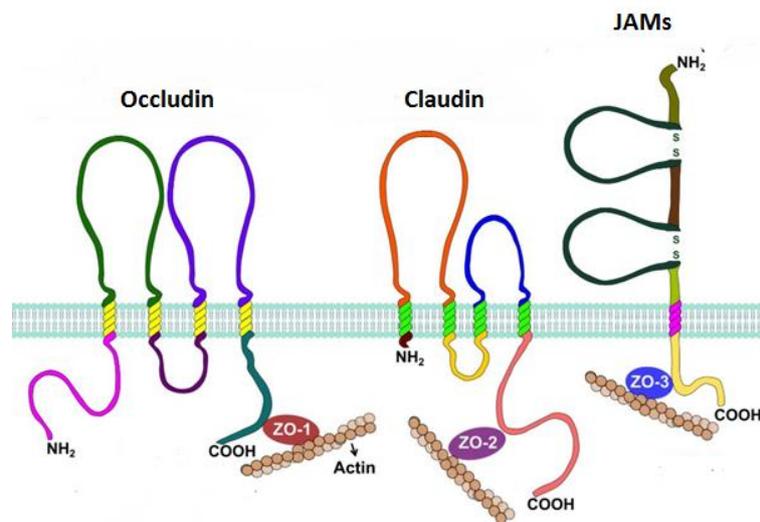
## IV- Gut permeability and its major role in metabolic disorders

### IV-1 Gut permeability: definition and main components

The GI tract is the main region of digestion and absorption of nutrients. To optimize digestion and absorption, the GI exhibits a large epithelial surface of approximately 300-400 m<sup>2</sup> in humans (Brandtzaeg, 2011). Thus, the intestinal epithelium represents the largest epithelial barrier in the body, separating the internal milieu from the external environment. It regulates the translocation of water, electrolytes, nutrients, and luminal compounds into the blood, in addition to preventing the entry of luminal poisons, toxic compounds, and microorganisms. The intestinal barrier consists, from the lumen to the serosa, of a mucus layer, a monolayer of intestinal epithelium cells (IECs) tightly connected by intercellular junctions (physical protection), an array of antimicrobial peptides produced by specific IECs (immune protection), and a pool of immune cells localized in the *lamina propria* (Peterson and Artis, 2014, Greenwood-Van Meerveld et al., 2017, Wells et al., 2017). One of the most important functional features of the intestinal barrier is intestinal permeability, which regulates the flow of materials through IECs from the lumen to the rest of the body (Bischoff et al., 2014). Two major types of intestinal permeability allow the flux of molecules across the IECs monolayer: transcellular and the paracellular permeability. One of the major consequences of alterations in gut barrier function is increased intestinal permeability. It is often associated with the loss of intestinal homeostasis, an increase in intestinal inflammation, and intestinal tissue destruction, and further induces physiopathological conditions, such as obesity, diabetes, and IBD (Bischoff et al., 2014).

#### IV-1.1 Transcellular permeability

Transcellular permeability is defined by the transportation of molecules across IECs into the blood or secretion system, and includes two major pathways: (1) the passive diffusion of molecules from the apical to the basolateral epithelium (*e.g.* transport of small hydrophilic molecules through aquaporins) and (2) The passage of large molecules by endocytosis, micropinocytosis, or exocytosis (Zimmer et al., 2016). The transport by endocytosis or micropinocytosis ensures the passage of large molecules (*e.g.* immunoglobulin) that are unable to cross the intercellular space (Keita and Soderholm, 2010) (**Figure 15A**).



**Figure 16. The tight junction protein structures  
(Adapted from (Torres-Flores and Arias, 2015))**

Occludin contains four transmembrane domains, two extracellular loops and two cytoplasmic domains. Claudins are characterized by four transmembrane regions, two extracellular loops, one intracellular loop and two intracellular regions. JAMs are transmembrane proteins constituted of an extracellular domain, a single intramembrane domain and a short cytoplasmic tail. JAMs: Junctional adhesion molecules.

### ***IV-1.2 Paracellular permeability***

Paracellular permeability is the transfer of molecules across the intestinal epithelium through the intercellular space between the IECs. It is regulated by intercellular adhesive junction complexes localized between adjacent IECs. Four types of intercellular junctions are localized from the apical to the basolateral level of the plasma membranes of neighboring IECs: tight junctions (TJs), adherent junctions (AJs), desmosomes, and gap junctions (**Figure 15B**). TJs are the limiting factor that regulates the passage of molecules through the intercellular space as a function of their diameter and charge. TJs are composed of transmembrane proteins that form complex protein-protein interactions between neighboring cells and with protein of the cytoskeleton, regulating the opening of the space between the cells (Turner et al., 2014).

#### ***IV-1.2.1 Tight junction proteins***

The TJs consist of three major families of trans-membrane proteins including claudins, occludin, junctional adhesion molecules (JAMs), and a cytoskeletal connector zonula occludens (ZO) family (Shen et al., 2011).

##### **IV-1.2.1.1 The claudin family**

The claudin family consists of 27 proteins with molecular weights ranging from 20 to 34 kDa (Mitic et al., 2003). They are characterized by four transmembrane regions, two extracellular loops, one intracellular loop, and two intracellular regions (**Figure 16**) (Suzuki et al., 2017). Claudins play a major role in sealing the paracellular passage and forming pores, allowing the passage of specific ions (Suzuki et al., 2017), due to their interactions with each other (homophilic interaction) and with other family members (heterophilic interaction) (Piontek et al., 2008). Thus, some claudins (claudins -1, -3, -4, -5, -8, -11, -14, and -19) can strengthen the epithelial barrier (Will et al., 2008) whereas others (claudins 2, 10, 15, and 17) participate in the formation of pores that allow the diffusion of selective ions and molecules across the paracellular space (Rosenthal et al., 2017).

##### **IV-1.2.1.2 Occludin**

Occludin is a 65 kDa protein with four transmembrane domains, two extracellular loops, and two cytoplasmic domains (**Figure 16**). The localization of occludin to TJs is regulated mainly by the phosphorylation of its serine, threonine, and tyrosine residues (Sakakibara et al., 1997). These phosphorylations are regulated by multiple kinases and phosphatases which can differentially regulate the structure and function of the TJ (Per review (Dorfel and Huber, 2012) and **Figure 16**). For example, phosphorylation of tyrosine residues weakens the TJs whereas phosphorylation of threonine by specific protein kinases strengthens TJ assembly. The major role of occludin in paracellular permeability was determined by observing increased transepithelial electrical resistance (TEER) measured in cells that overexpress occludin (Balda et al., 1996) and a major decrease in occludin expression in IBD patients (Gassler et al., 2001). However, occludin knockout mice show normal TJ formation (Saitou et al., 2000).

#### IV-1.2.1.3 The family of junctional adhesion molecules

JAMs are cell surface adhesion receptors of the immunoglobulin superfamily. The JAM family consists of five members: JAM-A, JAM-B, JAM-C, JAM-4, and JAM-like. JAMs are transmembrane proteins consisting of an extracellular domain, a single intramembrane domain, and a short cytoplasmic tail (**Figure 16**). All members are located in the intercellular space of epithelial cells and participate in the assembly and maintenance of TJ structures via homophilic interactions. The use of JAM-A knockout mice showed that JAM-A plays a fundamental role in maintaining the integrity of the intestinal barrier (Laukoetter et al., 2007, Vetrano et al., 2008).

#### IV-1.2.1.4 The family of zonula occludens

Occludin, claudins, and JAMs are considered as transmembrane components of the TJ, whereas members of the ZO family are considered as cytoskeletal connectors. The ZO family is composed of three proteins, ZO-1, ZO-2, and ZO-3, of 220, 160, and 130kDa, respectively. They are members of the Membrane Associated Guanylate Kinase Homologs (MAGUK) and they can bind to proteins of the adherent junction and other TJ proteins (almost all the claudins and occludin), as well as proteins of the cytoskeleton (Hartsock and Nelson, 2008). These interactions play a prominent role in the regulation of TJ function.

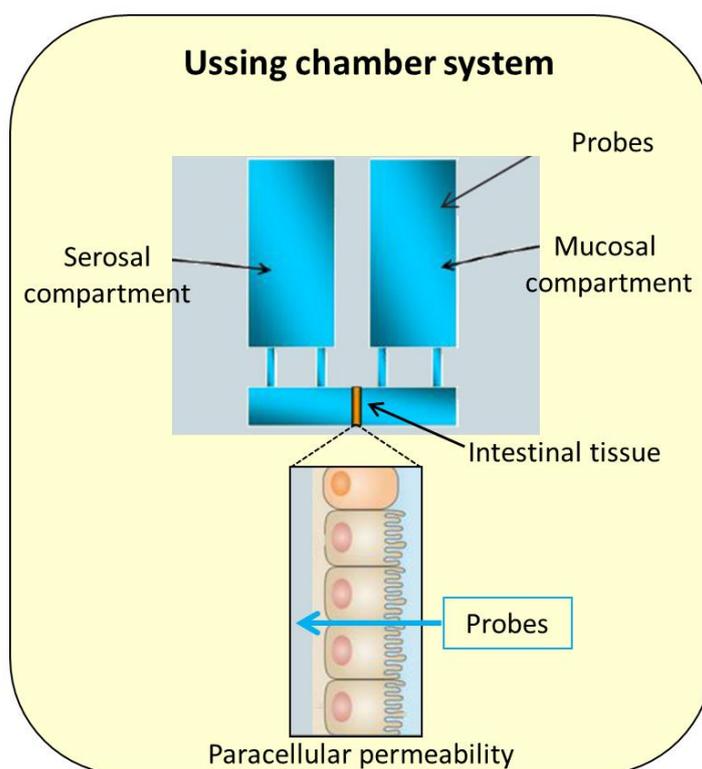
#### *IV-1.2.2 The regulation of paracellular permeability*

The regulation of paracellular permeability can be divided into two distinct pathways: the pore pathway and the leak pathway (Turner et al., 2014). The pore pathway is mainly controlled by the expression of claudins and involves charged molecules with a radius of below 4 Å (Anderson and Van Itallie, 2009). The leak pathway involves the transport of molecules with a radius above 4 Å without charge selectivity. The leak pathway requires cytoskeletal rearrangements, such as those induced by the phosphorylation of the myosin light chain (MLC) induced by MLC kinase (MLCK) (Graham et al., 2006). Phosphorylated MLC facilitates myosin binding to actin, contracts with actomyosin, and reorganizes the TJ proteins, including occludin and ZO proteins (Shen et al., 2006) (**Figure 15C**). MLCK can be activated by cytokines, such as tumor necrosis factor alpha (TNF $\alpha$ ), interferon gamma (IFN $\gamma$ ), and interleukin13 (IL13) (Graham et al., 2006, Cunningham and Turner, 2012, Cao et al., 2013, Turner et al., 2014). The role of MLCK in regulating intestinal permeability was confirmed using MLCK knockout mice (Clayburgh et al., 2005, Lorentz et al., 2017), as well as the use of mice constitutively expressing the active form of MLCK (Su et al., 2009). MLCK deletion prevents TJ disruption and improves gut barrier function (Clayburgh et al., 2005, Lorentz et al., 2017). In contrast, mice constitutively expressing the active form of MLCK show a marked increase in intestinal permeability (Su et al., 2009). Both mouse models show that MLCK expression is essential for the regulation of TJs.

Disruption of the architecture of TJs compromises barrier function and leads to the development of immune and inflammatory diseases. Many intestinal diseases are characterized by the loss of epithelial barrier function (Turner, 2009). The events involved in the loss of barrier function are not fully understood. However, cytokines, such as interleukin 1 beta (IL1 $\beta$ ) and IL13, IFN $\gamma$ , and TNF $\alpha$  are known to participate in the remodeling of TJ architecture, as well as bacterially produced SCFAs, and nutrients (Wachtershauser and Stein, 2000, Zeissig et al., 2007, Barreau and Hugot, 2014, Zhou et al., 2018).

#### **IV-2 The measurement of gut permeability**

*Ex vivo*, *in vivo*, and *in vitro* techniques have been used to assess intestinal permeability and integrity. Paracellular permeability can be evaluated by measuring TEER or tracing the passage of labeled molecules (< 600Da) (Watson et al., 2001) from the mucosal to the basolateral side of the epithelium. Transcellular permeability is determined by measuring the passage of large molecules (Menard et al., 2010).



**Figure 17. The *ex vivo* Ussing chamber system**

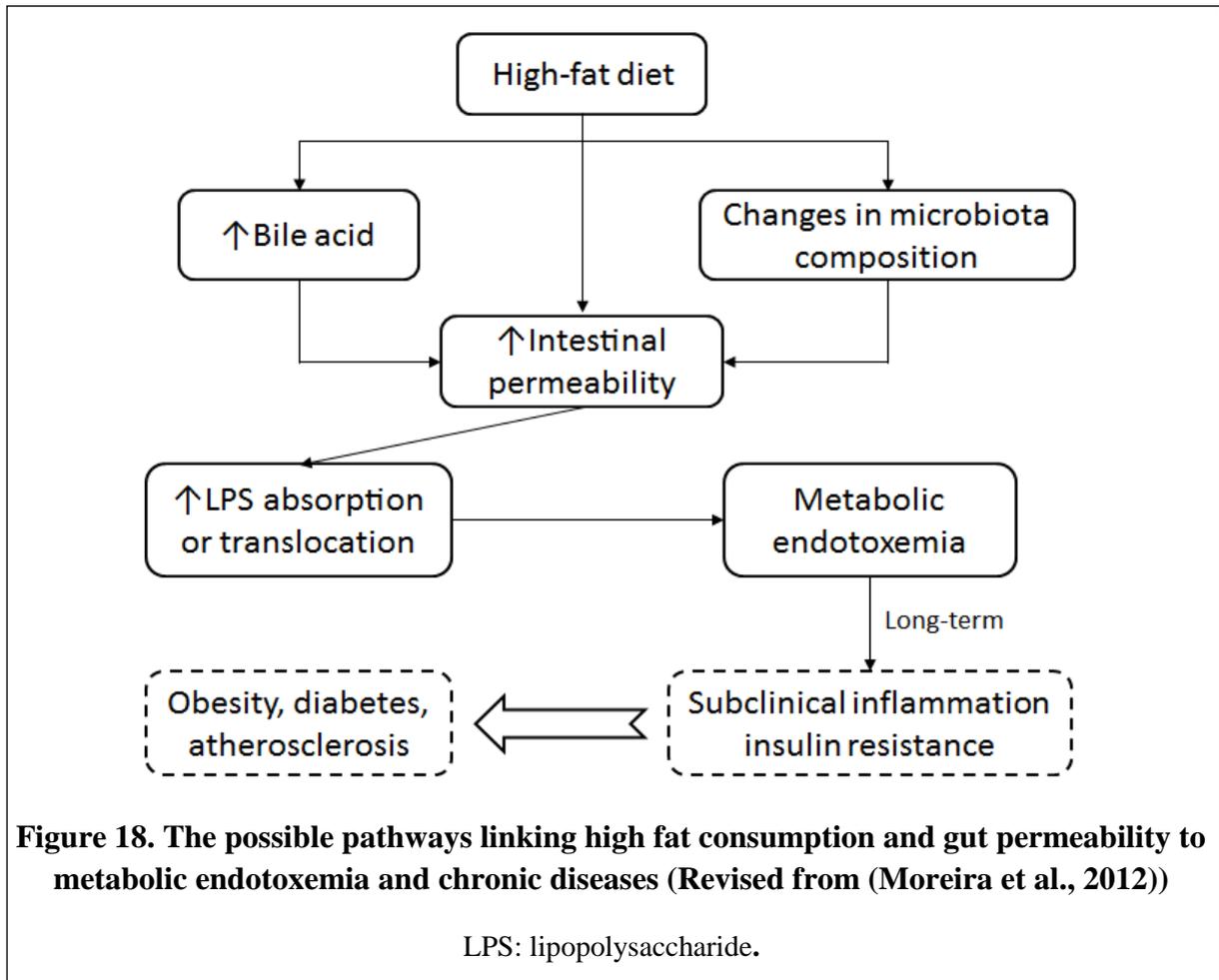
In the Ussing chamber system, intestinal tissue is inserted between two linkable chambers separating the serosal and mucosal compartments.

### ***IV-2.1 In vivo permeability assays***

Measurements of gut permeability *in vivo* are based on the utilization of various probes. These probes can be large molecules, such as inulin (75 kDa), cellobiose (30 kDa), or polyethylene glycol (PEG) (33-50 kDa) (Juby et al., 1989, Ammori et al., 1999), or small molecules, such as mannitol or  $^{13}\text{C}$ -Mannitol (182 Da), rhamnose (164 Da), or lactulose (342 Da) (Juby et al., 1989, Grover et al., 2016). Moreover, fluorescent tracers, such as fluorescein isothiocyanate (FITC)-dextran (3 kDa to 2000 kDa), FITC-sulfonic acid (478 Da), or fluorescein sodium salt (377 Da), are also largely used in animal models (Baxter et al., 2017, Dorshow et al., 2017, Vanhaecke et al., 2017, Malaise et al., 2018). Probes are generally orally ingested and detected in plasma or urine samples using an appropriate time course and detection methods as a function of the probe.

### ***IV-2.2 Ex vivo Ussing chamber system***

In 1954, the Ussing chamber method was established by Hans H. Ussing to study active sodium transport through the skin epithelium (Kalman and Ussing, 1955). This system allows the evaluation of paracellular permeability by simultaneously measuring the TEER and the passage of labeled molecules. Transcellular permeability across endothelial monolayers can also be measured by this technique using larger molecules (*e.g.* albumin, horseradish peroxidase (HRP)) (Kimm et al., 1996, Duffy and Murphy, 2001). The system is composed of two linkable chambers (Clarke, 2009, Vidyasagar and MacGregor, 2016) (**Figure 17**). Intestinal segments of various sizes are inserted between the two chambers. The labeled molecules (*e.g.* radioisotopes, fluorescent molecules, enzymatic markers) are added to the mucosal compartment and samples are taken from the serosal compartment at various timepoints to calculate the net flux per surface and per time unit. This flux reflects the pore size of the TJ in association with the paracellular water flow. The TEER can also be measured using the Ussing chamber system (Li et al., 2004). The TEER is a measurement of the electrical resistance across a cellular monolayer and reflects the ionic conductance of the paracellular pathway (Srinivasan et al., 2015). Ussing chambers have been used in both human and animal gut permeability studies under physiological and pathological conditions (Wallon et al., 2005, Menard et al., 2012, Hamilton et al., 2015).



**Figure 18. The possible pathways linking high fat consumption and gut permeability to metabolic endotoxemia and chronic diseases (Revised from (Moreira et al., 2012))**

LPS: lipopolysaccharide.

### ***IV-2.3 In vitro permeability measurements***

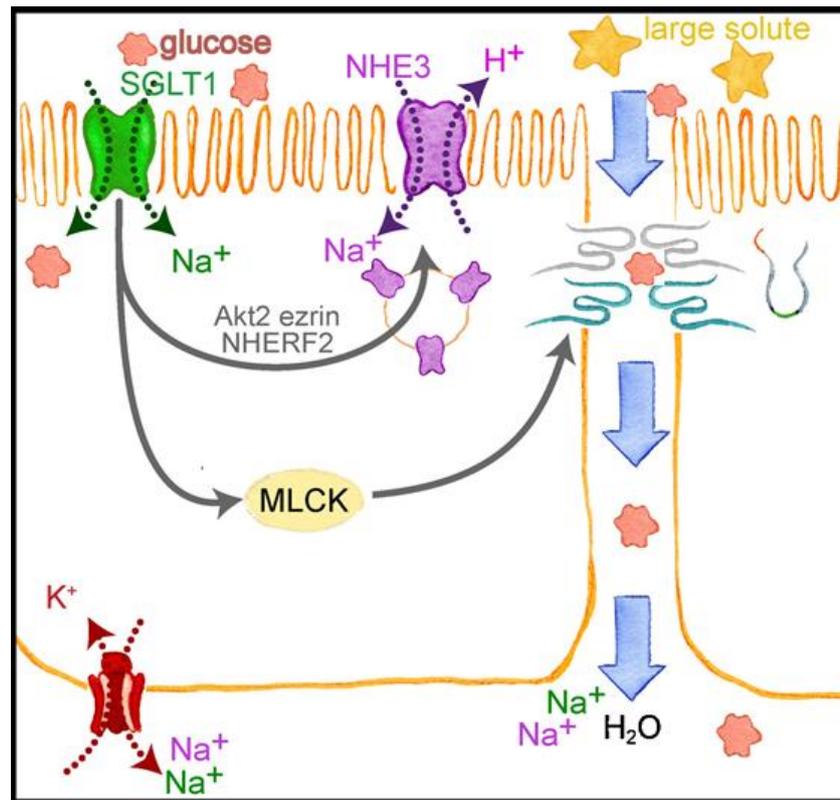
Gut permeability has also been explored *in vitro* using monolayers of colonic Caco-2 cell cultures in transwells. This model allows the measurement of the TEER, paracellular, and transcellular permeability. A high TEER reflects low permeability, as in *in vivo* systems (Shen et al., 2006). The same probes used *in vivo* can be used *in vitro*. They are delivered into the apical part of the monolayer and samples collected from the basal compartment (Cao et al., 2013).

## **IV-3 The effect of nutrients on gut permeability**

Recent studies have shown that nutrient-stimuli or chronic intake of specific diets can regulate the intestinal integrity. The effect of nutrients, mainly lipids and sugars, on gut permeability are discussed in this section.

### ***IV-3.1 Lipids and chronic exposure to a high-fat diet***

It is commonly accepted that obesity resulting from a HFD is linked to an alteration of gut barrier function by increasing intestinal para- or trans-cellular permeability (Cani et al., 2008, Stenman et al., 2012, Hamilton et al., 2015), impairing TJ protein expression (de La Serre et al., 2010, Suzuki et al., 2011), and increasing intestinal inflammation (Stenman et al., 2012). Furthermore, endotoxemia resulting from alteration of the intestinal barrier is believed to play an important role in the establishment of peripheral tissue inflammation (Cani et al., 2007, Erridge et al., 2007, Amar et al., 2008, Cani et al., 2008, Laugerette et al., 2011) (**Figure 18**). However, recent work has also shown the absence of an effect of very HFD on low-grade inflammation (Benoit et al., 2015), as well as a lack of an effect of chronic intake of a HFD on gut permeability, inflammation, and expression of TJ proteins (Kless et al., 2015). One study suggested a key role of the gut microbiota composition and bile acids in preventing gut barrier disruption (Muller et al., 2016). Probiotics or bacterially fermented products can also reduce high fat intake-dependent increased gut permeability (Lim et al., 2016, Matheus et al., 2017). In parallel, another study showed that the inhibition of adipose tissue development during exposure to a hyperlipidic diet rendered the intestine more susceptible to the establishment of experimental colitis (Wernstedt Asterholm et al., 2014). Overall, these data reveal the complex



**Figure 19. The sugar effects on paracellular permeability**  
(Adapted from (Turner et al., 2014))

The SGLT1 activates the NHE3 through the mediation of Akt2 and ezrin to increase the transcellular Na<sup>+</sup> transport. This also activates MLCK and therefore enhance the TJs permeability. SGLT1: Na<sup>+</sup>-glucose cotransport-dependent transporter; NHE3: Na<sup>+</sup>/H<sup>+</sup> exchanger; MLCK: Myosin light chain kinase.

array of interactions between the intestine, nutrients, and the intestinal ecosystem and peripheral organs in maintaining gut permeability.

#### *IV-3.2 Sugars*

Added sugars (glucose, fructose, and sucrose) are also associated with the development of metabolic diseases. Although less well-explored than for HFD, the role of intestinal permeability is also now believed to be essential for the development of metabolic disorders resulting from high sugar intake. Luminal glucose has long been known to regulate intestinal paracellular permeability through its transport into enterocytes by the apical Na<sup>+</sup>-glucose cotransport-dependent transporter (SGLT1). Briefly, the absorption of glucose through SGLT1 activates the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE3) and increases the phosphorylation of MLC, resulting in contraction of the perijunctional actomyosin ring, thus increasing TJ permeability (Turner et al., 1997, Turner et al., 2000) (**Figure 19**). In the context of hyperglycemia, plasma glucose has been shown to alter the transcription of TJ proteins and intestinal barrier function via its retrotransport into enterocytes by the basolateral glucose transporter GLUT2 (Thaiss et al., 2018). This glucose-dependent alteration of gut barrier function may be important in the aggravation of hyperglycemia associated outcomes.

The effect of dietary fructose on intestinal permeability is less clear. Eight weeks of chronic intake of a fructose solution (30%) decreased the expression of the TJ proteins ZO-1 and occludin in the mouse duodenum (Spruss et al., 2012, Wagnerberger et al., 2013). More recently, chronic intake of a 30% fructose solution was associated with decreased occludin, ZO-1, and claudin-2 mRNA levels in the ileum and decreased claudin-2 expression in the colon (Volynets et al., 2017). This study also showed an overall greater gut permeability (measured by FITC-dextran gavage) of mice receiving fructose in combination with a HFD than those fed a HFD without fructose or those fed fructose without a HFD. In humans, an eight-day study comparing beverages that provide 25% of their energy from fructose, glucose, or high fructose corn syrup (HFCS) showed that fructose- and glucose- (but not HFCS-) containing beverages slightly increased gut permeability (lactulose : mannitol test) (Kuzma et al., 2016). Nondigestible polysaccharides can also affect gut permeability. Apple polysaccharides have been shown to dramatically reduce the increase in gut permeability induced by a HFD (Wang et al., 2017).

Similarly, oligofructose reduces the increased gut permeability induced by a HFD and of genetically obese (*ob/ob*) mice fed a chow diet (Cani et al., 2009, Steensels et al., 2017).

The role of gut homeostasis in health is now believed to be essential. However, numerous questions are yet to be clarified. Although increased intestinal permeability has been correlated with inflammation (intestinal and systemic), it is still unclear whether the loss of barrier function is a cause or consequence of intestinal inflammation. In the context of sugar-induced gut barrier defects, one important missing piece of information in these studies is the region of the gut where permeability is impaired, as mono- and di-saccharides are absorbed in the proximal small intestine.

## **V-Enteric nervous system**

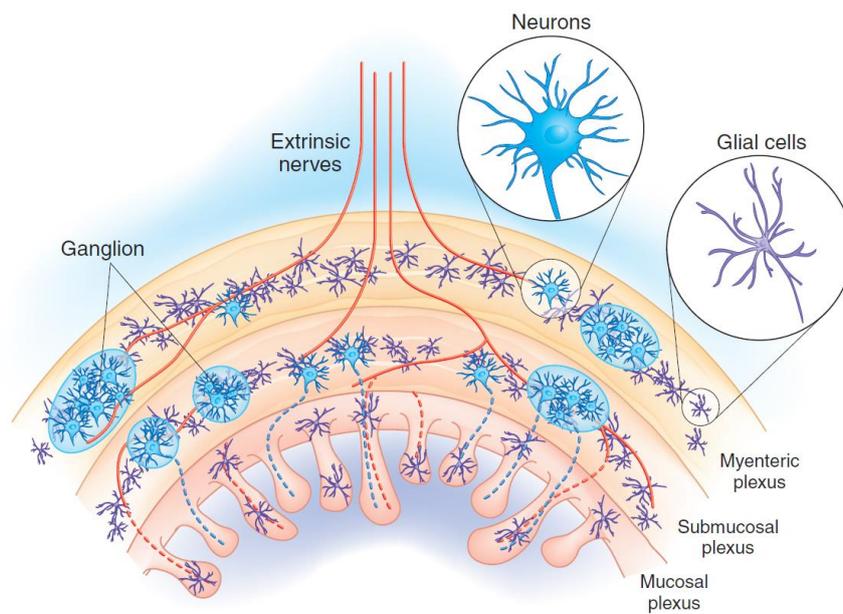
Recently, numerous studies discovered that the enteric nervous system plays an essential role in the regulation of intestinal barrier functions, the secretion of gut peptides secretions and that it could interact with the gut microbiota.

### **V-1 The basic structure and functions of enteric nervous system**

The ENS is one of the divisions of the autonomic nervous system and is located within the gut wall to support the intrinsic innervation in the GI tract (Furness, 2008, 2012). It consists of two major networks or plexuses including myenteric plexus and submucosal plexus, which are respectively located between the longitudinal and circular muscle, and around the muscularis mucosa (Furness, 2006, Grubisic et al., 2018). The ENS in humans is constituted of about 200~600 million neurons (Furness et al., 2014) which are surrounded by large populations of enteric glial cells (EGCs), about 6-7 times more numerous than neurons in the myenteric plexus and 1.3-1.9 times in the submucosal plexus (Gabella and Trigg, 1984, Hoff et al., 2008) (**Figure 20**). A major component of the ENS, EGCs consist of four morphological subtypes (type I to type IV) (Boesmans et al., 2015) which can be distinguished through glial markers glial fibrillary acidic protein (GFAP) and/or S100 $\beta$  (a type of calcium binding protein) contents (Neunlist et al., 2014). The neurons present in the gut are mainly cholinergic, serotonergic and dopaminergic. Nitric oxide is also an important mediator. Collectively, all of the cell types support the ENS to contribute alone or in concert with extrinsic neurons to regulating multiple intestinal functions including gut motility (Grubisic et al., 2018), blood flow controlling (Neunlist and Schemann, 2014), intestinal barrier functions (Savidge et al., 2007), immune system interactions (Margolis et al., 2011), nutrients absorption and endocrine responses (Neunlist et al., 2014), gut microbiota interactions (Hyland and Cryan, 2016). The ENS functions on the gut barrier, interactions with inflammatory response and microbiota, nutrients sensing will be described below.

### **V-2 The regulation of enteric nervous system on intestinal barrier function**

The ENS is involved in the regulation of the intestinal barrier functions mainly through its major component EGCs (Neunlist et al., 2014). In vivo, the transgenic mice with the ablation of GFAP-expressing EGCs showed severe jejunitis and ileitis, resulting from the disruption of



**Figure 20. The structure overview of enteric nervous system  
(Adapted from (Rescigno, 2008))**

The enteric nervous system (ENS) consists of two major ganglionated plexuses including myenteric plexus and submucosal plexus which are respectively located between the longitudinal and circular muscle, and around the muscularis mucosa. Millions of neurons and enteric glial cells (EGCs) form the neural network of the ENS.

intestinal barrier integrity (Bush et al., 1998, Cornet et al., 2001). The disruption of enteric glia has also been associated with the induction of paracellular permeability increase (Aube et al., 2006). In vitro, EGCs could increase the intestinal barrier resistance and decrease the paracellular permeability via the upregulation of tight junction markers ZO-1 and occludin when cocultured with the monolayer of intestinal epithelial cells (Savidge et al., 2007). Moreover, EGCs might contribute to the restoration of intestinal barrier resulting from bacterial infection (Flamant et al., 2011, Xiao et al., 2011) or inflammation (Cheadle et al., 2013). Taken together, the ENS might be considered “protective” for the intestinal barrier from the impairment chiefly through the regulation of EGCs.

### **V-3 The inflammation induced modification in enteric nervous system**

Likewise, EGCs essentially contribute to the interactions between the inflammation and the ENS. Pro-inflammatory stimuli IFN $\gamma$  and TNF- $\alpha$  activated the enteric glia via the elevation of GFAP and S100 $\beta$  expression coupled with the upregulation of c-fos level (von Boyen, 2004, Cirillo et al., 2011, Gougeon et al., 2013). Inflammatory mediators such as IL1 $\beta$  could reduce EGCs proliferation in a dose-way (Ruhl et al., 2001). Indeed, IL-1 receptor was activated in EGCs, reflecting the potential role of EGCs in inflammatory response (Stoffels et al., 2014). The density of enteric neurons also contributes to the severity of inflammation (Margolis et al., 2011). In addition, clinical studies found out that the expression of GFAP and S100 $\beta$  was strikingly higher in ulcerative colitis (UC) patients, emphasizing the role of EGCs or ENS in the mediation of inflammation (Cirillo et al., 2009, von Boyen et al., 2011).

### **V-4 The effects of gut microbiota on enteric nervous system**

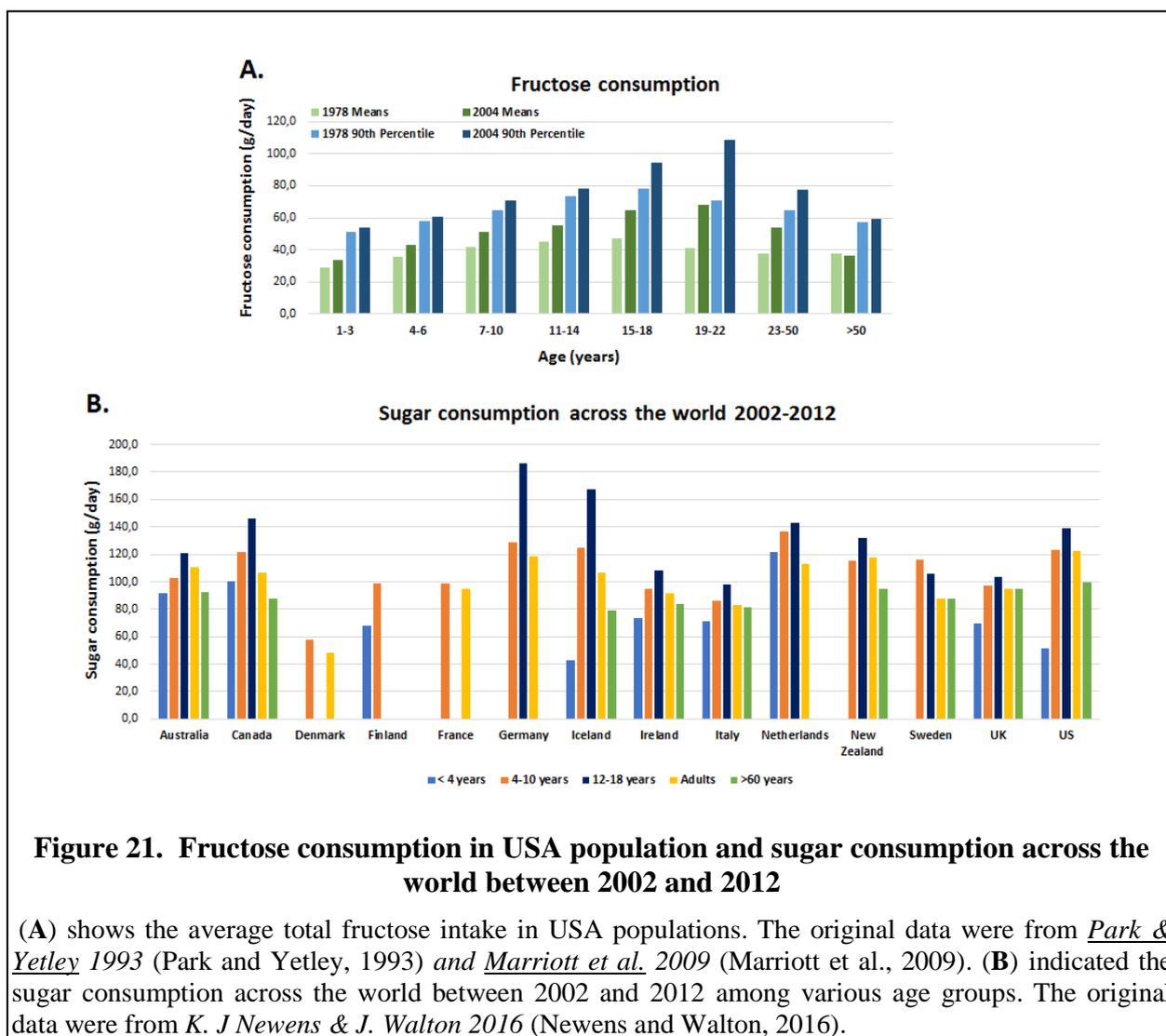
In recent decades, numerous studies illustrated the essential role of gut microbiota or microbial products/metabolites on the ENS, either enteric neurons or EGCs. For instance, in GF animals an abnormal myenteric plexus architecture has been described, characterized by a significant decrease in the number of myenteric neurons per ganglion in jejunum and ileum (Collins et al., 2014, Hyland and Cryan, 2016, Endres and Schafer, 2018). EGCs of the submucosal plexus are also less developed in GF mice (Kabouridis et al., 2015). Excessive use of antibiotics also induced a reduction of the number of enteric neurons in mice (Anitha et al., 2012, Kabouridis and Pachnis, 2015) which emphasizes the effect of gut microbiota on the homeostasis of ENS. In vitro studies showed that pathogens such as *E. coli* activated the EGCs via upregulation of c-

fos (a marker of neuronal activity) expression, and strikingly induced the overexpression of S100 $\beta$  suggesting the essential role of EGCs in host-bacteria interactions (Turco et al., 2014). Moreover, LPS, a major component of gram-negative bacteria, also systematically induced the upregulation of enteric markers GFAP and S100 $\beta$ , characterizing the activation of EGCs in the distal gut (da Cunha Franceschi et al., 2017). Microbial metabolites such as SCFAs directly or indirectly can modulate the ENS. The direct evidence indicated that SCFAs such as butyrate could induce the neuroplasticity and increase the proportions of enteric neurons in the gut (Hamodeh et al., 2004, Soret et al., 2010, Suply et al., 2012). The expression of SCFAs receptors including GPR41/FFAR3 in myenteric and submucosal neurons indirectly emphasized the interactions between gut microbiomes and ENS (Nohr et al., 2013). It has been shown recently that the gut microbiota could modify ENS maturation through an effect on serotonergic neurons (De Vadder et al., 2018). Overall, the microbiota or its major component, the microbial metabolites directly or indirectly interacted with the ENS through the mediation of EGCs or enteric neurons.

#### **V-5 The function of enteric nervous system in nutrients sensing**

The ENS has been identified as a peripheral target in response to the presence of multiple nutrients including fatty acids/ high fat, glucose, amino acids in the lumen. For instance, the infusion of the unsaturated fatty acid oleate elevated the expression of c-fos in myenteric and submucosal neurons in the proximal gut including duodenum and jejunum, but not ileum (Sayegh et al., 2004). Glucose perfusion induced the increase of c-fos expression is only in the myenteric neurons in the jejunum but in all three regions of submucosal neurons (Sayegh et al., 2004). Amino acids glutamate or glycine mostly increased the excitability of myenteric neurons (Liu et al., 1997, Neunlist et al., 2001). Although the activation of neurons is different in response to oleate, glucose or amino acids, the nutrients seem to act directly on the ENS. Yet, the effects of oleate on enteric neurons were also indirect, mediated by enteroendocrine CCK through the activation of the CCK1R located in the submucosal plexus (Gulley et al., 2005, Raboin et al., 2008). Since the enteric neurons express the SGLT1, the direct sensing of the ENS in response to glucose has been suggested (Balén et al., 2008). In addition, HFD suppressed the activation of enteric neurons (both myenteric and submucosal) during the oleate infusion test (Covasa et al., 2000), and also damaged the mucosal EGCs populations indicating the deleterious effects of HFD on the ENS functions (Stenkamp-Strahm et al., 2013). Chronic fructose consumption stimulated the impairment of submucosal plexus as well (Lowette et al.,

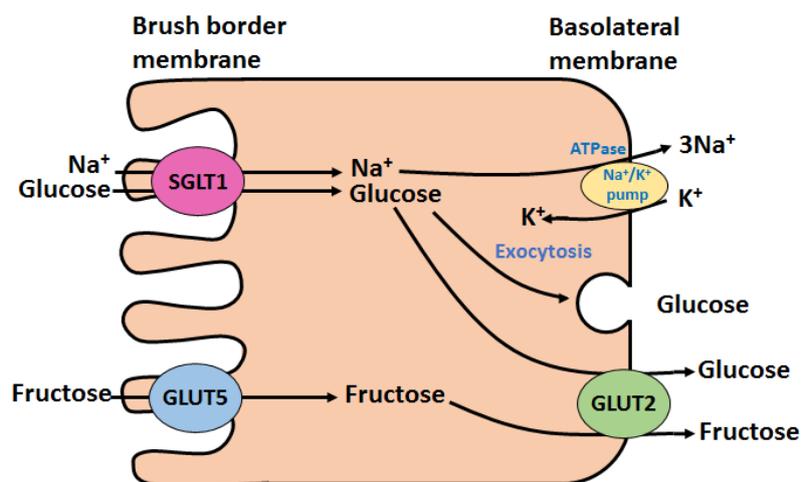
2016). Although direct or indirect evidence have been shown to support the role of nutrients on the function of enteric neurons and the deleterious effects of western diet consumption, the potential modifications of EGCs induced by nutrients such as glucose or fructose still need to be clarified.



## **VI- Fructose consumption, transport and metabolism – health consequences of its excessive consumption**

### **VI-1 The pattern of fructose consumption**

Fructose is a six-carbon ketonic monosaccharide (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) that has the same formula to glucose. Naturally, fructose is present in honey and most fruit, either as free fructose or in combination with glucose (as sucrose, table sugar) and is mainly responsible for the sweet taste. It is the most soluble and sweetest of all natural sugars. Fructose is widely used in the food and beverage industry because of its low price and relatively high sweetness. Since the 1970s, fructose has increasingly been consumed in developed countries due to the global increase in table sugar consumption and the advent of HFCS. Glucose and fructose are not bonded in HFCS, as they are in sucrose, which eliminates the requirement of hydrolysis by sucrase prior to its absorption by the small intestine, making fructose readily available for immediate absorption. For thousands of years, humans consumed < 5 g of fructose per day, coming mostly from fruit and honey (Douard and Ferraris, 2013). In the USA, mean total fructose consumption increased between 1978 (37 g/day) and 2004 (49 g/day) (Marriott et al., 2009, Powell et al., 2016). The top 10% of fructose consumers ingested approximately 75 g/day in 2004 compared to 64 g/day in 1978 (**Figure 21A**). This rise in fructose intake is not limited to the USA. However, precise fructose consumption data are often unavailable for many countries. For example, consumption reaches 35 to 60 g/day in the Netherlands (Sluik et al., 2015). Nevertheless, data on average total sugar intake is more frequently accessible. For adults, average total sugar intake ranged from 95 g/d in the UK, 102 g/d in Canada, 117g/d in New Zealand, to 118g/d in Germany and the US between 2004 and 2012 (Newens and Walton, 2016). The consumption of total sugar, added sugar, or fructose in the general population, as well as in the 90th percentile group of fructose consumers, is remarkably higher in the 15 to 22 age group relative to other age groups (**Figure 21B**) (Douard and Ferraris, 2013, Newens and Walton, 2016), which puts this age group at a greater risk for disorders associated with high fructose consumption.



**Figure 22. Intestinal transport of fructose and glucose**  
(Revised from (Wright et al., 2004))

SGLT1: Na<sup>+</sup>-glucose cotransport-dependent transporter.

## VI-2 Intestinal transport of fructose

### VI-2.1 The main intestinal fructose transporters GLUT5 and GLUT2

Fructose is mainly transported across the brush border membrane (BBM) via GLUT5 (Burant et al., 1992, Burant and Saxena, 1994, Douard and Ferraris, 2008, Cura and Carruthers, 2012) and subsequently exits enterocytes to enter the blood via GLUT2 in the basolateral membrane (**Figure 22**). Unlike glucose transported by SGLT1, fructose uptake by GLUT5 does not require ATP and is independent of sodium transport. GLUT5 and GLUT2 belong to the GLUT family, which is encoded by Slc2 genes and composed of membrane proteins primarily responsible for the transport of various monosaccharides, polyols, and small carbon compounds (Uldry et al., 2002, Mueckler and Thorens, 2013). Amongst the 14 members of the GLUT family, seven possess the ability to transport fructose (**Table 3**) (Manolescu et al., 2007b). Of these seven, GLUT5 is the only fructose-specific transporter, as it has no ability to transport glucose or galactose at physiological concentrations (Kane et al., 1997, Manolescu et al., 2005) whereas GLUT2 is can transport the three monosaccharides (glucose, fructose, and galactose) (Uldry and Thorens, 2004).

The  $K_m$  of GLUT5 for fructose ranges between 6 and 14 mM (Drozdowski and Thomson, 2006) which is the likely concentration found in the lumen of rats fed medium- to high-fructose diets (Jiang and Ferraris, 2001). GLUT5 is highly expressed in the brush border membrane of the small intestine and renal proximal tubule cells (Kayano et al., 1990, Rand et al., 1993). It is also modestly expressed in the brain, skeletal muscle, fat, bone marrow, and heart (Hajduch et al., 1998, Hajduch et al., 2003, Sasaki et al., 2004). However, the functions of GLUT5 in these organs remain unclear. Within the small intestine, the highest expression of GLUT5 is found in the duodenum >> jejunum > ileum (Lenzen et al., 1996). GLUT2 has a lower affinity ( $K_m \sim 76$  mM) for fructose and a higher one ( $K_m \sim 15$  mM) for glucose (Gould et al., 1991, Drozdowski and Thomson, 2006). In addition to its role in the exit of fructose from enterocytes (Cheeseman, 1993), GLUT2 is responsible for fructose entry into hepatocytes (Mueckler, 1994, Thorens, 2015) where fructose is primarily metabolized. GLUT2 is also one of the main glucose transporters, it is expressed in numerous tissues (pancreatic cells, brain, and kidney) (Leturque et al., 2009). But its role in fructose transport in these tissues is still uncertain due to its low affinity for fructose and the generally low fructose concentration in systemic circulation (Patel et al., 2015c). Within the intestine, GLUT2 expression is high from the duodenum to mid-

**Table 3. GLUT family proteins properties (Revised from (Manolescu et al., 2007a))**

Class	GLUT family	<i>K<sub>m</sub></i> (Oocyte)	Substrate Specificity	Special Feature	Tissue
<b>Class I</b>	GLUT1	5mM	Glucose/galactose		Ubiquitous, red cells
	<b>GLUT2</b>	76mM fructose/ 15mM glucose	Glucose/galactose /fructose	Mediates both uptake and efflux	Intestine, kidney, liver, beta-cell
	GLUT3 (GLUT14)	1mM	Glucose/galactose		Neurons
	GLUT4	5mM	Glucose	Trafficking regulated by insulin (LL motif)	Fat, muscle including heart
<b>Class II</b>	<b>GLUT5</b>	6-14mM	Fructose (glucose)	Found primarily in fructose-metabolizing tissues	Intestine, sperm
	GLUT7	0.3mM	Glucose/fructose	Apical membrane targeting	Intestine
	GLUT9a and 9b	0.3mM	Glucose/fructose	Liver expression co-localizes with GLUT2 but not kidney	Kidney BLM
	GLUT11	0.2mM	Glucose/fructose	Three isoforms (A, B, and C) with differing tissue distribution	Muscle, heart, fat, placenta, kidney, and pancreas
<b>Class III</b>	GLUT6 (GLUT9)	High <i>K<sub>m</sub></i>	Glucose?	Has LL targeting motif	Brain and spleen
	GLUT8	2.4mM	Glucose/(fructose )	Has LL targeting motif	Testis, brain, fat, liver, and spleen
	GLUT10	0.3mM	Glucose/fructose		Heart and lung
	GLUT12	4-5mM	Glucose/galactose /fructose	Has LL targeting motif	Insulin-sensitive tissues
	GLUT13	0.1mM	Myoinositol	Proton-coupled substrate movement	Brain

jejunum and then decreases in the distal jejunum to the ileum (Lenzen et al., 1996). GLUT2 can also rapidly translocate to the apical membrane of enterocytes (Gouyon et al., 2003, Cottrell et al., 2006, Caccia et al., 2007, Barone et al., 2009, Ait-Omar et al., 2011). This phenomenon has been observed in rodents in response to high glucose intake, leading to an increased luminal glucose concentration ( $> 1$  mM) and saturation of the glucose transporter SGLT1 (Gouyon et al., 2003). GLUT2 is an unsaturable transporter and thus its translocation to the apical membrane supports glucose transport across this membrane. The translocation of GLUT2 has also been observed in the small intestine of diabetic rats (Corpe et al., 1996), the jejunum of obese humans (Ait-Omar et al., 2011), and the small intestine of GLUT5 knockout (GLUT5<sup>-/-</sup>) mice (Barone et al., 2009). However, apical GLUT2 does not appear to play a major role in supporting apical fructose transport, as GLUT5<sup>-/-</sup> mice display a 99% reduction in fructose transport in the duodenum (Patel et al., 2015b).

### ***VI-2.2 Other potential transporters***

Other GLUT transporters that can potentially transport fructose include GLUT7, GLUT8, and GLUT12. GLUT7 is a newly discovered, facilitated hexose transporter present in the BBM of the intestine and can transport glucose and fructose (Cheeseman, 2008). It likely does not play a vital role in fructose and glucose transport, as its regional distribution in the small intestine does not match the corresponding luminal fructose and glucose concentrations (Cheeseman, 2008). Moreover, the physiological substrate of GLUT7 has not been yet identified (Manolescu et al., 2005, Cheeseman, 2008). GLUT8 and GLUT12 can also transport fructose (DeBosch et al., 2012). However, their expression, relative to that of GLUT5, is almost nonexistent in the small intestine (Patel et al., 2015a). Hence, even though there are many GLUTs that can transport fructose, GLUT5 appears to be the fructose transporter present in the BBM of the small intestine responsible for most fructose uptake by enterocytes.

### ***VI-2.3 The regulation of GLUT5 in intestinal tissue***

GLUT5 is a limiting factor of fructose absorption and its regulation has been intensively studied. Various physiological (age, circadian rhythm), hormonal (leptin, thyroid, glucocorticoids), pathological (diabetes, cancer, hypertension), dietary (fructose/glucose), and immune (TNF $\alpha$ ) factors have been shown to affect the expression and/or activity of GLUT5 in *in vivo* and *in*

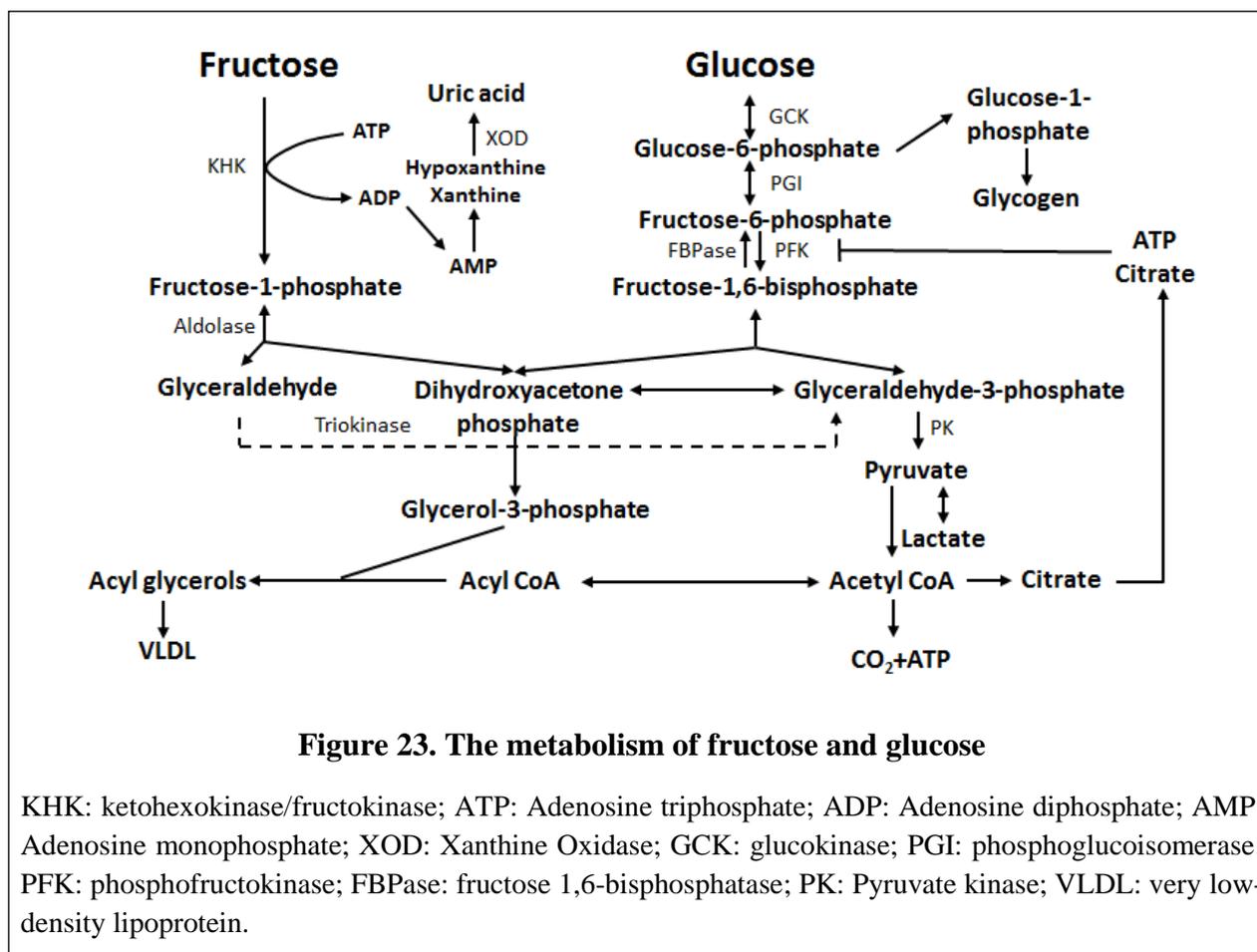
*vitro* studies (Tolozza and Diamond, 1992, David et al., 1995, Monteiro and Ferraris, 1997, Cui et al., 2004a, Sakar et al., 2009, Fanjul et al., 2012, Barrenetxe et al., 2013). We will describe here in detail the developmental regulation of GLUT5 and the effect of dietary fructose on its regulation.

#### ***VI-2.4 Developmental Regulation of GLUT5***

Unlike GLUT2 and SGLT1, which are expressed in the small intestine of newborn rodents and rabbits, GLUT5 expression remains very low at birth and increases only after the completion of weaning (at 28 days of age for rodents) (Buddington and Diamond, 1989, Tolozza and Diamond, 1992, Castello et al., 1995, Cui et al., 2003). This corresponds with a diet based on mother's milk, which contains lactose (dimers of galactose and glucose, both transported by SGLT1 and GLUT2) but not fructose, until the introduction of solid food. The increase in GLUT5 expression after weaning is believed to be hardwired, as it does not require the presence of fructose in the lumen. However, GLUT5 expression and activity can be induced early in the small intestine (between 14 and 28 days) by introducing fructose into the lumen (Cui et al., 2004b). However, GLUT5 expression and activity cannot be induced by luminal fructose during the suckling period (< 14 days of age) (Douard et al., 2008b). This highlights a clear difference in the way GLUT5 is regulated by fructose between the suckling and weaning periods of rat development. The striking developmental regulation of GLUT5 had been largely elucidated by demonstrating the major role of glucocorticoids and the glucocorticoid receptor in the regulation of GLUT5 expression (Douard et al., 2008b). Indeed, fructose-induced GLUT5 upregulation during the suckling period requires translocation of the glucocorticoid receptors to the nucleus and histone acetylation in the GLUT5 promoter regions (Douard et al., 2008a, Suzuki et al., 2011). This occurs naturally around 12 days of age, while a surge of corticosterone is observed in the plasma and primes the gut. The question that remains is that of the developmental regulation of GLUT5 in humans. Despite the absence of clear measurements of GLUT5 expression in the proximal intestine of babies, fructose malabsorption data by the hydrogen breath test support poor fructose intestinal absorption from birth to six to eight years of age (Jones et al., 2013). This aspect of fructose malabsorption will be detailed later in the introduction.

### ***VI-2.5 The regulation of GLUT5 by luminal fructose***

The mRNA level of SGLT1, an important apical glucose transporter, can be enhanced in the small intestine of rats by chronically feeding them high glucose, galactose, glycerol, xylose, mannose, or sucrose diets (Miyamoto et al., 1993, Dyer et al., 1997). Unlike SGLT1 regulation, GLUT5 expression in adults can only be induced by its substrate fructose (Ferraris and Diamond, 1997) and expression increases in response to increasing levels of luminal fructose (Ferraris, 2001). This ability of fructose to increase GLUT5 expression is specific as the expression of SGLT1 and GLUT2 are unaltered when mice are exposed to isocaloric diets enriched in either fructose or glucose (Gouyon et al., 2003, Douard et al., 2008b). This regulation is rapid, as GLUT5 mRNA levels increased within 2 h after the small intestine of adult rats was exposed to fructose (Kishi et al., 1999, Jiang et al., 2001). The upregulation of GLUT5 by its substrate involves *de novo* GLUT5 protein synthesis (Jiang and Ferraris, 2001, Douard et al., 2008a). The upregulation of GLUT5 expression and function can be accomplished by several methods that introduce fructose into the lumen: feeding, gavage, or *in vivo* perfusion (Douard et al., 2008a, Douard et al., 2008b, Douard et al., 2013, Patel et al., 2015a). However, the presence of fructose in the lumen is essential, as exposure of the basolateral side of enterocytes to fructose does not lead to GLUT5 induction (Cui et al., 2003). In enterocytes, the main pathway of fructose metabolism involves ketohexokinase (KHK or fructokinase), which is specific for fructose metabolism and highly expressed in enterocytes (Diggle et al., 2009). KHK is the first enzyme of fructose metabolism, converting fructose into fructose-1-P. Its role in fructose metabolism is described below and in **Figure 23**. Recently, the use of a KHK knockout (KHK<sup>-/-</sup>) mouse model demonstrated that the suppression of intracellular fructose metabolism prevents fructose-induced upregulation of GLUT5 in the small intestine (Patel et al., 2015a, Patel et al., 2015b). Thus, although basal GLUT5 expression permits a low level of fructose absorption, KHK<sup>-/-</sup> mice cannot adapt to increased luminal fructose concentrations. These mice have made it possible to uncover an essential missing element of the regulation of GLUT5 by fructose. They are also a good moderate fructose malabsorption model and were used for this reason in the experimental part of the present PhD project.

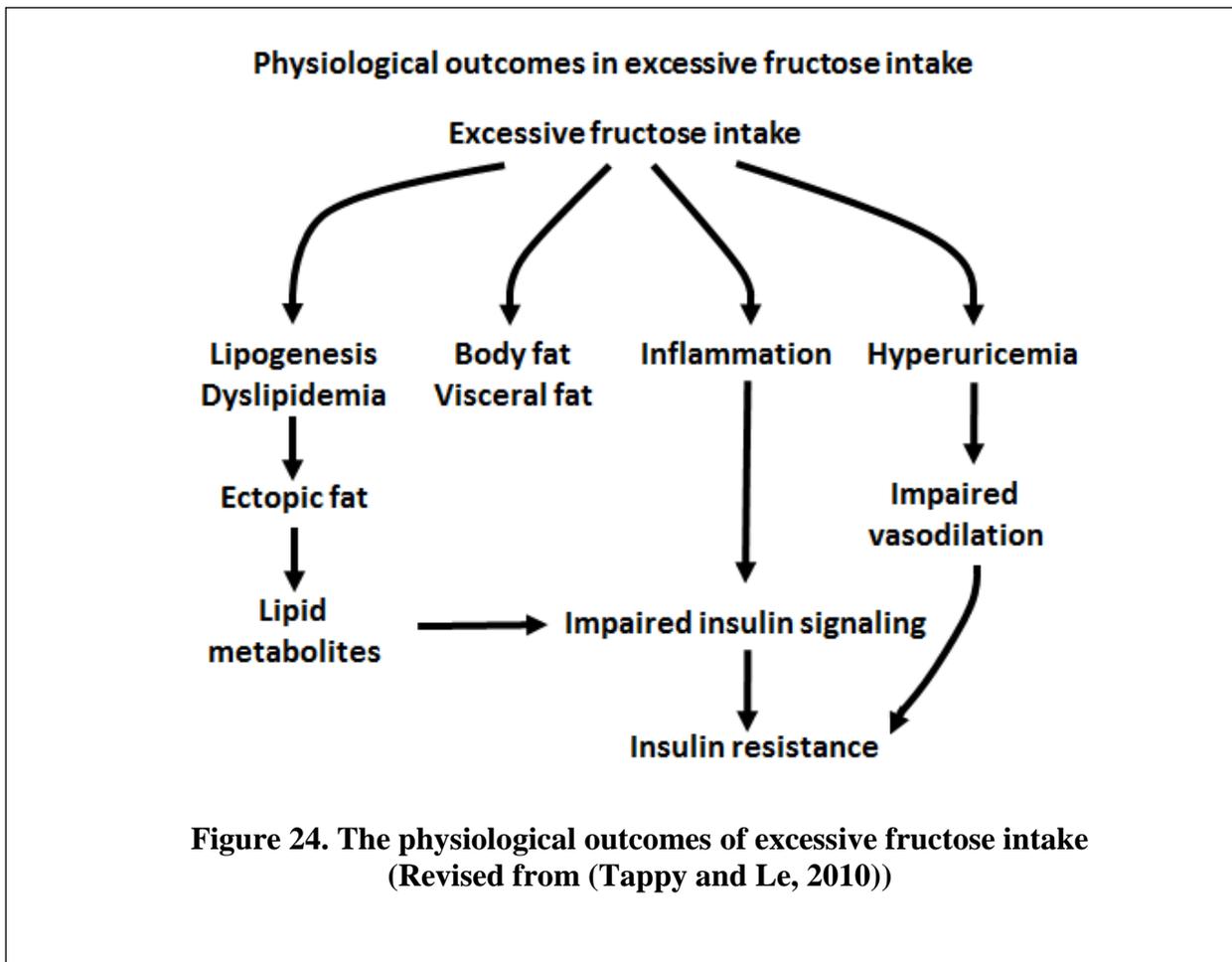


**Figure 23. The metabolism of fructose and glucose**

KHK: ketohexokinase/fructokinase; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate; AMP: Adenosine monophosphate; XOD: Xanthine Oxidase; GCK: glucokinase; PGI: phosphoglucoisomerase; PFK: phosphofructokinase; FBPase: fructose 1,6-bisphosphatase; PK: Pyruvate kinase; VLDL: very low-density lipoprotein.

### VI-3 Kethexokinese: the key enzyme in fructose metabolism

The main pathway of fructose metabolism involves KHK, aldolase, and triokinase (Mayes, 1993), which are specific for fructose metabolism (Rippe and Angelopoulos, 2013) and highly expressed in hepatocytes and intestinal and renal cells (Douard and Ferraris, 2013). As mentioned previously, KHK is the primary metabolizing enzyme of fructose in mammalian cells. KHK is mutually spliced into two isoforms A and C, which arose by an intragenic duplication (Asipu et al., 2003, Diggle et al., 2009). KHK-C is primarily found in hepatocytes, enterocytes, and renal proximal tubule and white adipose tissue cells and has the highest affinity for fructose of any hexokinase (Diggle et al., 2009). KHK-A is ubiquitously expressed, except in the gut, liver, and kidney (Diggle et al., 2009). As mentioned previously, the metabolism of fructose via KHK in enterocytes contributes to the regulation of GLUT5 expression and therefore controls intestinal fructose transport. After its intestinal absorption, fructose is directed to the liver via the portal vein, where it is transported by GLUT2 into hepatocytes and metabolized by KHK. Knocking out both KHK-A and KHK-C in mice abolishes fructose breakdown, leaving the sugar intact until it is cleared in the urine (Diggle et al., 2009). The metabolism of fructose is unique compared to that of glucose, as it bypasses a major regulatory check point, phosphofruktokinase (**Figure 23**), an enzyme inhibited by ATP and citrate, which allows regulation of glucose metabolism depending on the energy status of the cell. In addition, compared to the ubiquitous nature of glucose metabolism, fructose is primarily metabolized in the liver, as very little fructose reaches the systemic circulation. Lacking feedback inhibition like glucose, the conversion of fructose to fructose-1-phosphate results in the rapid accumulation of several metabolic intermediates (triose-phosphate (also known as di-hydroxy-acetone-phosphate or 3-phosphoglyceraldehyde)) and pyruvate or acetyl-CoA (Woods et al., 1970, van den Berghe et al., 1977). Subsequently, triose-phosphate is either converted into lactate (Miller et al., 2018) or glucose and glycogen through gluconeogenesis (Bode et al., 1981, Miller et al., 2018). Pyruvate and acetyl-CoA are used as precursors of fatty acid synthesis in hepatocytes through the process of *de novo* lipogenesis (Mayes, 1993, Tappy and Le, 2010). Such uncontrolled metabolism of fructose mediated by KHK is in part responsible for the adverse outcomes that arise from the chronic consumption of fructose.



## **VI-4 Health outcome of excessive fructose intake**

Initially, fructose was suggested to be useful for diabetic patients due to its low glycemic index (19 *versus* 100 for glucose). However, over the past decades, the use of fructose as a sweetener has become a controversial issue and although fructose and glucose contain the same number of calories, fructose is much more lipogenic than glucose (Delhotal-Landes et al., 1987). As an added sugar, fructose is now largely suspected to promote obesity, NAFLD, type 2 diabetes (T2D), dyslipidemia, hypertension, and other symptoms of metabolic syndrome, as well as fructose malabsorption and gout (Reed et al., 1994, Mate et al., 2004, Sanchez-Lozada et al., 2007, Brown et al., 2008, Stanhope et al., 2009, Dekker et al., 2010, Madero et al., 2011, Fan et al., 2014, Stanhope et al., 2015).

### ***VI-4.1 Liver: the central target***

The liver is the organ in which 90% of fructose is metabolized (Tappy and Le, 2010) and thus its functions are strongly affected by excessive fructose consumption. Both human and animal models have shown increased hepatic lipid content following the high intake of fructose or fructose-containing sweeteners (Lustig, 2010, Basaranoglu et al., 2013). This accumulation of lipids in the liver results from the accumulation of fatty acid synthesis precursors, described above, as well as fructose-induced synthesis of the sterol response element binding protein (SREBP1), which in turn upregulates the synthesis and activity of fatty acid synthase and other lipogenic enzymes (Denechaud et al., 2008, Koo et al., 2008). High fructose diets also markedly increase the plasma levels of very-low-density lipoproteins (VLDL) (Faeh et al., 2005), which increases the levels of circulating triglycerides and may contribute to fat accumulation in adipose tissue. This may contribute to the increase in visceral adiposity observed in humans after ten week's consumption of fructose-sweetened beverages (Stanhope et al., 2009). Leptin resistance is also apparent in rats fed 60% fructose for six months (Shapiro et al., 2008) and may result from the hepatic accumulation of lipids and increased adiposity (**Figure 24**).

### ***VI-4.2 Fructose intake and functional alteration of other organs***

Fructose increases the concentration of uric acid in liver cells in rats due to the rapid phosphorylation of fructose to fructose-1 phosphate and the subsequent increase in adenosine

monophosphate (AMP) concentrations (Reiser, 1985, Karczmar et al., 1989), which in turn increases the activity of rate-limiting enzymes in uric acid synthesis (**Figure 23**). This finding links fructose to various disorders associated with uric acid metabolism, including gout and kidney stones (Choi and Curhan, 2008, Taylor and Curhan, 2008). In addition, uric acid may also participate in the activation of lipogenesis in the liver (Kratzer et al., 2014). In the kidney, several studies in rats have linked excessive and chronic consumption of fructose with renal injury, as well as high blood pressure (Nakayama et al., 2010, Kretowicz et al., 2011). Fructose has also been observed to cause vascular inflammation and dysfunction in rats (Glushakova et al., 2008, Tan et al., 2008).

### **VI-5 Fructose malabsorption**

Here, we will not review hereditary fructose intolerance, which is an autosomal recessive disease caused by the deficiency of aldolase B (fructose-1, 6-bisphosphate aldolase), and is associated with a very severe outcome. We will focus our attention on nonhereditary fructose malabsorption. Fructose malabsorption is defined by an increase in hydrogen and/or methane levels above 20 ppm in the breath in response to a fructose load (Romagnuolo et al., 2002). Of note, the occurrence of GI symptoms defines fructose intolerance but not fructose malabsorption (Melchior et al., 2014). Despite the abundant literature connecting the excessive intake of fructose to the altered function of numerous organs, more than 50% of the adult population cannot fully absorb a 25g fructose load (Jones et al., 2011b). Thus, fructose malabsorption affects ~34% of healthy adults and its occurrence and severity, measured by positive breath hydrogen tests, correlated with the level of ingested dietary fructose (Gomara et al., 2008, Jones et al., 2011b). Interestingly, the prevalence of fructose malabsorption is higher in patients with functional GI disorders (Barrett et al., 2009, Melchior et al., 2014, Major et al., 2017). More importantly, toddlers and children display the greatest susceptibility to fructose malabsorption (Jones et al., 2011a), despite being the age group that consumes the highest amount of fructose (Douard and Ferraris, 2013, Svensson et al., 2014), likely because of the developmental regulation of GLUT5 described previously. Fructose malabsorption is not associated with low expression levels of GLUT5 or GLUT2 in the intestine (Wilder-Smith et al., 2014). However, the role of KHK, of which deletion in mice inhibits fructose absorption, has not yet been investigated in human fructose malabsorption. Fructose malabsorption is commonly associated with bloating, diarrhea, and visceral hypersensitivity, as well as depression (Putkonen et al., 2013). It has been suggested that distension of the small bowel and abdominal pain may result

from the remaining luminal fructose that attracts fluid into the lumen and increases intestinal motility (Spiller, 2006, Gomara et al., 2008, Major et al., 2017). Another hypothesis is that fructose accumulates in the distal GI tract and is fermented by colonic bacteria (Rumessen, 1992, Born et al., 1995). However, the fundamental mechanisms by which fructose leads to these symptoms are still unknown.



# **OBJECTIVES**

## OBJECTIVES

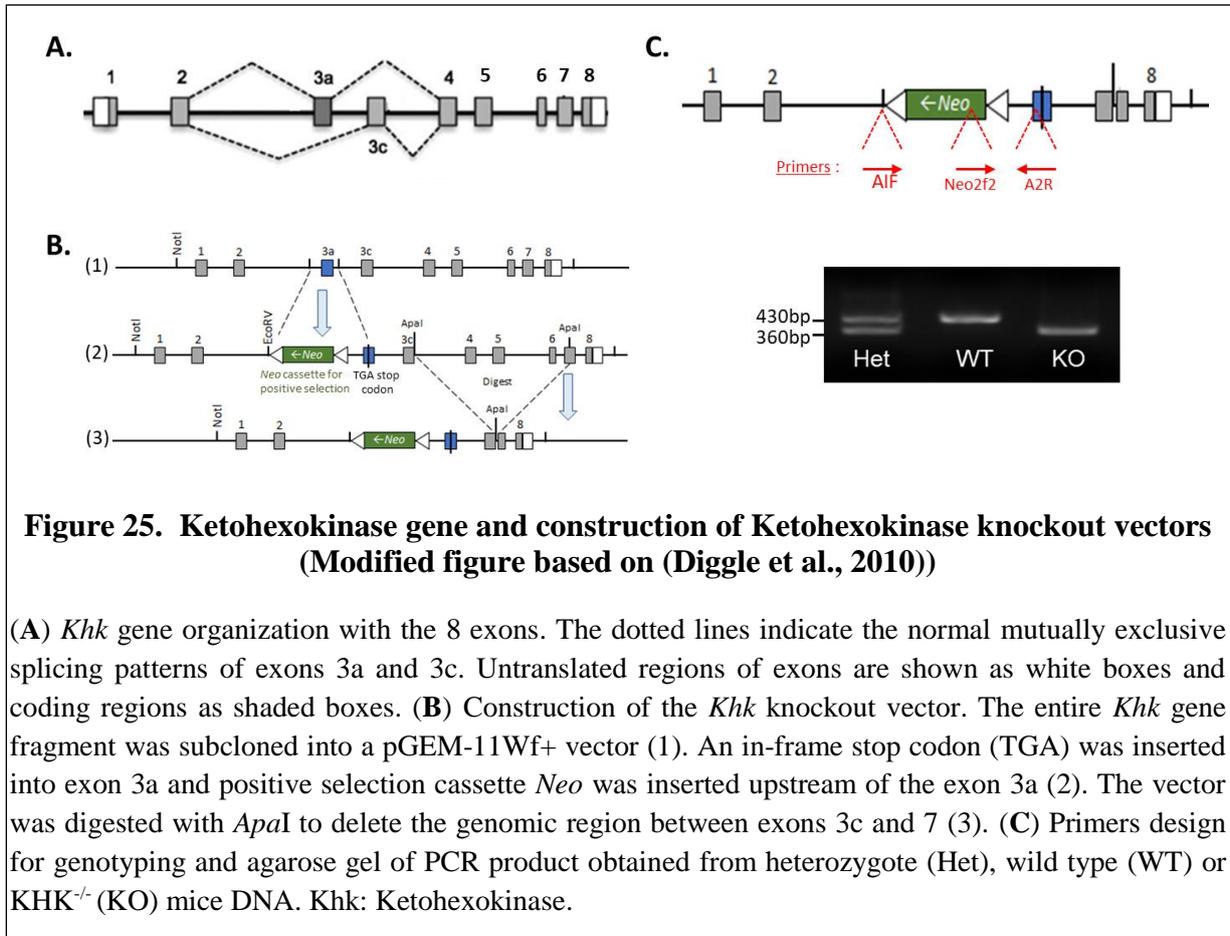
As described in the introduction, fructose consumption patterns in humans have dramatically changed over the past few. This is a result of unrestrained ability by the food industry to add sugars such as HFCS or sucrose to a variety of foods to an extent that now the per capita fructose consumption is 50-80 g/d in Unites States and in some European countries. Because fructose is relatively poorly absorbed in the small intestine (the fructose transport is 10 times less efficient than that of glucose), more than 50% of the adult population cannot fully absorb a 25 g fructose load, i.e. less than half of the average consumption. Moreover, both fructose absorption and consumption are age-dependent: although children under 10 years can least absorb it, they are the age group consuming the highest amounts of fructose. High fructose intake, together with fructose mal-absorption, likely leads to fructose overspill into the distal part of the intestine. There, fructose interacts with various epithelial cells of the intestinal mucosa and very likely with the bacterial population of the distal intestine. For a long time, gut was viewed mainly a nutrient absorbing organ. Recent insight, however, has generated an entirely new perspective suggesting that the homeostasis of the GI tract (including barrier functions, endocrine system, microbiota) plays a central role in the physiology of the entire body. The overall objective of my PhD was to determine whether fructose-induced changes in GI homeostasis regulate the outcomes resulting from chronic fructose intake in both, fructose malabsorption or normo-absorption, conditions.

Fructose malabsorption is associated with a wide range of symptoms: diarrhea, abdominal pain, or even anxiety. The fundamental mechanisms by which fructose leads to these symptoms have not yet been investigated. Mice models (ketoheokinase knockout,  $KHK^{-/-}$ ) of fructose malabsorption are now available. In the  $KHK^{-/-}$  mice, loss of KHK function prevents fructose-induced upregulation of fructose transporter GLUT5 and leads to a moderated malabsorption of fructose. Thus, the first part of the PhD explored the impact of fructose diet on the gut of the mice models of fructose malabsorption. We focused our attention on the mechanisms by which malabsorbed fructose reaching the distal part of the GI tract regulates its endocrine function. We also evaluated the impact of fructose malabsorption on gut inflammation, permeability and visceral pain and discuss their links with the endocrine changes.

Numerous studies focusing on HFD found a major role of fat-induced impairment of intestinal homeostasis (barrier defects, mucosal inflammation and dysbiosis) in the development of metabolic disorders. At the same time, the increase in consumption of sweets (mainly fructose-containing soft drinks) has also been tightly linked to the surge in metabolic diseases. Yet, the direct effect of fructose on the physiology and overall functionality of the GI tract had been poorly explored. Thus, the second objective of the PhD was to study the effects that chronic intake of fructose has on the different regions of the gut and on systemic metabolic parameters. Since intestinal barrier defects contribute to the development of obesity and metabolic disorders, and because the enteric nervous system controls the gut permeability and is a potential target of dietary and microbiota changes, we investigated the effects of fructose on intestinal permeability and on the ENS. To determine whether fructose-rich diets alter the gut bacterial profile (known as dysbiosis) we characterized interactions between sugar and the gut microbiota.



# RESULTS



## FRUCTOSE MALABSORPTION - KETOHEXOKINASE KNOCKOUT MICE MODEL

According to the introduction and objectives, KHK knockout mice is a good model for the study of fructose malabsorption. Before illustrating the results in this thesis project, we would like to give some background information about KHK knockout mice construction.

As mentioned previously, KHK is the primary enzyme that catalyzes the phosphorylation of fructose into fructose-1-phosphate in the intracellular metabolism pathway (Raushel and Cleland, 1977). In mice *Khk* genes are composed of nine exons (1-2, 3a, 3c, 4-8) in which exons 3a and 3c are mutually exclusively spliced into two distinct mRNA that encode for KHK-A and KHK-C isoforms, respectively (Hayward and Bonthron, 1998) (**Figure 25A**). Those two isoforms differ in their expression profile (see introduction § VI-3) and their affinity for fructose (Asipu et al., 2003, Ishimoto et al., 2012). KHK-C with a  $K_m$  of 0.8mM has a high affinity for fructose and is mainly expressed in liver, kidney and intestine. KHK-A has a lower affinity for fructose with a  $K_m$  of 8mM. KHK-A is expressed ubiquitously and represents the main isoform except in liver, kidney and intestine.

In 2010, Diggle, et al built a KHK knockout mouse models, named *Khk* $\Delta$  null homozygous type, that had a deficiency for both isoforms, KHK-A and KHK-C (Diggle et al., 2009). *Khk* $\Delta$  null mice were generated by engineering an in-frame stop codon into exon 3 and by inserting a neo cassette at the level of a EcoRV site 5' to exon 3a. Then a 3.6-kb genomic region between exons 3c and 7 was deleted after digestion with *ApaI* (see **Figure 25B** and (Diggle et al., 2009) for detailed vector construction). The *Khk* $\Delta$  null homozygous mouse type (names KHK<sup>-/-</sup> in the rest of the PhD manuscript) were mated with WT C57BL/6 mice for reproduction. Mouse generation began with pairing one KHK<sup>-/-</sup> males with WT females. Their heterozygous progeny was then bred to get the homozygote KHK<sup>-/-</sup> parents of the experimental animals. Genetic shift between WT and KHK<sup>-/-</sup> populations was minimized by the shared lineage. Pups were genotyped at 3 weeks old, with PCR on DNA extracted from 2-mm ear punches. PCR was conducted using the primers neo2f2 (CGGTAGAATTTTCGACGACCT), A2R (AGAATGTTGGCGGAGGTCA), and AIF (GGGAGGGGTCCAAAGTATTACC), in which neo2f2 is specific to knock-out mice, AIF is specific to WT mice, and A2R is common to both (**Figure 25B**). The PCR generate products of 360bp and 430bp for KHK<sup>-/-</sup> and WT

respectively (**Figure 25C**). The comparison between WT and *Khk* $\Delta$  mice revealed that the deletion of both, KHK-A and KHK-B, had no impact on the growth and development of the mice and displayed minimal biochemical abnormalities under basal dietary condition suggesting that when not exposed to fructose, both isoforms of KHK are not essential (Diggle et al., 2009).

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**FRUCTOSE MALABSORPTION MODIFIES THE ENDOCRINE RESPONSE OF  
THE LOWER INTESTINE BY MODULATING MICROBIOTA COMPOSITION  
AND METABOLISM**

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**Running title: Effects of fructose malabsorption in the lower intestine**

## **Abbreviations**

AB: Antibiotics; AIN: American Institute of Nutrition; BCFAs: Branched-chain fatty acids; CCK: Cholecystokinin; EECs: Enteroendocrine cells; FDR: False Discovery Rate; FFAR3: Free fatty acid receptor 3; FROGS: Find, Rapidly, OTUs with Galaxy Solution; GCG: Pre-proglucagon; GI: Gastrointestinal; GIP: Glucose dependent insulinotropic peptide; GLP-1: Glucagon-like peptide-1; GPCR: G-protein coupled receptors; KHK<sup>-/-</sup>: Kethexokinase knockout mice; LcdA: Lactoyl-CoA dehydratase subunit alpha; Logfc: Log-fold changes; Math1: Atonal bHLH transcription factor 1; MTC: Monocarboxylate transporters; Neurod1: Neuronal Differentiation 1; Neurog3: Neurogenin-3; NMR: Nuclear magnetic resonance; NTS: Neurotensin; O-PLS-DA: Orthogonal projection on latent structure-discriminant analysis; OTU: Operational taxonomic unit; Pax4: Paired Box4; Pax6: Paired box6; PCA: Principal Component Analysis; PCoA: Principal coordinates analysis; PYY: Peptide YY; SBS: short bowel syndrome; SCFAs: Short chain fatty acids; SCT: Secretin; SST: Somatostatin; TPH1: Tryptophan hydroxylase 1; WT: Wild type.

## ABSTRACT

We investigated the impact of fructose malabsorption on the endocrine function of the lower intestine and addressed the role of the microbiota in this process. To answer this question, a mouse model of moderate fructose malabsorption (ketoheokinase-knockout, KHK<sup>-/-</sup>) and WT littermates mice were used and received 20%-fructose (KHK-F and WT-F) or 20%-glucose diet. Cholecystokinin mRNA (*Cck*) and protein expression in the ileum and caecum increased dramatically in KHK-F mice as well as glucagon (*Gcg*) and neurotensin (*Nts*) mRNA expression in the caecum relative to the 3 other groups. In KHK-F, triple-label immunohistochemistry showed major upregulation of CCK in enteroendocrine cells (EECs) that were GLP-1<sup>+</sup>/PYY<sup>-</sup> in the ileum and colon, and GLP-1<sup>-</sup>/PYY<sup>-</sup> in the caecum. The caecal microbiota composition was drastically modified in the KHK-F in association with an increase in glucose, propionate, succinate and lactate concentrations when compared to the WT-F. Antibiotic treatment abolished fructose malabsorption-dependent induction of caecal *Cck* mRNA expression and, in GLUTag cells *Cck* mRNA expression levels increased in response to propionate; both suggesting a microbiota-dependent process. Fructose reaching the lower intestine can modify the composition and metabolism of the microbiota, thereby stimulating the secretion of CCK from the EECs possibly in response to propionate.

**Key words : CCK, microbiota, fructose, KHK, propionate**

## 1 INTRODUCTION

Fructose intake increased substantially over the past few decades, raising to the current per capita consumption of 50-80 g/day in the USA (2) and also increasing in most of the continents (3). Meanwhile, > 50% of the adult population cannot fully absorb a 25g fructose load (4). Moreover, despite being the age group that consumes the highest amount of fructose (1), children display the greatest susceptibility to fructose malabsorption (5). Fructose malabsorption is commonly associated with bloating, diarrhea, visceral hypersensitivity as well as depression (6). However, the fundamental mechanisms by which fructose leads to those symptoms remain unknown.

Under conditions of normal fructose intake, fructose absorption occurs mainly in the proximal intestine: in the duodenum and proximal jejunum. Transepithelial fructose transport is mediated by two members of the facilitative glucose transporter family, GLUT5 and GLUT2, which are expressed in the enterocytes and are localized to the apical and basal membranes, respectively (7, 8). GLUT5 expression is strongly up-regulated by luminal fructose in a ketohexokinase (KHK)-dependent manner and is essential for intestinal fructose transport (9, 10). Yet, intestinal transport of fructose is less efficient than glucose transport (11) and substantial fraction of fructose reaches the distal regions of the gut (ileum to colon) following excessive fructose intake (12).

Enteroendocrine cells (EECs) distributed along the gastrointestinal (GI) tract release gut peptides in response to luminal stimuli and regulate GI and peripheral physiological functions. Numerous studies have shown that changes in nutrient flow in the intestine can modify the endocrine function along the GI tract through modifications in the distribution or transcriptional activity of EECs. For instance, the number of glucagon-like peptide-1 (GLP-1)-, glucose-dependent insulinotropic polypeptide (GIP)- and peptide YY (PYY)-positive cells as well as the density of cholecystokinin (CCK)- and neurotensin (NTS)-positive cells increased in rodent models or in human patients after roux-en-Y gastric bypass (13-16). Animal models of short bowel syndrome (SBS), a strong malabsorption disorder resulting from the resection of the small intestine, exhibited major endocrine adaptations in the GI tract including elevated *Pyy* and *Gcg* transcription levels in the colon (17). Interestingly, the transfer of SBS patient microbiota into germ-free rats increased the density of GLP-1-positive cells in the colon of recipient animals when compared to rats colonized with conventional

microbiota, emphasizing the role of the intestinal ecosystem in the intestinal EECs proliferation and differentiation (18). Numerous studies have highlighted the ability of microbiota-derived products to regulate *Gcg* expression, GLP-1 or PYY secretion or L-cell (EECs cells secreting GLP-1 and PYY) transcriptional activities (19) (20, 21). However, while it is now well established that most of the EECs secrete more than one peptide (22), the ability of the microbiota to modulate specifically the panel of secreted hormones in individual EECs remains unclear.

Recently, the use of KHK knockout ( $KHK^{-/-}$ ) mouse model demonstrated that although basal GLUT5 expression permits low levels of fructose absorption, the  $KHK^{-/-}$  mice, cannot adapt to increased luminal fructose concentration (9, 23). Therefore, they represent a good model for moderate fructose malabsorption. Here, we used the  $KHK^{-/-}$  mice to investigate the impact of fructose malabsorption on the endocrine function of the lower intestine regions (ileum, caecum as well as colon) and to study the role of the intestinal microbiota in mediating fructose effects. We found that fructose reaching the distal regions of the GI tract is able to modify the panel of peptides secreted by the EECs of the ileum, caecum and colon (normally dedicated to the secretion of PYY and GLP-1), particularly by stimulating the transcription and secretion of CCK. We showed that this effect is, at least in part, mediated by the fructose-induced changes in the microbiota populations and metabolism in the lower intestine.

## 2 MATERIALS AND METHODS

### 2.1 ANIMALS

The present protocol received written agreement from the local animal ethics committee (COMETHEA at Jouy-en-Josas, France, APAFIS#1620-2015102618572930v2).  $KHK^{-/-}$  (background: C57/BL6) mice were obtained from RP Ferraris, Rutgers University of New Jersey with permission from R Johnson (24). The mice were a global KHK knock out and were lacking both isoforms, A and C, of the ketohexokinase as previously described (25). All  $KHK^{-/-}$  and wild type (WT) mice were littermates. Only males were used and all feeding experiments started with mice aged from 4 to 7 weeks.

### 2.2 EXPERIMENTAL DESIGN

In the first experiment, WT and KHK<sup>-/-</sup> mice (6-8 mice/group) received for 8 weeks, either 20% of fructose (WT-F and KHK-F groups respectively), or 20% of glucose (WT-G and KHK-G) experimental iso-caloric diets which were based on a standard American Institute of Nutrition (AIN)-93G formula (Research Diets, New Brunswick, NJ) and contained in addition to starch, either 20% fructose or 20% glucose respectively.

In the second experiment, same aged chow-fed WT and KHK<sup>-/-</sup> mice were divided into 2 groups (5-7 mice/group) of similar body weight and received for 7 days either plain water or antibiotic (AB) mix in their bottle. The AB mix was composed of vancomycin (45µg/mL), streptomycin (1mg/mL), colistin (1mg/mL) and ampicillin (1mg/mL). The mice were individually housed. Body weight and food/drink intake measurements as well as AB mix and litter changes were done every 2 days. After the first 7 days, the antibiotic treatment was maintained and both antibiotics and control water groups received 20% fructose diet for 3 weeks. The shortening of this fructose feeding period to 3 weeks was first validated by showing that the effects on peptide expression levels and the microbiota composition were similar after 3 or 8 weeks of fructose feeding (**Fig.S1A and 1B**).

For all experiments, non-starved mice were euthanized under anesthesia. Blood was collected at the level of the portal vein. Caecum was sampled and weighed. The entire small intestine and colon were sampled and flushed with cold PBS. Sections of 1 cm of duodenum, mid-jejunum, ileum and proximal colon were stored with RNAlater at -80°C until RNA extraction or fixed in 4% paraformaldehyde for immunohistology. The caecal content was sampled and stored at -80°C for bacterial DNA and metabolite analysis. The caecum epithelium was flushed sectioned and stored as described above for RNA extraction, immunohistology or CCK content analysis.

### **2.3 TISSUE RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (qRT-PCR)**

mRNA from duodenum, jejunum, caecum and colon sections was extracted using MirVana isolation kit (Invitrogen, ThermoFisher Scientific, Villebon-Sur-Yvette, France). 2µg of total RNA were reverse transcribed using High Capacity Complementary DNA Reverse Transcription Kit (Applied-Biosystems, ThermoFisher Scientific, Villebon-Sur-Yvette, France). To quantify gut peptide mRNA, qRT-PCR using SybrGreen chemistry was performed (Life Technologies, ThermoFisher Scientific, Villebon-Sur-Yvette, France). β-

actin housekeeping gene was used to normalize the mRNA abundance. Primers are listed in **Table S1**.

## 2.4 IMMUNOHISTOCHEMISTRY

Ileum, caecum and colon were fixed in fresh 4% paraformaldehyde in PBS (pH 7.3) overnight at 4°C and then embedded in paraffin. The 5µm sections were obtained. After paraffin removal, heat-induced antigen retrieval was performed in 10mM tri-sodium citrate buffer (pH 6.0) for 40 min at 96°C. The sections were rinsed in PBS and blocked in 1/100 mouse IG blocking reagent (Vector Laboratories, Burlingame, USA) diluted in 1% normal Donkey serum for 1 hr at room temperature. The sections were then incubated with primary antibodies (**Table S2**) for 16 hrs at 4°C, and after PBS rinses, the slides were incubated with the appropriate secondary antibodies for 1 hr at room temperature. Stained sections were imaged using laser scanning confocal microscopy (Leica, SP8). All sections stained with the same marker combination were imaged using the same settings. Non-specific staining with secondary antibodies was consistently negligible. For counting cells, the immune-stained sections were scanned using high-capacity digital slide scanner (Pannoramic SCAN, 3DHISTECH, Budapest, Hungary) and images were analyzed using CaseViewer software. The counts were performed blindly. Counts were based on averages of two to four intestinal cross-sections from each limb for each animal. For each section, the surface of the tissue section was measured and the data are presented as the number of cells/mm<sup>2</sup>.

## 2.5 CAECAL CONTENT ANALYSIS

CCK peptide concentrations in caecal tissue extracts were measured using a specific “in-house” radioimmunoassay as previously detailed (26).

The caecal contents from WT and KHK<sup>-/-</sup> mice fed fructose with or without antibiotics were analyzed for short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) by gas chromatography as previously described (27).

Caecal contents were analyzed by Nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR). The detailed protocol for sample extraction and spectra acquisition is described in the **Supplementary Material and Methods**.

## 2.6 MICROBIOTA DNA EXTRACTION, QRT-PCR QUANTIFICATION

All bacteria DNA was extracted from caecum content using GNOME DNA isolation kit (MP Biomedicals, Strasbourg, France). Quantifications of the total bacteria DNA and specific phyla were performed on 10ng of DNA by qRT-PCR following the procedure described previously (28) and detail in **Supplementary Materials and Methods**.

## **2.7 16S rRNA SEQUENCING**

V3-V4 region of the 16S rRNA genes was amplified using MolTaq (Molzym, Plaisir, France) and the primers V3F: TACGGRAGGCAGCAG and V4R: ATCTTACCAGGGTATCTAATCCT (29). Purified amplicons were sequenced using the MiSeq sequencing technology (Illumina) at the GeT-PLaGe platform (Toulouse, France). Paired-end reads obtained from MiSeq sequencing were analyzed using the Galaxy-supported pipeline named FROGS (Find, Rapidly, OTUs (operational taxonomic units) with Galaxy Solution) (30). For the preprocessing, reads with length  $\geq 380$  bp were kept. The clustering and chimera removal tools followed the guidelines of FROGS (30). Assignment was performed using SILVA 16 S. OTUs with abundances lower than 0.005% were removed from the analysis (31).

## **2.8 GLUTAG CELL CULTURE AND PEPTIDE mRNA EXPRESSION**

The GLUTag cell line was kindly provided by D. Drucker (Mount Sinai Hospital, Toronto, Ontario, Canada). Cells were kept at 37°C, 5% CO<sub>2</sub>, 95% humidity until 80% confluent, then split and transferred to fresh T75 flasks. Cell medium composition was DMEM with 1g/L glucose (Gibco, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL) (PS). For the assay, cells were plated onto 6-well plates precoated with matrigel basement membrane (BD Biosciences, Rungis, France) 24-48h before the study with the same medium as for the maintenance of the cell line. For the experiments, cells were incubated in no glucose DMEM (Gibco, ThermoFisher Scientific, Villebon-Sur-Yvette, France) supplemented with 1% FBS, 1% PS under 1mM glucose as a control, 25mM glucose, 25mM fructose, 10mM lactate, 2mM propionate or 2mM succinate for 24 h. Then, the cells were trypsinized and the cell pellets were used for mRNA extraction with TRIzol (Ambion, Life Technologies, ThermoFisher Scientific, Villebon-Sur-Yvette, France) following the manufacturer protocol. These experiments were repeated 3 times and for each experiment, each condition was done in triplicate. Reverse Transcription and qRT-PCR were performed as described above.

## **2.9 STATISTICAL ANALYSIS**

Statistical analysis was performed with GraphPad Prism software (v5; La Jolla California), and data were expressed as means  $\pm$  SEM. For all the means obtained from in vivo experiments with more than two groups, one-way ANOVA were performed followed by Tukey's post-hoc test after initial ANOVA showed significant effects. For means of two groups, a t-test was performed. Means of peptide expression data from GLUTag were compared by Kruskal-Wallis test followed by Dunn's multiple comparison test.

16S sequencing data were analyzed using the Phyloseq (29), ggplot2 (32) R packages in additions to custom scripts. Samples were rarefied to even sampling depths before computing within-samples compositional diversities (observed richness and Inverse Simpson) and between-samples compositional diversity (Bray-Curtis). Principal Coordinate Analysis was also performed on Bray-Curtis dissimilarities to obtain a 2D representation of the samples. Raw, unrarefied OTU counts were used to produce relative abundance graphs and to find taxa with significantly different abundances in KHK-F and WT-F. A negative binomial model was fit to each OTU, using DESeq2 (33) with default parameters, to estimate abundance log-fold changes (logfc). p-values were corrected for multiple testing using the BH procedure to control the False Discovery Rate (FDR) and significant OTUs were selected based on effect size ( $|\logfc| > 3$ , corresponding to an 8 folds increase or decrease in abundance), adjusted p-value ( $< 1e-3$ ) and prevalence (relative abundance  $> 0.1\%$  in at least half the samples).

$^1\text{H-NMR}$  data were mean-centered prior to analysis using Principal Component Analysis (PCA) and orthogonal projection on latent structure-discriminant analysis (O-PLS-DA) as previously described (34) and detailed in **Supplementary Materials and Methods**.

### 3 RESULTS

#### 3.1 FRUCTOSE MALABSORPTION IMPACTS INTESTINAL PARAMETERS

As expected, in the WT mice, fructose diet (WT-F) induced a significant up-regulation of *Glut5* expression in the jejunum compared to the mice fed glucose diet (WT-G) (**Fig. S2A**). The deletion of *KHK* gene prevented this induction of *Glut5* expression by fructose. In the *KHK*<sup>-/-</sup> mice, fructose feeding (KHK-F) was associated with a significant increase in the caecum weight and an enlargement of caecum epithelium relative to the glucose-fed *KHK* mutants (KHK-G) or the WT-F or WT-G (**Fig. S2B**). Morphological changes in the ileum and caecum tissues were also observed such as significant increase in villus lengths in the ileum and crypt+villus lengths in the caecum of the KHK-F compared to the WT-F (**Table S3**). No differences in the colon crypt depth were observed. At the animal level, neither the deletion of *KHK* nor the fructose diet affected body weight, daily food intake or epididymal adiposity (**Table S4**).

#### 3.2 FRUCTOSE MALABSORPTION IMPACTS GUT PEPTIDE EXPRESSION PATTERN ALONG THE GI TRACT

Previous studies demonstrated that changes in nutrient flux could modify gut peptide expression in specific areas of the gut (13-16). Thus, we investigated the impact of fructose malabsorption on the expression pattern of the main gut peptides along the GI tract. In the duodenum and jejunum, no significant changes in the gut peptide expression were observed among the 4 groups except for a mild decrease in *Pyy* mRNA expression in the jejunum of KHK-F when compared to WT-F and WT-G (**Fig. 1A and B**). In contrast, in the ileum and the caecum, fructose malabsorption was associated with major changes in peptide mRNA expression (**Fig. 1C and D**). The expression levels of *Cck* mRNA was 5-fold and 9-fold greater in the ileum and caecum respectively of KHK-F than in the 3 other groups of mice. The expression of *Gcg* was 1.8-fold and 3-fold higher in the ileum and caecum respectively of KHK-F compared to the 3 other groups. While *Nts* transcript levels were similar in the ileum in the 4 groups, its expression was up-regulated 6-fold in the caecum of KHK-F when compared to the 3 other groups. More modest increases in *Pyy* (1.6-fold) and *Sst* (encoding for somatostatin) (1.7-fold) mRNA expression were also measured in the caecum of KHK-F

mice compared to the KHK-G and WT-F group. In the colon, a more variable expression was observed for most of the peptides (**Fig. 1E**) and only *Pyy* mRNA expression was significantly higher in the KHK-F when compared to WT-G mice. *GIP*, *Sct* (encoding for secretin) and *Tph1* (encoding for tryptophan hydroxylase 1, an enzyme of the serotonin synthesis pathway) mRNA expression levels did not change in response to fructose or fructose malabsorption in any of the GI segments.

### 3.3 FRUCTOSE MALABSORPTION IMPACTS CCK-POSITIVE CELL TYPE POPULATION IN THE LOWER INTESTINE

The L-cells can synthesize GLP-1 (expressed from *Gcg* gene) and PYY. These cells are mainly localized in the lower intestine (ileum, caecum and colon) while CCK is normally synthesized by the I-cells, mainly localized in the duodenum and the proximal jejunum. However, recent studies demonstrated that more than one peptide can be expressed in each L- or I-cells (35). To determine whether the changes in peptide expression were supported by an increase in EEC number and/or a shift in the pattern of peptide expression in existing cells, we quantified the number of CCK-, GLP-1- and PYY-positive cells (CCK<sup>+</sup>, GLP-1<sup>+</sup> and PYY<sup>+</sup>) in the ileum, caecum and colon of the KHK-F and WT-F mice. Triple labeling by immunohistochemistry of CCK, GLP-1 and PYY was also performed to determine which cell type supports the drastic CCK increase in KHK-F. The density of CCK<sup>+</sup>-cells increased significantly in the ileum, caecum and colon of KHK-F relative to WT-F mice (**Fig. 2A**). Importantly, the increased *Cck* mRNA levels and CCK<sup>+</sup>-cell density translate to a 7-fold increase in the CCK peptide content in the caecal tissue of the KHK-F when compared to the WT-F (**Fig. 2D**). In the ileum, fructose malabsorption was also associated with a significant increase in GLP-1<sup>+</sup>-cell density (**Fig. 2B**) while the PYY<sup>+</sup>-cell density significantly decreased (**Fig. 2C**). No change in density was observed in the caecum and colon for GLP-1<sup>+</sup>- and PYY<sup>+</sup>-cells. The increase of CCK<sup>+</sup>-cell density in the ileum, caecum and colon in the KHK-F compared to WT-F is illustrated in **Fig. 2E-G**.

Triple staining used to investigate the colocalization of CCK, GLP-1 and PYY showed four cell types of CCK-immunoreactive cells in the distal GI tract: CCK<sup>+</sup>-cells co-expressing neither GLP-1 nor PYY (CCK<sup>+</sup>/GLP-1<sup>-</sup>/PYY<sup>-</sup>)(**Fig. 3A**), CCK<sup>+</sup>-cells co-expressing PYY but not GLP-1 (CCK<sup>+</sup>/GLP-1<sup>-</sup>/PYY<sup>+</sup>) (**Fig. 3B**), CCK<sup>+</sup>-cells co-expressing GLP-1 but not PYY

(CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>-</sup>) (**Fig. 3C**), and CCK<sup>+</sup>-cells co-expressing both, GLP-1 and PYY (CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>+</sup>) (**Fig. 3D**).

In the WT-F the predominant CCK<sup>+</sup>-cell population in the ileum, caecum and colon was the triple-positive cells (CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>+</sup>) (**Fig. 3E-G and Table S5**). In the KHK-F the density of this cell type remained the same as in WT-F (**Fig. 3E-G**). Rather, the expansion of the CCK<sup>+</sup>-cell population was due to density changes in other types of CCK<sup>+</sup>-EEC types. In the ileum and the colon, this expansion was primarily due to the increased density of CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>-</sup>-cells, while in the caecum it was almost entirely due to a drastic increase in density of CCK<sup>+</sup>/GLP-1<sup>-</sup>/PYY<sup>-</sup>-cells (**Fig. 3E-G**).

Differentiation of intestinal progenitor cells in the enteroendocrine lineage involves coordinated expression of various transcription factors. We tested a number of them for changes in expression (**Fig. S3**). *Neurod1* (Neuronal differentiation 1), a late marker for the differentiation of intestinal progenitor cells in the EEC lineage, was significantly up-regulated in the caecum of the KHK-F when compared to WT-G mice. In addition, *Pax6* (Paired box 6) which acts downstream of *Neurod1* and is a key transcription factor for GLP-1-positive cell differentiation was significantly upregulated in the caecum of the KHK-F when compared to the other 3 groups.

### 3.4 FRUCTOSE MALABSORPTION IMPACTS THE CAECAL MICROBIOTA COMPOSITION AND METABOLISM

Our data supports the idea that malabsorbed fructose reaches the lower intestinal tract. This could potentially affect the local microbiota populations. To address this possibility, we investigated the caecal microbial composition based on the sequencing of V3-V4 regions of microbial 16S rRNA. At the OTU level, no significant change in richness was observed among the 4 groups of mice (**Fig. 4A**), although the inverse Simpson index indicated a mildly significant higher number of effective taxa in the KHK-F mice compared to WT-G (**Fig. 4B**). Those differences were confirmed by  $\beta$ -diversity analysis based on the Bray-Curtis dissimilarity, which revealed a strong modification of the caecal microbial community in the presence of fructose malabsorption (**Fig. 4C**). The relative abundance of the 5 main phyla, Actinobacteria, Bacteroidetes, Deferribacteres, Firmicutes and Proteobacteria, was strongly affected by fructose malabsorption while the deletion of KHK by itself or the fructose feeding in WT mice had a very modest impact on the microbial community composition (**Fig. 4C-D**).

At the phylum level, in KHK-F the relative abundance of the Bacteroidetes increased while the relative abundance of Firmicutes slightly decreased in comparison with the other 3 groups. The impact of fructose malabsorption was striking at the level of minor phyla with a marked relative enrichment of Actinobacteria and a major decrease of the relative abundance of the Proteobacteria and the Deferribacteres (**Fig. 4D**). The differences in Actinobacteria and Proteobacteria were validated by qRT-PCR (**Supplementary data Fig. S1B**). At lower taxonomic levels, main differences were also observed when comparing KHK-F mice to the 3 other groups. Within Actinobacteria, at the family level, KHK-F mice displayed a major increase in the relative abundance of *Coriobacteriaceae* and *Corynebacteriaceae*, this latest family being almost undetectable in the 3 other groups (**Fig. 4E**). Within Bacteroidetes, we observed a higher abundance of *Bacteroidaceae* and *Bacteroidales*, and a decreased abundance of *Rikenellaceae* in KHK-F mice when compared to the 3 other groups (**Fig. 4F**). Within Firmicutes, despite no overall change in relative abundance of the two major families, *Lachnospiraceae* and *Ruminococcaceae*, at the genera and species level, the relative abundance of members of both families were strongly increased in the KHK-F when compared to WT-F (**Fig. 4J**). In addition, relative abundances of *Lactobacillaceae* increased drastically in KHK-F (**Fig. 4G**) when compared to the 3 other groups. Differential abundance analysis at the OTU level revealed that this enhancement in the family of *Lactobacillaceae* is largely supported by increase in the relative abundance of *Lactobacillus johnsonii* (**Fig. 4J**). Within Deferribacteres, the decrease observed was supported by a drop in *Deferribacteraceae* family (**Fig. 4H**). In line with the observations at the level of Proteobacteria phylum, the abundance of *Desulfovibrionaceae* family was drastically reduced in the KHK-F as the result of a major decrease in *Desulfovibrio simplex* and despite greater *Desulfovibrio desulfuricans* abundance (**Fig. 4I-J**). Differential abundance analysis identified 25 highly significant species or OTUs (adjusted  $p < 1e-3$ ) that were both strongly (fold change  $>8$ ) positively differentially abundant between KHK-F and WT-F (**Fig. 4J**) and very prevalent (abundance  $> 0.1\%$  in at least half the samples). Among them, 6 species or OTUs exhibited a fold change  $>1000$ , meaning that they were almost exclusively found in KHK-F mice. Similarly, 17 species or OTUs had highly reduced abundance (fold change  $< 1/8$ ) in KHK-F relative to WT-F.

These changes in the composition of the bacteria community were associated with changes in the metabolic activity of the gut microbiota, as first assessed using gas chromatography (**Fig. 5A**). Among the major SCFA detected, the amount of propionate increased by 50% in the caecum content of the KHK-F when compared to the WT-F. Otherwise only minor SCFA (*e.g.*

valerate) or branched SCFA such as isobutyrate and isovalerate displayed a lower concentration in KHK-F than in WT-F.  $^1\text{H-NMR}$  based metabolic profiling of the same caecal extracts confirmed a very strong shift in the metabolic activity of the microbiota upon fructose malabsorption as illustrated by the separation of the caecal metabolic profiles of WT-F vs KHK-F in the PCA plot (**Fig. 5B**). A supervised PLS-DA model was then fit to unravel the metabolites significantly changed between the 2 groups (**Fig. 5C**). The most prominent discriminating signals belong to glucose, which was strongly increased in KHK-F when compared to WT-K mice (**Fig. 5C and D**).  $^1\text{H-NMR}$  also confirmed the significant increase in propionate levels detected by gas chromatography and revealed the increase in succinate in the KHK-F when compared to WT-F (**Fig. 5C and D**). A number of other metabolites were also higher in KHK-F mice than WT-F, such as lactate, alanine, uracil, fumarate and hypoxanthine. One bile acid signal and other signals belonging to unidentified metabolites were lower in KHK-F than in WT-F mice (**Fig. 5D**). Of note fructose itself was not detected in the caecum of KHK-F or WT-F.

### 3.5 ANTIBIOTICS PARTIALLY REVERSE THE FRUCTOSE MALABSORPTION EFFECTS ON PEPTIDE EXPRESSION IN THE LOWER INTESTINE.

Since fructose feeding alters the gut microbiota in KHK<sup>-/-</sup> mice, we treated them with antibiotics to test whether a reduced or modified bacterial population would prevent or cause further changes in expression of peptides in the distal GI tract in response to fructose malabsorption. The antibiotic treatment did not affect body weight, food consumption or drink intake in the WT or KHK<sup>-/-</sup> mice fed fructose when compared to water (no antibiotic) controls (**Fig S4A-C**). However, 4 weeks of antibiotic treatment drastically reduced the total amount of bacteria in the caecum as the result of a major decrease in the main four phyla (**Fig. S4D**). In the ileum, the antibiotic treatment, independently of the KHK status, had no significant effect on the expression of any of the peptides except for a mild decrease in *Sst* mRNA levels (**Fig. 6A**). In the KHK-F+AB mice, antibiotic treatment partially prevented the KHK mutation-dependent increase in *Cck* expression in the ileum but had no effect on *Gcg* expression when compared to KHK-F (**Fig. 6A**). In the caecum, the antibiotic treatment by itself (WT-F+AB) had a significant positive effect on the expression of *Nts* and *Gcg* (**Fig. 6B**). Antibiotic treatment reduced, by 50% and 40%, respectively, the upregulation of *Cck* and *Nts* in the caecum of KHK-F mice leading to no significant differences between WT-F+AB and KHK-F+AB. The antibiotic treatment in KHK-F+AB abolished completely the increase in *Sst*

expression observed in the KHK-F group but had no effect on *Gcg* expression level. In the colon, expression demonstrated greater variability and no differences were found (**Fig. 6C**).

### 3.6 ANTIBIOTIC TREATMENT PARTIALLY PREVENTED FRUCTOSE MALABSORPTION-INDUCED CHANGES IN METABOLITES OF THE CAECAL CONTENT

Both antibiotic groups (KHK-F+AB and WT-F+AB) had almost undetectable SCFA contents except for acetate, for which the caecal content was reduced in the same proportion relative to KHK-F and WT-F (**Fig. 7A**). The antibiotic treatment created a shift in the metabolic profiles of the caecum content obtained by <sup>1</sup>H-NMR analysis as shown by PCA results (**Fig. 7B**) and by two supervised PLS-DA models (**Fig. 7C and D**). Antibiotics almost completely prevented the fructose-induced increase in caecal glucose levels in the KHK-F+AB mice when compared to KHK-F (**Fig. 7E**). Conversely, antibiotic treatment did not reduce the increase in succinate observed in the caecum of the KHK-F mice. In both genotypes, antibiotic treatment led to greater lactate levels.

### 3.7 ACTIVATION OF PEPTIDE EXPRESSION BY MICROBIAL METABOLITES IN GLUTAG CELL LINE

GLUTag cells are an *in vitro* model of EECs capable of secreting GLP-1, NTS and CCK (36). In order to determine whether the metabolites (glucose, lactate, succinate and propionate) identified as increased in the caecum of KHK-F mice could regulate *Cck*, *Gcg* and *Nts* mRNA expression, we tested the impact of those metabolites on enteroendocrine peptide expression in the GLUTag cells. Exposure of GLUTag cells to 2mM propionate increased significantly mRNA expression of *Cck* when compared to the control, whereas glucose, fructose, succinate and lactate had no effect (**Fig 8A**). GLUTag cells cultured with 25mM glucose significantly increased *Gcg* mRNA expression when compared to the control while cells cultured with 25mM fructose also increased *Gcg* mRNA level, but not significantly ( $P = 0.08$ ) (**Fig 8B**). *Nts* mRNA expression remained unchanged after exposure to any of the metabolites tested (**Fig 8C**) except for 10mM lactate where a tendency for upregulation ( $P = 0.06$ ) was observed.

## 4 DISCUSSION

In our model of KHK mutant mice fed fructose diet, fructose malabsorption has a significant impact on the lower intestine (ileum, caecum and colon), inducing major changes in the intestinal ecosystem composition, microbiota metabolism and intestinal endocrine response.

We found that fructose malabsorption regulates the functional identity of the specific subtype of EECs by stimulating the expression of *Cck* (and to a lesser extent *Nts* and *Gcg*) in the ileum and caecum. The signal(s) (e.g. propionate) able to activate peptide expressions in the EECs of the lower intestine likely originate from byproducts of the fructose metabolism by the microbiota that is drastically modified by fructose malabsorption.

#### 4.1 CHANGES IN CCK EXPRESSION, CCK<sup>+</sup> CELL DENSITY AND CCK<sup>+</sup> EEC SUBTYPES IN RESPONSE TO FRUCTOSE MALABSORPTION

It has long been known that *Cck* expression and secretion takes place in the upper intestinal EECs. However, recent results (37) have highlighted a potential secretion of this hormone also in the lower intestine. We confirmed the presence of CCK<sup>+</sup>-cells in the lower intestine of WT mice and using triple-labeling with anti-CCK, anti-GLP-1 and anti-PYY antibodies, we identified several subtypes of CCK<sup>+</sup>-cells. Thus, under normal fructose absorption conditions (WT-F, **Fig. 3**) the predominant EEC subtype present in the lower intestine was CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>+</sup> although we also detected a number of minority subtypes (**Fig. 3 and Table S5**). Other peptides, not tested here, could also be co-expressed with CCK in the lower intestine. Nevertheless, our data clearly corroborates the new paradigm that each EEC can express multiple gut peptides (35, 38-41).

The change in the CCK<sup>+</sup>-cell number plays a significant role in the intestinal response to fructose malabsorption without excluding the possible increase in CCK production per cell. Interestingly, the density of the normally predominant CCK-expressing cell type, the triple positive CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>+</sup> cells, did not increase in the KHK-F mice. In fact, the three regions of the lower intestine responded differently through expansion of different CCK<sup>+</sup>-cell subtypes. Whether this is due to intrinsic differences in the three intestinal regions or different environments created by fructose or fructose-microbiota interactions is an important question that remains to be addressed.

Our results further support the idea that the endocrine system in the intestine is plastic (42). Although it remains undetermined which stages of EEC differentiation (stem cells, progenitor cells, or differentiated cells) are affected by the luminal environment, in the caecum the changes in peptide pattern expression were associated with an increase in *Neurod1* and *Pax6* but not in *Neurog3* (Neurogenin-3) and *Math1* (Atonal bHLH transcription factor 1) expression. Both *Neurod1* and *Pax6* are transcription factors known to regulate the

differentiation of CCK and GLP-1 expressing cells respectively and they are both considered late markers for the differentiation of intestinal progenitor cells in the EEC lineage (43-45). In contrast, Neurog3 is an early marker that controls the commitment of *Math1* positive progenitors to the endocrine cell fate (46). Thus, the changes in the luminal environment resulting from fructose malabsorption may regulate the differentiation process downstream of Neurog3.

#### **4.2 THE ROLE OF THE MICROBIOTA IN THE REGULATION OF THE GUT PEPTIDE EXPRESSION IN THE ILEUM, CAECUM AND COLON**

It is unlikely that fructose itself changes the gut peptide expression in the ileum, caecum and colon. First, the fructose content is likely substantially increased in the duodenum and jejunum in KHK-F mice (23). Yet, no changes in *Cck* or other peptide expression were detected in these regions. Second, fructose was not detected in the caecum content of KHK-F mice suggesting its rapid metabolism by the bacteria of the lower intestine. On the other hand, the drastic changes in gut microbiota composition in KHK-F mice and the effect of antibiotic treatment suggest that the microbiota, and more likely the compounds originating from the bacterial metabolism of fructose, are involved in the EEC response to fructose malabsorption. Several studies have shown that changes in the intestinal ecosystem can affect L-cell density and function (19-21). Our study uncovered the potential of the gut microbiota to regulate *Cck* expression. This role of the microbiota is reinforced by the fact that we showed here that propionate, a SCFA produced by bacteria, can activate specifically the expression of *Cck* in GLUTag cell line.

#### **4.3 THE POTENTIAL ROLE OF PROPIONATE, GLUCOSE AND LACTATE IN REGULATING GUT PEPTIDES IN RESPONSE TO FRUCTOSE MALABSORPTION**

The concomitant increase in *Cck*, *Gcg* and *Nts* expression in the caecum raises the idea of a possible common activation mechanism. Since propionate, lactate, glucose and succinate were the main microbial metabolites that increased in the caecum in response to fructose malabsorption, they were plausible candidates for activation of *Cck*, *Gcg* and *Nts* expression. Interestingly, our *in vitro* results indicated that the expression of each peptide might be regulated by different metabolites that are nonetheless all resulting from the fructose metabolism by the gut microbiota.

From our metabolite analysis and the *in vitro* experiment using GLUTag cells, propionate is the strongest candidate for the activation of *Cck* expression. It has been shown to regulate GLP-1 and PYY secretion *in vivo* and *in vitro* (47, 48) and here we show for the first time that it can also up-regulate *Cck* expression. Propionate effects on intestinal cells are mediated primarily through two receptors FFAR2 (Free fatty acid receptor 2) and FFAR3 (Free fatty acid receptor 3) (48, 49). Interestingly, FFAR3 was found in CCK-positive cells in the duodenum and in NTS-positive cells in the ileum and colon (50). To our knowledge, no study has focused on the regulation of CCK by SCFA, likely because, until recently, the abundance of CCK in the ileum, caecum and colon was thought to be insignificant (51).

We also found that fructose malabsorption is associated with a dramatic increase in glucose content in the caecum of the KHK-F which is likely to participate to the increase in *Gcg* expression and GLP-1<sup>+</sup>-cell density in the ileum and caecum. Indeed, even in the lower intestine L-cells seems to be able to sense glucose (35) and as shown by others and confirmed here in GLUTag cells, glucose is a potent activator of *Gcg* expression. Whether glucose participates in changing CCK expression and CCK<sup>+</sup>-cell density in the lower intestine of KHK-F remains to be clarified. *In vivo*, duodenal I-cells secreting CCK did not seem to be equipped to sense sugar (52). Based on this and on the lack of increase in *Cck* expression in response to glucose in GLUTag cells, the regulation of CCK by glucose in the lower intestine appears unlikely. However, the regulation of CCK<sup>+</sup>-cells in the lower intestine may differ from that in the upper small intestine as strongly conjectured for the L-cells (35).

The lactate, which was also increased in the caecum of the KHK-F mice, is able to stimulate *Nts* expression in GLUTag cells. Thus, it might be the regulatory component activating *Nts* expression in the caecum of the KHK-F. Lactate signals through the G-protein-coupled receptor GPR81 (53) or is transported into intestinal cells by the monocarboxylate transporters (MTC) (54). Expression of GPR81 in the intestine is not well documented (55). Members of the MTC family are broadly expressed along the intestine, however their specific presence and potential function in EECs has not been explored yet. The succinate, which after glucose shows the strongest increase in the caecum of the KHK-F mice, did not stimulate *Cck*, *Gcg* nor *Nts* mRNA expression in the GLUTag cells. Succinate signals via GPR91 receptor (56), and it is not known whether GPR91 is expressed in the GLUTag cells, thus the signaling role of succinate *in vivo* cannot be excluded.

#### 4.4 CHANGES IN MICROBIOTA COMPOSITION AND FUNCTION IN RESPONSE TO FRUCTOSE MALABSORPTION

We propose that glucose, lactate, propionate, and succinate are byproducts of the fructose metabolism by the intestinal bacteria populations that are expanded in the distal intestine in response to fructose malabsorption. The most striking and significant effect we observed was the increase in abundance of *Lactobacillaceae* family, especially *Lactobacillus johnsonii*, in the caecum of the KHK-F. Since carbohydrates, including sucrose and fructose, are the major energy source favoring growth of *Lactobacillaceae* (57), these results support the hypothesis that unabsorbed fructose was moving into the caecum to become a key carbon source for the bacteria of the lower intestine. Fructose itself was not detected in the caecum of the KHK-F mice and was likely converted to glucose (12) which increased drastically. Three metabolic pathways can convert fructose into glucose in bacteria (**Fig. S5**) and all three can be deployed by the bacteria that increased in relative abundance in the KHK-F group.

Among the various SCFA of the caecal content, only propionate increased in response to fructose malabsorption. Normally SCFAs originate from fermentation of non-digestible carbohydrates by the gut bacteria (58). In the KHK-F mice, the increase in propionate concentration likely arises from the fermentation of the malabsorbed fructose *via* glucose (**Fig. S5**). Two pathways could contribute to bacterial propionate formation from carbohydrates: the succinate pathway and the acrylate pathway (**Fig. S5**) (59). In the caecum of KHK-F mice, the level of some intermediates of the succinate pathway (*e.g* fumarate and succinate) or acrylate pathway (*e.g* lactate) were higher than in the WT-F confirming that both synthetic pathways were stimulated by the fructose malabsorption. However, the accumulation of succinate in the caecal content of KHK-F suggests that the part of the succinate pathway acting downstream of succinate was inefficient. On the other hand, the *Lachnospiraceae*, which increased in the KHK-F group (**Fig. 4J**), have the *lcdA* (lactoyl-CoA dehydratase subunit alpha) gene (59), and may be the main bacteria supporting the propionate production through the lactate pathway.

Interestingly, the role of CCK in mediating the visceral pain has been suggested in mice and humans (60-62). Thus our results provide a possible explanation for the pain experienced by fructose mal-absorbing patients, and identify a number of possible therapeutic entry points to alleviate this condition.

#### 5 CONCLUSIONS

We showed that, in response to fructose malabsorption, CCK is the highest upregulated gut hormone in the ileum and caecum. We also demonstrated for the first time that microbial metabolites such as propionate can regulate CCK expression. Our results confirmed the adaptability of the EECs to the luminal environment. Importantly, we showed that the response to the changes in the luminal environment can be EEC-subtype specific. In our system, only some CCK<sup>+</sup>-EEC subtypes, as defined by the combination of hormone that they express, increased in their density in response to fructose malabsorption. Whether the CCK<sup>+</sup>/GLP-1<sup>-</sup> or CCK<sup>+</sup>/GLP-1<sup>+</sup>-cells differ markedly in their functional responsiveness and physiological role will be interesting topics for future evaluation. Thus, the nature of the EEC response to specific luminal environment changes may be one of the defining characteristics of EEC subtypes with distinct functional identities.

### **AUTHOR CONTRIBUTIONS**

The authors' responsibilities were as follows: XZ, EW, AG, RF, JCF, and VD designed the research; XZ, AG, EW, SB, LS, FD, CP, ND, JFR, SES and VD conducted the research; XZ, AG, CP, SES, JFR, MM, DV and VD analyzed the data; DV, CH, SES, AG and VD wrote the manuscript; CM, GG, PG, DV, HMB, RF, JCF and VD contributed to the discussion. VD has primary responsibility for the final content; and all authors read and approved the final manuscript. The authors have declared no conflicts of interest.

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**FIGURE LEGEND**

**Figure 1:** KHK<sup>-/-</sup> and WT mice were fed 20% fructose (KHK-F or WT-F) or 20% glucose diet (KHK-G or WT-G) for 8 weeks. The mRNA expression levels of the main entero-hormones were measured in KHK-F, KHK-G, WT-F and WT-G mice in (A) the duodenum; (B) the jejunum; (C) the ileum; (D) the caecum and (E) the proximal colon. The relative values were normalized to WT-G levels (n=6-8/group). All values are means ± SEM. Means were compared by one-way ANOVA followed by Tukey's post-hoc test; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. *Gip*: Glucose-dependent insulinotropic peptide; *Cck*: Cholecystokinin; *Pyy*: Peptide YY; *Gcg*: preproglucagon; *Nts*: Neurotensin; *Sst*: Somatostatin; *Sct*: Secretin; *Tph1*: tryptophan hydroxylase 1.

**Figure 2.** KHK<sup>-/-</sup> and WT mice were fed 20% fructose (KHK-F and WT-F respectively) for 8 weeks. Distribution of (A) CCK-, (B) GLP-1-, (C) PYY-positive enteroendocrine cells, per section area (mm<sup>2</sup>) of mucosa in the ileum, caecum and colon of KHK-F and WT-F. (D) CCK peptide content in caecum tissue of KHK-F and WT-F. Representative immunofluorescence staining of CCK-positive cells (red) in (E) ileum, (F) caecum and (G) colon of KHK-F (left panels) and WT-F (right panels) mice (n=5-6/group). All values are means ± SEM. Means were compared with *t*-test; \*p < 0.05, \*\*p < 0.01, \*\*\* p<0.001.

**Figure 3.** KHK<sup>-/-</sup> and WT mice were fed 20% fructose (KHK-F or WT-F) for 8 weeks. (A to D) Representative triple immunofluorescence staining of CCK (red), GLP-1 (green) and PYY (blue) in the ileum of KHK-F mice. Each set of panels (e.g A, A', A'', A''') shows the same field of view with CCK, GLP-1, PYY channels separately and a merged image. Red arrows show EEC in which CCK only is expressed (CCK<sup>+</sup>/GLP-1<sup>-</sup>/PYY<sup>-</sup>), yellow arrows show EEC in which CCK and PYY are both expressed (CCK<sup>+</sup>/GLP-1<sup>-</sup>/PYY<sup>+</sup>), green arrows show EEC1 in which CCK and GLP-1 are both expressed (CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>-</sup>) and blue arrows show EEC in which CCK, GLP-1 and PYY (CCK<sup>+</sup>/GLP-1<sup>+</sup>/PYY<sup>+</sup>) are expressed. Quantification of the density of the different populations of CCK<sup>+</sup>-cells in the (E) ileum, (F) caecum and (G) colon of KHK-F and WT-F (n=5-6/group). All values are means ± SEM. The counts and statistics are presented in **Supplementary data Table S5**.

**Figure 4.** Microbiota composition of the caecum content collected from KHK<sup>-/-</sup> or WT mice fed 20% fructose (KHK-F or WT-F) or 20% glucose (KHK-G or WT-G) diet for 8 weeks (n = 6-8/group). Analysis was based on 16S rDNA sequencing (region V3-V4). **(A)** Observed species richness and **(B)** Inverse Simpson Index as indicators of  $\alpha$ -diversity. **(C)** Principal coordinates analysis (PCoA) of Bray-Curtis compositional dissimilarity at the operational taxonomic unit (OTU) level. Relative distribution of **(D)** phyla and **(E-I)** families within the 5 most abundant phyla. **(J)** Graphic representation of differentially abundant OTUs having a large ( $|\log_2\text{Fold change}| > 3$ ) and significant ( $P < 0.001$ ) effect size in addition to high relative abundances ( $> 0.1\%$  in at least half the samples). Each OTU is represented by a dot and colored according to its taxonomic classification at the Family level. Taxonomy at the Genus or species level is also indicated, when available, next to each OTU. Observed species richness and Inverse Simpson Index values are means  $\pm$  SEM. Means were compared by one-way ANOVA followed by Tukey's post-hoc test; \* $p < 0.05$ .

**Figure 5.** Metabolite composition of the caecum content collected from KHK<sup>-/-</sup> or WT mice fed 20% fructose (KHK-F or WT-F) diet for 8 weeks (n=5-6/group). **(A)** Short fatty acid quantification in caecum content by gas chromatography. **(B)** PCA score plots obtained from <sup>1</sup>H-NMR spectra of caecum content extracts of KHK-F or WT-F. **(C)** Plot of O-PLS-DA coefficients related to the discrimination between <sup>1</sup>H-NMR spectra from caecum content extracts of WT-F vs KHK-F. **(D)** Bar-graph representation of the relative integral in arbitrary units (a.u) for different metabolites (glucose, lactate, alanine and succinate) in caecum content. All values are means  $\pm$  SEM; means were compared with *t*-test; \* $p < 0.05$ , \*\* $p < 0.001$ . AUC = area under the curve. UK= unknown.

**Figure 6.** KHK<sup>-/-</sup> and WT mice were fed 20% fructose for 3 weeks with (KHK-F+AB or WT-F+AB) or without (KHK-F or WT-F) antibiotic treatment (n=5-7/group). The mRNA expression levels of gut peptides were measured in WT-F, KHK-F, WT-F+AB, and KHK-F+AB mice in **(A)** the ileum; **(B)** the caecum and **(C)** the proximal colon. Data were normalized to levels in WT-F. All values are means  $\pm$  SEM. Means were compared by one-way ANOVA followed by Tukey's post-hoc test; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Figure 7.** Metabolite composition of the caecum content collected from KHK<sup>-/-</sup> or WT mice fed 20% fructose diet for 3 weeks with (KHK-F+AB or WT-F+AB) or without (KHK-F or WT-F) antibiotic treatment (n = 5-7/group). **(A)** Short-chain fatty acid (SCFA) quantification

in caecum content by gas chromatography **(B)** PCA scores plots obtained from  $^1\text{H-NMR}$  spectra of caecum content extracts from KHK-F, KHK-F+AB, WT-F and WT-F+AB. **(C)** Plots of O-PLS-DA coefficients related to the discrimination between  $^1\text{H NMR}$  spectra of caecum content extracts from WT-F vs KHK-F. **(D)** Plots of O-PLS-DA coefficients related to the discrimination between  $^1\text{H-NMR}$  spectra of caecum content extracts from WT-F+AB vs KHK-F+AB. **(E)** Bar-graph representation of the relative integral for different metabolites (glucose, lactate, alanine and succinate) in caecum content. All values are means  $\pm$ SEM. Means were compared by one-way ANOVA followed by Tukey's post-hoc test; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

**Figure 8.** **(A)** *Cck*, **(B)** *Gcg* and **(C)** *Nts* mRNA expression in GLUTag cells incubated for 24h with 1mM glucose (control) or 25mM glucose, 25mM fructose, 2mM succinate, 10mM lactate, or 2mM propionate (n=3/group with each experiment done in triplicates). The relative values were normalized to control levels. All values are means  $\pm$  SEM. Means were compared by Kruskal-Wallis test followed by Dunn's multiple comparison test; \*  $p < 0.05$ , \*\*  $p < 0.01$ .

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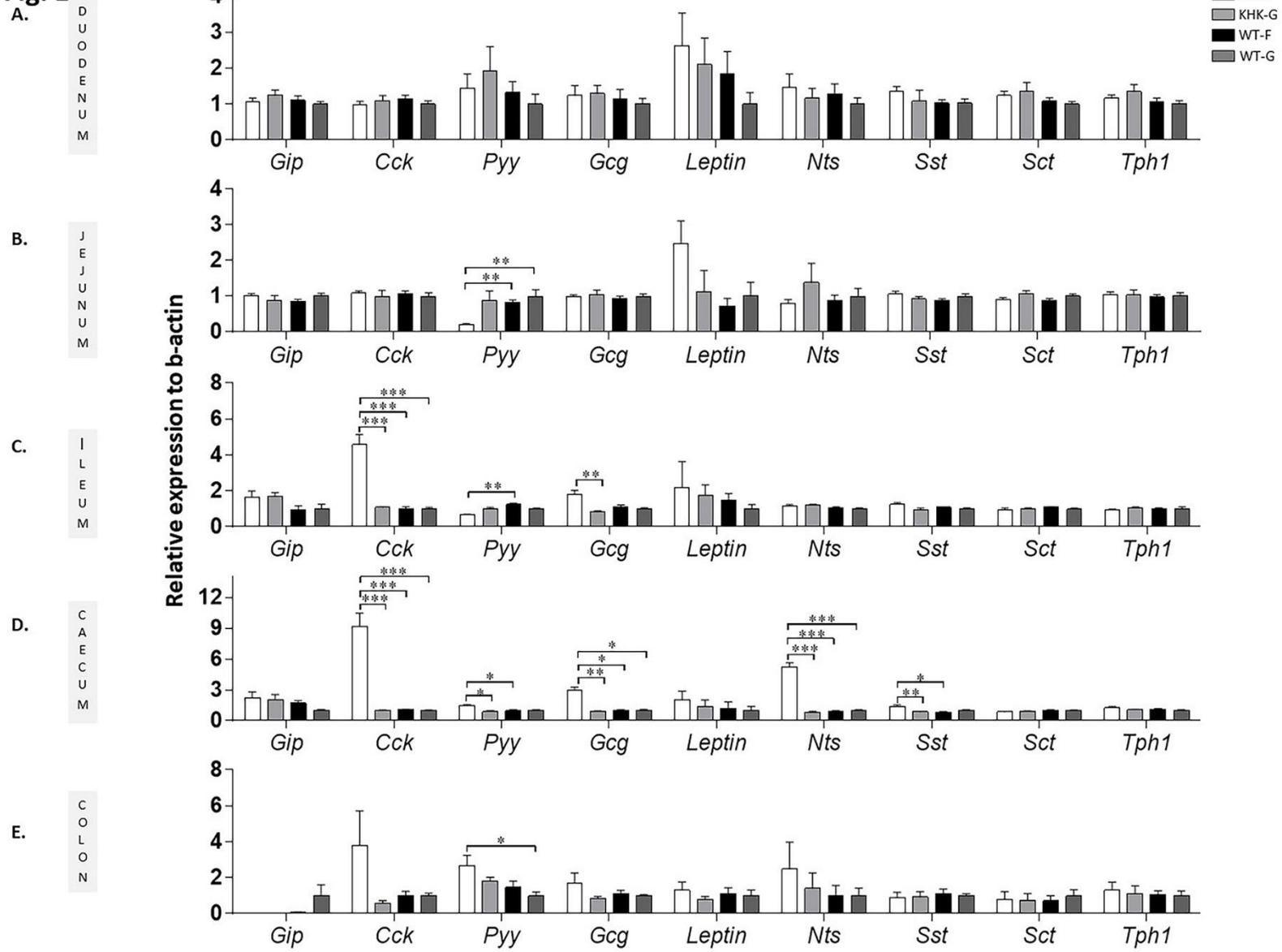
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**Fig. 1**



**Fig. 2**

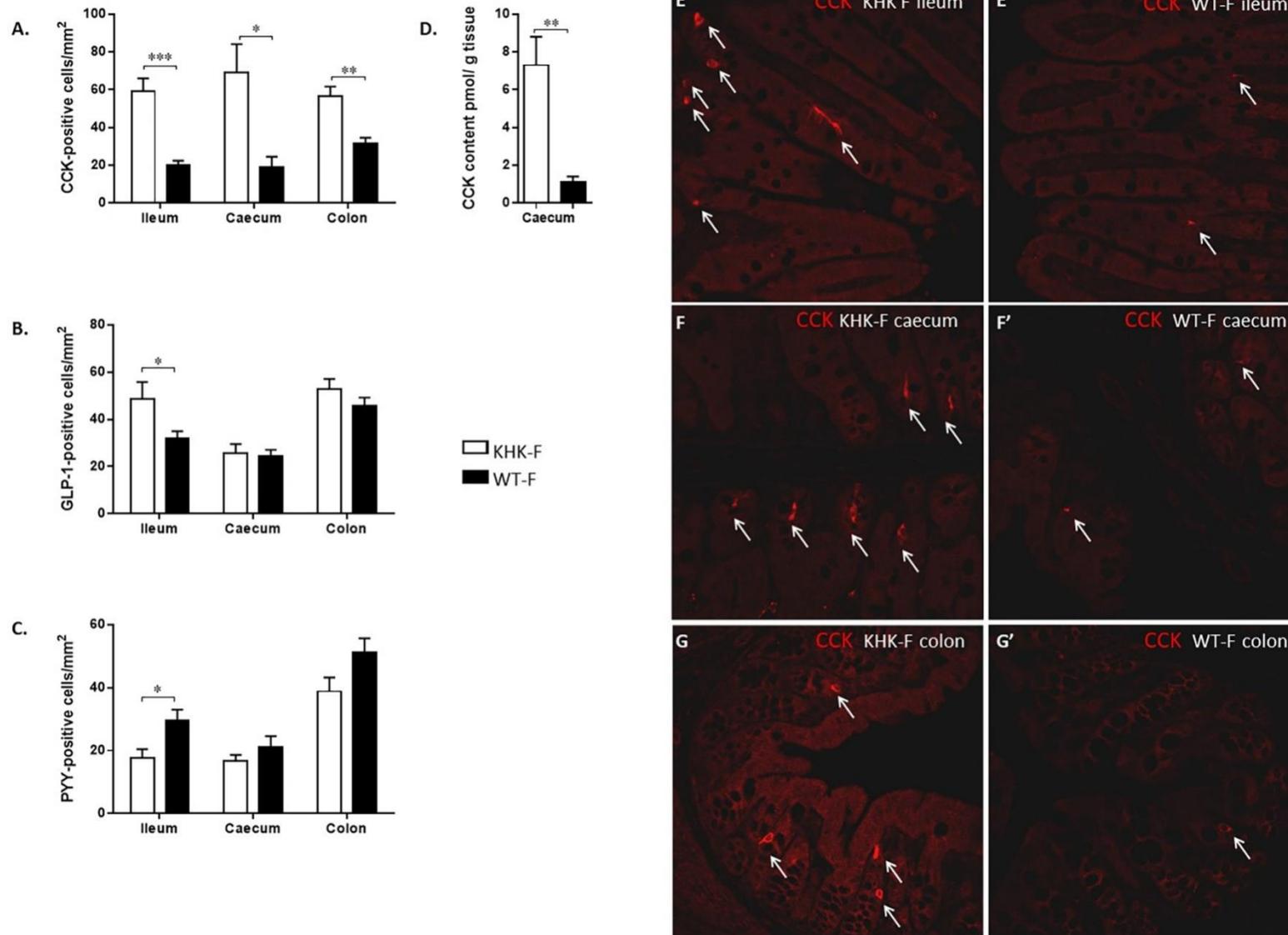


Fig. 3

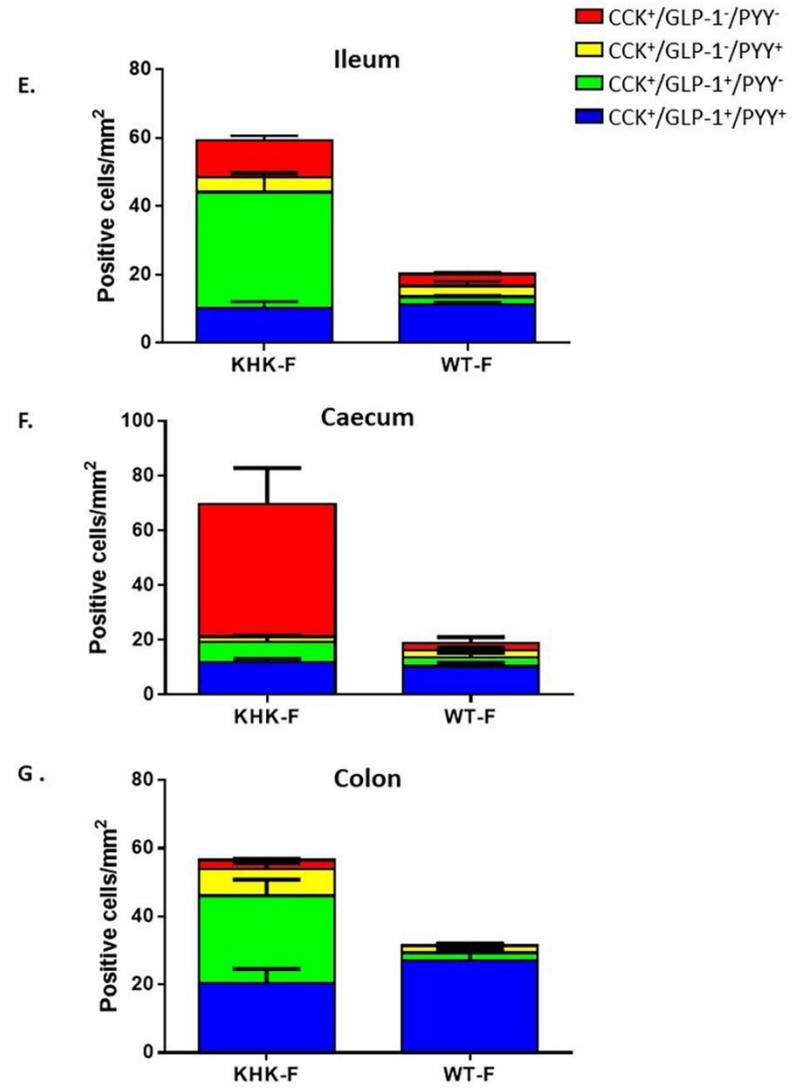
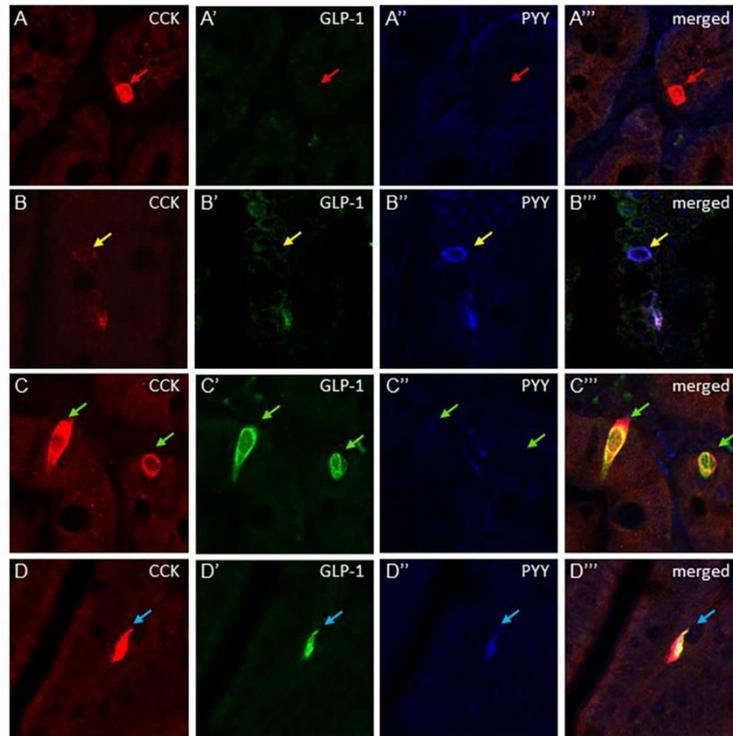


Fig. 4

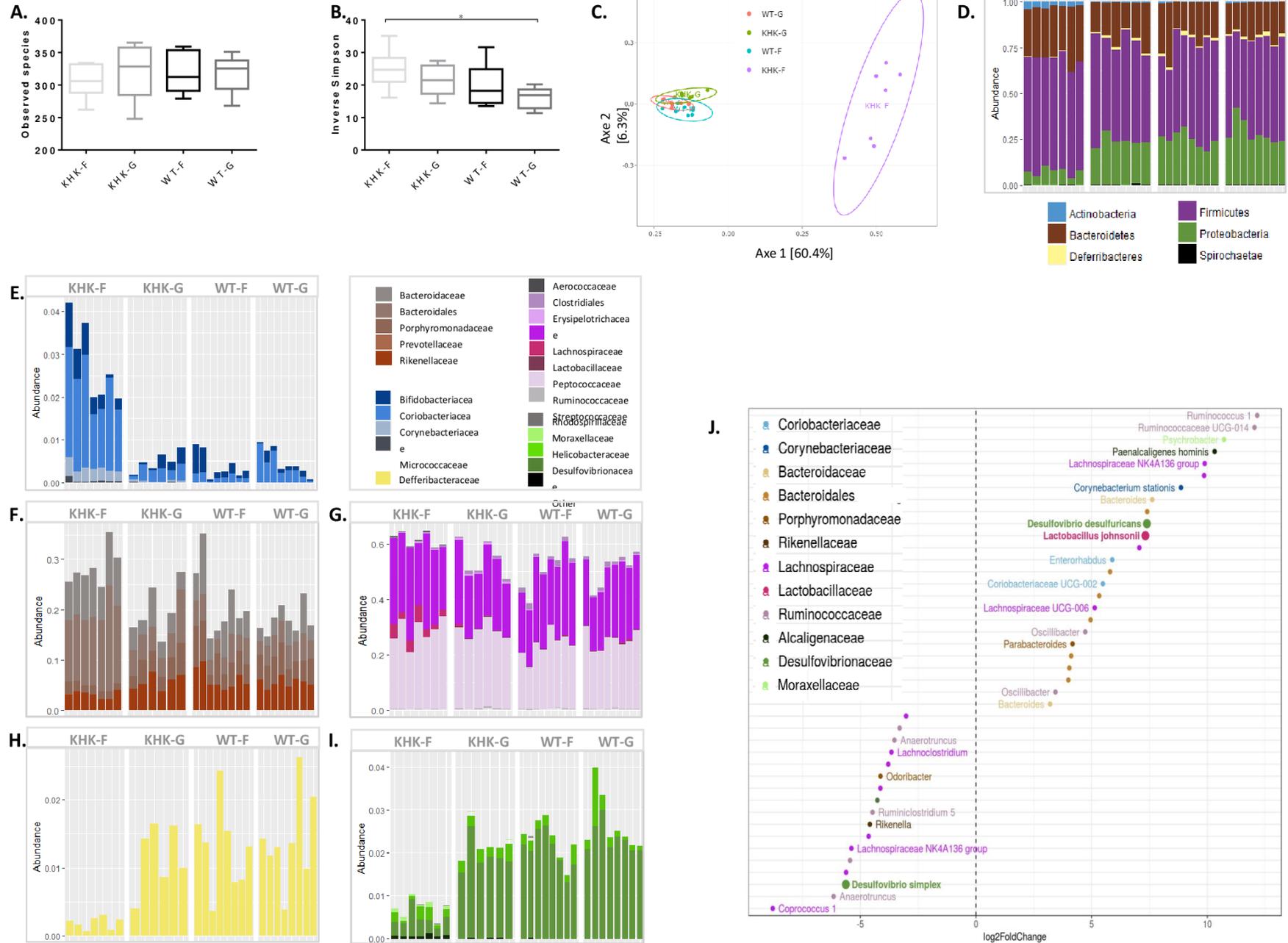
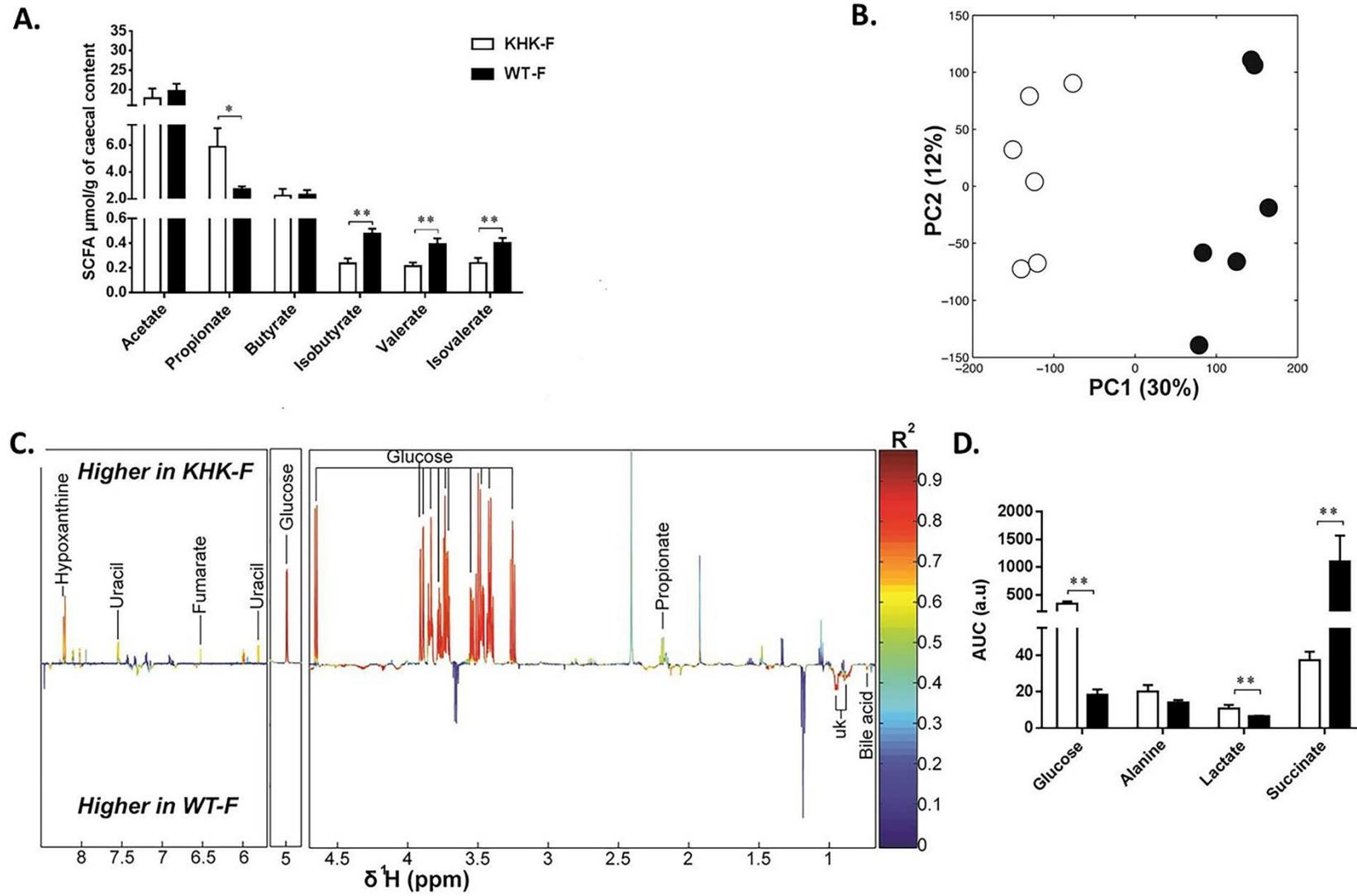


Fig. 5



**Fig. 6**

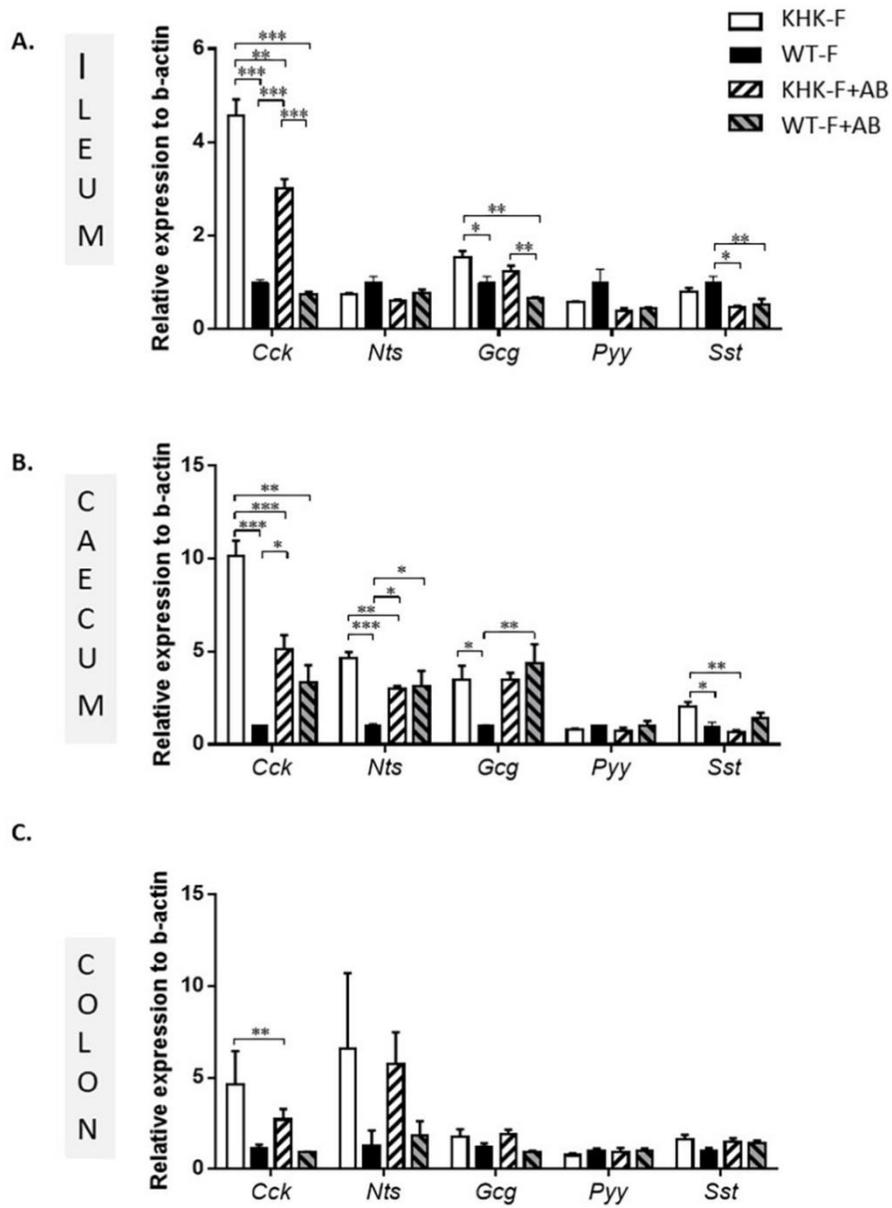
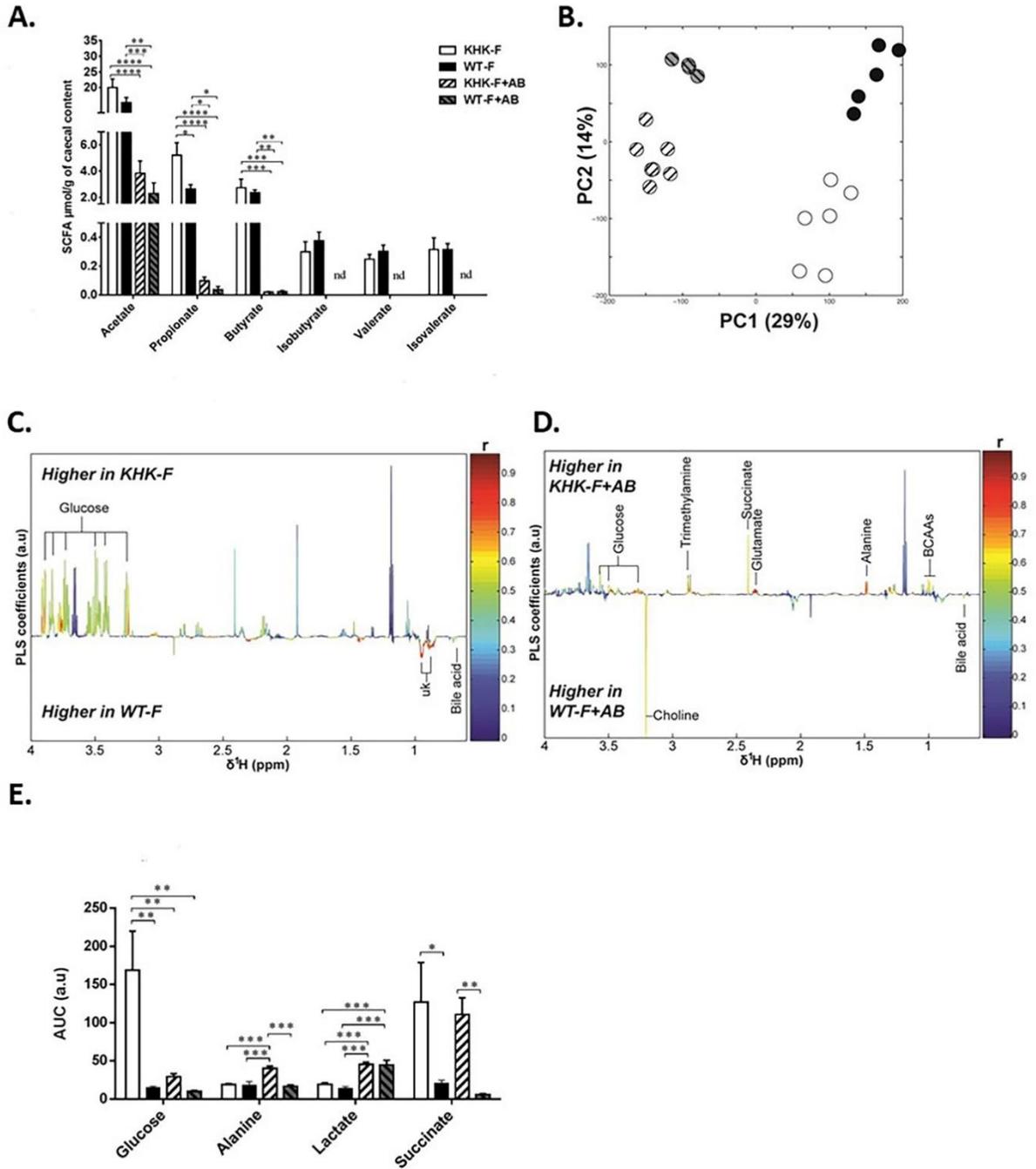
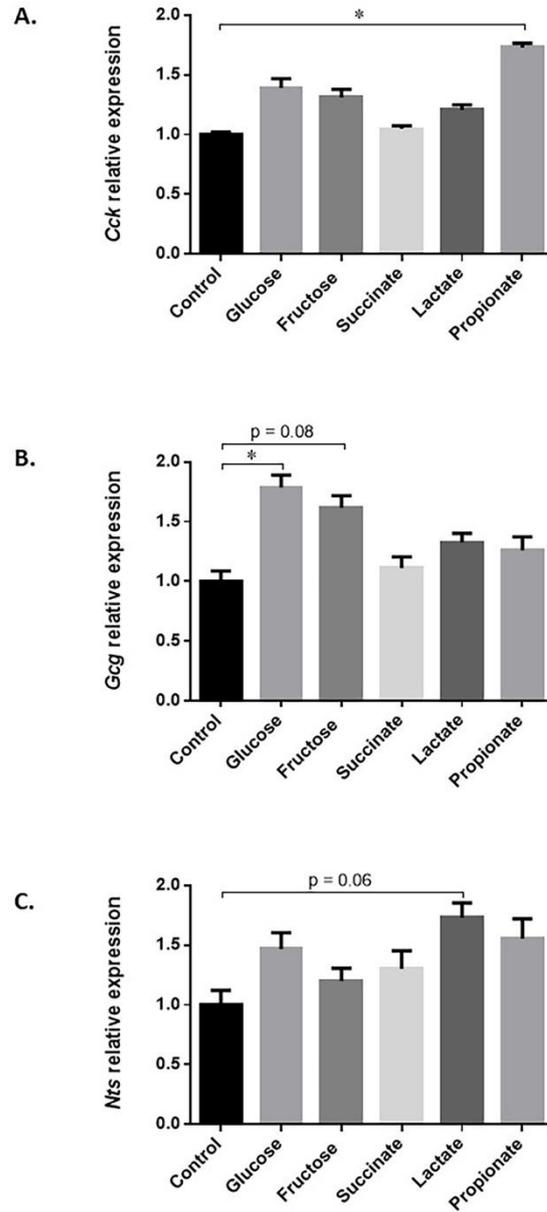


Fig. 7



**Fig. 8**

## SUPPLEMENTARY MATERIALS AND METHODS

### PREAMPLIFICATION AND qRT-PCR OF TRANSCRIPTION FACTOR MRNA IN THE CAECUM

For the EEC differentiation markers (*Math1*, *Neurog3*, *NeuroD1*, *Pax6* and *Pax4*), a preamplification was performed with predesigned TaqMan primers (Assays-on-Demand™, Gene Expression Products; Applied Biosystems, France) (**Table S1**) using TaqMan PreAmp Master Mix (Applied Biosystems, Victoria). The quantification of the preamplified targeted genes was achieved using quantitative RT-PCR based on TaqMan gene expression assays with the same predesigned Taqman primers (Applied Biosystems, France).  $\beta$ -actin housekeeping gene was used to normalize the mRNA abundance.

### MICROBIAL qRT-PCR ASSAYS

Quantitative PCR procedures were performed as described (1). qRT-PCR were performed using the following probes specific of the 4 main phyla: *Firmicutes* (928F-Firm-TGAAACTYAAAGGAATTGACG and 1040FirmR-ACCATGCACCACCTGTC), *Bacteroidetes* (MIBF-GGCGACCGGCGCACGGG and MIBR-GRCCTTCCTCTCAGAACCC), *Proteobacteria* (1080 $\gamma$ F-TCGTCAGCTCGTGTYGTGA and  $\gamma$ 1202R-CGTAAGGGCCATGATG) and *Actinobacteria* (Act920F3-TACGGCCGCAAGGCTA and act920F3 TACGGCCGCAAGGCTA) with SYBR-Green® PCR 2 $\times$  Master Mix (Applied-Biosystems) as previously described (1-3). All qRT-PCR reactions were conducted in a final volume of 25  $\mu$ L with 0.2  $\mu$ M final concentration of each primer and 5  $\mu$ L of appropriate dilutions of DNA samples. Amplifications were carried out using the following ramping profile: 1 cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 61 °C for 1 min. The total number of bacteria was inferred from averaged standard curves as previously described (1). For the quantification of *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* standard curves were generated from serial dilutions of a known concentration of genomic DNA from *Lactobacillus acidophilus*, *Bacteroides fragilis*, *Bifidobacterium adolescentis* and *Escherichia coli* respectively.

### H<sup>1</sup>-NMR EXTRACTION AND ANALYSIS

Caecum content extracts were prepared by mixing 80-100 mg of the caecal content with 500  $\mu$ L of phosphate buffer (0.2 M, pH 7.4) in D<sub>2</sub>O containing 1% (w/v) of sodium 3-(trimethylsilyl) propionate (TSP). After vortexing, each sample was subjected to a freeze-thaw cycle in liquid nitrogen and subsequently homogenized with a tissue lyser (QIAGEN,

Hilden, Germany) at 20 Hz for 40 s followed by centrifugation at 10000×g for 10 min at 4°C. The supernatants were collected, and the remaining pellet was extracted once more as described above. Supernatants obtained from the two extractions were combined and centrifuged at 10000×g for 10 min at 4°C. A total of 600 µL of supernatant was transferred into an NMR tube (outer diameter, 5 mm) pending NMR analysis. All <sup>1</sup>H NMR spectra were obtained on a Bruker DRX-600-Avance NMR spectrometer (Bruker, Wissembourg, France) on the AXIOM metabolomics platform (MetaToul, Toulouse, France) operating at 600.13 MHz for <sup>1</sup>H resonance frequency using an inverse detection 5-mm <sup>1</sup>H-<sup>13</sup>C-<sup>15</sup>N cryoprobe attached to a cryoplatfrom (the preamplifier cooling unit). The <sup>1</sup>H NMR spectra were acquired at 300K using the Carr-Purcell-Meiboom-Gill spin-echo pulse sequence with pre-saturation and a total spin-echo delay (2nτ) of 100 ms. A total of 128 transients were collected into 64,000 data points using a spectral width of 12 ppm, a relaxation delay of 2.5 s, and an acquisition time of 2.28 s. Data were analyzed by applying an exponential window function with a 0.3-Hz line broadening prior to Fourier transformation. The resultant spectra were phased, baseline corrected, and calibrated to TSP (δ 0.00) manually using Mnova NMR (v9.0, Mestrelab Research). The spectra were subsequently imported into MATLAB (R2014a, MathsWorks, Inc.) All data were analyzed using full-resolution spectra. The region containing the water resonance (δ 4.6–5.2ppm) was removed, and the spectra were normalized to the probabilistic quotient (4) and aligned using a previously published function (5).

<sup>1</sup>H-NMR data were used as independent variables (X matrix) and regressed against a dummy matrix (Y matrix) indicating the class of samples. O-PLS-derived model was evaluated for goodness of prediction (Q<sup>2</sup>Y value) using 7-fold cross-validation. The reliability of each model was established using a permutation test of the Y vector (1000 permutations) in order to determine a p-value for each Q<sup>2</sup>Y. To identify metabolites responsible for discrimination between the animal groups, the O-PLS-DA correlation coefficients (r<sup>2</sup>) were calculated for each variable and back-scaled into a spectral domain, so that the shape of NMR spectra and the sign of the coefficients were preserved (6). The weights of the variables were color-coded, according to the square of the O-PLS-DA correlation coefficients. Correlation coefficients extracted from significant models were filtered so that only significant correlations above the threshold defined by Pearson's critical correlation coefficient (P < 0.05; |r| > 0.71) were considered significant. For illustration purposes, the area under the curve of several signals of interest was integrated and statistical significance was tested using univariate tests.

**Table S1:** Primers used for qRT-PCR with intestinal mRNA

Primers for SYBR Green		
Name(Gene)	Gene Bank accession n°	Primers
$\beta$ Actin ( <i>beta-actin</i> )	NM_007393	Forward: CTAAGGCCAACCGTGAAAAG Reverse: ACCAGAGGCATACAGGGACA
GLUT5 ( <i>Slc2A5</i> )	NM_019741	Forward: TGCAGAGCAACGATGGAGAAA Reverse: ACAGCAGCGTCAGGGTGAAG
Glucose-dependent insulinotropic peptide ( <i>Gip</i> )	NM_008119	Forward: CAGGTAGGAGGAGAAGACCTCAT Reverse: CCTAGATTGTGTCCCCTAGCC
Ccholecystokinin ( <i>Cck</i> )	NM_031161	Forward: GCTGATTTCCCATCCAAA Reverse: GCTTCTGCAGGGACTACCG
Peptide YY ( <i>Pyy</i> )	NM_145435	Forward: CCTACCCTGCCAAACCAG Reverse: GGACATCTCTTTTTCATACCG
Preproglucagon ( <i>Gcg</i> )	AF276754	Forward: CACGCCCTTCAAGACACAG Reverse: GTCCTCATGCGCTTCTGTC
Leptin ( <i>Lep</i> )	NM_008493	Forward: CAGGATCAATGACATTTACACA Reverse: GCTGGTGAGGACCTGTTGAT
Neurotensin ( <i>Nts</i> )	NM_024435	Forward: AGCTGGTGTGCCTGACTCTC Reverse: CCAGGGCTCTCACATCTTCT
Somatostatin ( <i>Sst</i> )	NM_009215	Forward: CCCAGACTCCGTCAGTTTCT Reverse: GGGCATCATTCTCTGTCTGG
Secretin ( <i>Sct</i> )	NM_001309439	Forward: CGATGCTACTGCTGTTGCTG Reverse: TCTGAGTGTCTTGGGGTCTCT
Tryptophan hydroxylase 1 ( <i>Tph1</i> )	NM_009414	Forward: CACAGTTCAGATCCCCTCTACA Reverse: GAACGTGGCCTAGGAGTTCA
Assays-on-Demand™ for Taqman		
Name (Gene)	Gene Bank accession n°	Assay ID
$\beta$ Actin ( <i>Actb</i> )	NM_007393	Mm00607939_s1
Neurogenic differentiation 1 ( <i>Neurod1</i> )	NM_010894	Mm01946604_s1
Paired box 4 ( <i>Pax4</i> )	NM_001159925	Mm01159036_m1
Paired box 6 ( <i>Pax6</i> )	NM_001244198	Mm00443081_m1
Atonal bHLH transcription factor 1 ( <i>Atoh1 = Math1</i> )	NM_007500	Mm00476035_s1
Neurogenin 3 ( <i>Neurog3</i> )	NM_009719	Mn00437606_s1

**Table S2:** Table of primary and secondary antibodies.

Peptide/protein target	Source/Name of AB	Species raised in	Dilution used
Primary antibody			
CCK	SantaCruz- sc20937	Rabbit	1/100
GLP-1	Abcam- ab23472	Mouse	1/400
PYY	SantaCruz- sc47318	Goat	1/50
Secondary antibody			
anti rabbit	Alexa 555 (Molecular Probes)	Donkey	1/750
anti mouse	Alexa 488 (Molecular Probes)	Donkey	1/700
anti goat	Alexa 647 (Molecular Probes)	Donkey	1/500

## SUPPLEMENTARY RESULTS

**Table S3: Morphological parameters of the ileum, caecum and colon.** KHK-F: KHK<sup>-/-</sup> mice fed fructose; WT-F: WT mice fed fructose. Mice were fed 20% fructose diet for 8 weeks. Values are mean  $\pm$  SEM. Means were compared with *t*-test.

	KHK-F	WT-F	P value
<b>Ileum</b>			
Villi length ( $\mu$ m)	267.5 $\pm$ 15.8	221.6 $\pm$ 7.6	<b>0.041</b>
Crypt depth ( $\mu$ m)	83.6 $\pm$ 3.0	82.6 $\pm$ 1.7	0.898
<b>Caecum</b>			
Crypt depth( $\mu$ m)	152.3 $\pm$ 7.1	111.9 $\pm$ 7.3	<b>0.015</b>
<b>Colon</b>			
Crypt depth ( $\mu$ m)	119.2 $\pm$ 7.6	138.6 $\pm$ 13.2	0.246

**Table S4: Physiological parameters.** KHK-F: KHK<sup>-/-</sup> mice fed fructose; KHK-G: KHK<sup>-/-</sup> mice fed glucose; WT-F: WT mice fed fructose; WT-G: WT mice fed glucose. Mice were fed 20% fructose diet for 8 weeks. Values are mean  $\pm$  SEM. Means were compared with one-way ANOVA.

	KHK-F	KHK-G	WT-F	WT-G	P value
Initial body weight (g)	13.8 $\pm$ 2.1	13.6 $\pm$ 2.1	11.6 $\pm$ 2.1	12.5 $\pm$ 1.7	0.276
Final body weight (g)	29.8 $\pm$ 2.2	29.5 $\pm$ 2.2	29.8 $\pm$ 2.8	28.2 $\pm$ 1.9	0.489
Daily food intake (g)	2.60 $\pm$ 0.27	2.80 $\pm$ 0.36	2.42 $\pm$ 0.31	2.58 $\pm$ 0.34	0.081
Epididymal fat tissue (g)	1.02 $\pm$ 0.19	0.85 $\pm$ 0.12	0.87 $\pm$ 0.11	0.79 $\pm$ 0.08	0.675

**Table S5: Cell density of various EEC populations identified by immunohistochemical staining in the ileum, caecum and colon.** KHK-F: KHK<sup>-/-</sup> mice fed fructose; WT-F: WT mice fed fructose. Mice were fed 20% fructose diet for 8 weeks. Values are mean ± SEM. Means were compared with *t*-test. ns = non-significant.

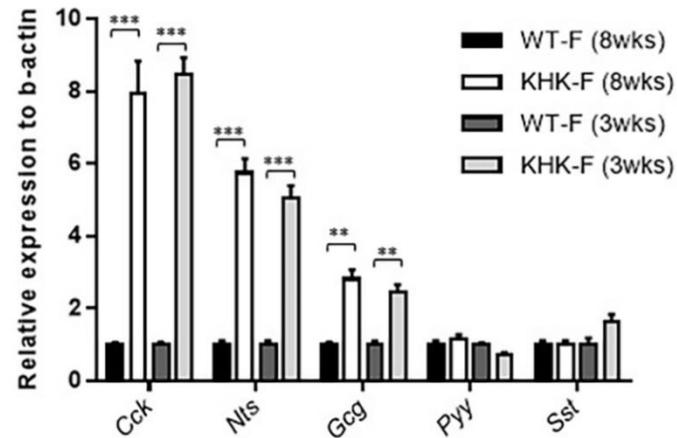
	Cell density: number of cells/mm <sup>2</sup>		P value
	KHK-F	WT-F	
<b>Ileum</b>			
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>-</sup>	10.8 ± 1.3	3.5 ± 0.6	0.002
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	33.8 ± 5.3	2.3 ± 0.5	0.002
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	4.3 ± 1.5	3.2 ± 1.3	ns
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	10.3 ± 1.8	11.2 ± 0.8	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	4.5 ± 0.6	5.9 ± 1.3	ns
CCK <sup>-</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	3.2 ± 1.8	3.0 ± 0.9	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	0.2 ± 0.2	12.2 ± 1.6	0.002
Total CCK <sup>+</sup> cells	59.1 ± 7.0	20.2 ± 2.3	0.002
Total GLP-1 <sup>+</sup> cells	48.7 ± 7.2	31.8 ± 3.2	0.049
Total PYY <sup>+</sup> cells	17.7 ± 2.8	29.7 ± 3.3	0.030
<b>Caecum</b>			
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>-</sup>	48.4 ± 13.2	2.6 ± 2.4	0.016
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	7.6 ± 2.0	3.2 ± 1.7	ns
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	1.8 ± 0.4	2.6 ± 1.1	ns
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	11.8 ± 1.4	10.4 ± 1.4	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	5.0 ± 1.3	3.6 ± 0.2	ns
CCK <sup>-</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	1.8 ± 0.6	1.4 ± 0.7	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	1.4 ± 0.7	6.8 ± 1.8	0.008
Total CCK <sup>+</sup> cells	69.2 ± 14.9	18.8 ± 5.7	0.016
Total GLP-1 <sup>+</sup> cells	25.6 ± 3.9	24.2 ± 2.9	ns
Total PYY <sup>+</sup> cells	16.6 ± 2.0	21.2 ± 3.4	ns
<b>Colon</b>			
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>-</sup>	2.7 ± 0.4	0.3 ± 0.3	0.007
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	25.5 ± 4.8	2.2 ± 0.5	0.002
CCK <sup>+</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	7.8 ± 1.8	2.0 ± 0.6	0.021
CCK <sup>+</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	20.5 ± 4.1	27.2 ± 3.2	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>-</sup>	5.0 ± 0.5	6.0 ± 1.3	ns
CCK <sup>-</sup> -GLP-1 <sup>-</sup> -PYY <sup>+</sup>	8.5 ± 1.9	12.2 ± 4.3	ns
CCK <sup>-</sup> -GLP-1 <sup>+</sup> -PYY <sup>+</sup>	2.0 ± 0.8	10.5 ± 1.7	0.002
Total CCK <sup>+</sup> cells	56.5 ± 5.2	31.5 ± 3.1	0.002
Total GLP-1 <sup>+</sup> cells	52.7 ± 4.5	45.7 ± 3.6	ns
Total PYY <sup>+</sup> cells	38.8 ± 4.4	51.3 ± 4.3	0.084

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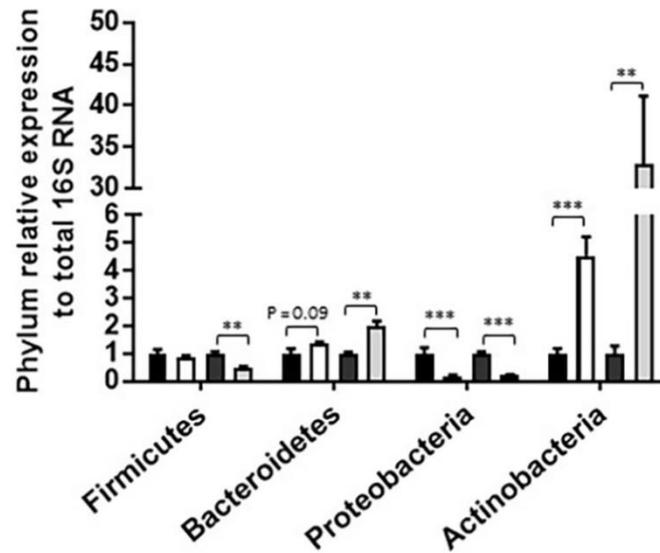
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Fig. S1

A.



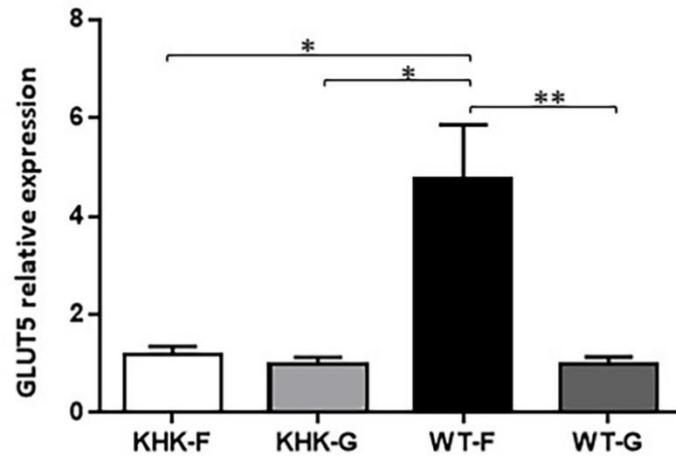
B.



**Figure S1: (A)** Comparison of peptide expression profile in the caecum after 3 wks or 8 wks of 20% fructose feeding in WT (WT-F) or KHK<sup>-/-</sup> (KHK-F) mice. mRNA levels were analyzed by qRT-PCR using  $\beta$ -actin as a reference. Data KHK-F (3wks) were normalized to levels in WT-F (3wks). Data KHK-F (8wks) were normalized to levels in WT-F (8wks). **(B)** Relative abundance of the main 4 phyla in caecal content of WT-F and KHK-F mice fed 20% fructose for 3 or 8 weeks. Data were obtained by qRT-PCR using primer targeting conserved regions of 16S rRNA for each taxonomic group. Data KHK-F (3 or 8wks) were normalized to levels in WT-F (8 or 3wks) respectively and universal 16S primer was used as a reference. The data confirmed the changes in microbiota composition obtained by pyrosequencing after 8 weeks of feeding (Fig. 4). n=6-8/group. All values are means  $\pm$  SEM. Means were compared with t-test; \*p < 0.05, \*\*p < 0.01, \*\*\* p < 0.001. WT-F (8wks): wild type mice fed 20% fructose for 8 wks; WT-F (3wks): wild type mice fed 20% fructose for 3 wks; KHK-F (8wks): KHK<sup>-/-</sup> mice fed 20% fructose for 8 wks; KHK-F (3wks): KHK<sup>-/-</sup> mice fed 20% fructose for 3 wks. Cck: Cholecystokinin; Gcg: pre-proglucagon; Nts: Neuropeptide Y; Pyy: Peptide Y; Sst: Somatostatin.

Fig. S2

A.



B.

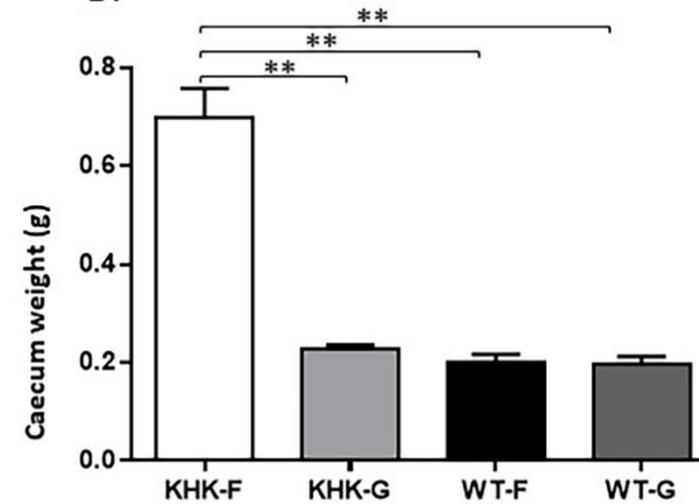
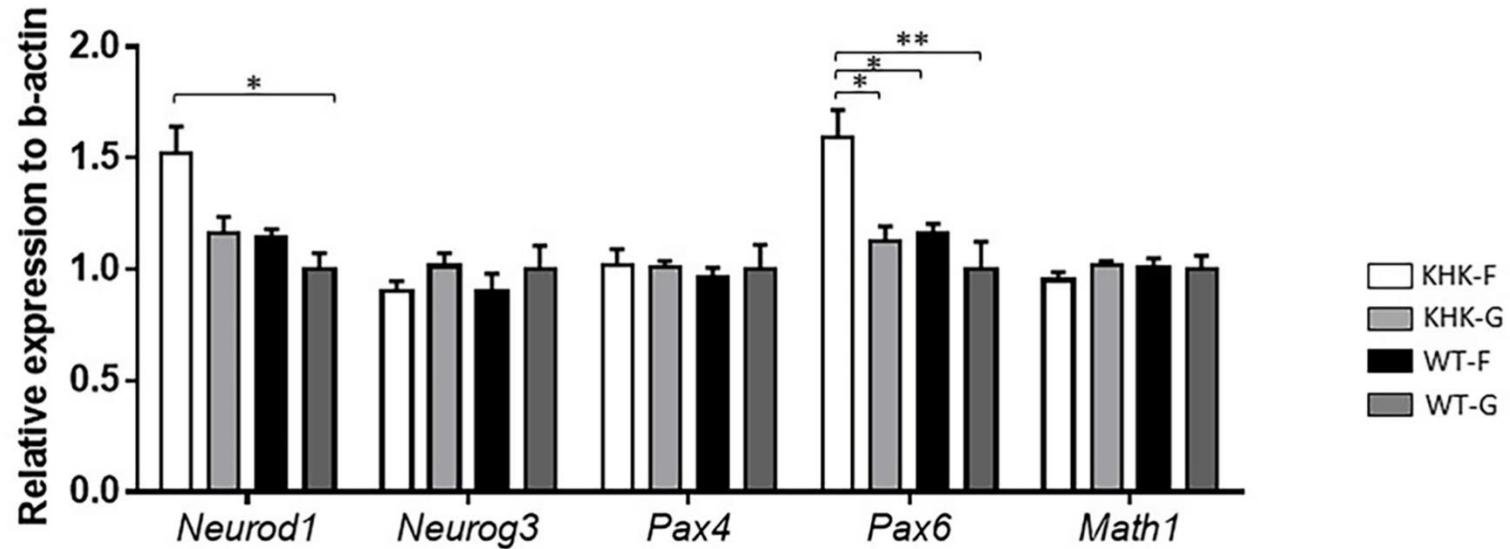


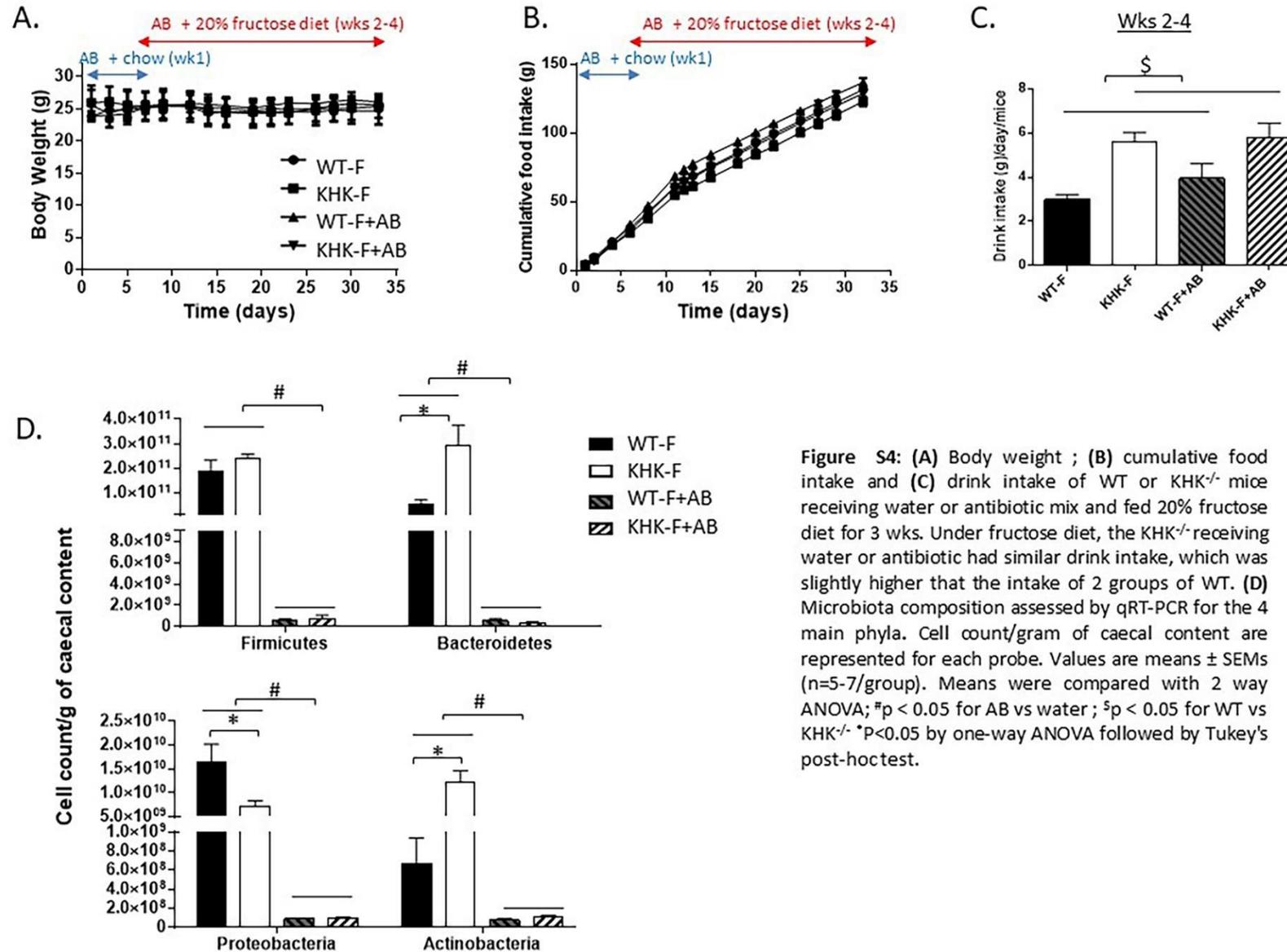
Figure S2: KHK<sup>-/-</sup> and WT mice were fed 20% fructose (KHK-F or WT-F) or 20% glucose diet (KHK-G or WT-G) for 8 weeks. (A) Relative mRNA abundance of GLUT5 in the jejunum normalized to  $\beta$ -actin levels (B) Caecum total weight in KHK-F, KHK-G, WT-F and WT-G. n=6-8/group; all values are means  $\pm$  SEM; Means were compared by one-way ANOVA followed by Tukey's post-hoc test. \*p < 0.05, \*\*p < 0.01

Fig. S3



**Figure S3:** KHK<sup>-/-</sup> and WT mice were fed 20% fructose (KHK-F or WT-F) or 20% glucose (KHK-G or WT-G) for 8 wks. Relative mRNA abundance of transcription factors involved in EEC proliferation and differentiation was measured by qRT-PCR after preamplification in the caecum of KHK-F, KHK-G WT-F and WT-G. *Neurod1*: Neuronal Differentiation 1 ; *Neurog3*: Neurogenin-3 ; *Pax4*: Paired Box4 ; *Pax6*: Paired box6 ; *Math1*: (also known as *Atoh1*) Atonal bHLH transcription factor 1. n=6/group. All values are means  $\pm$  SEM. Means were compared by one-way ANOVA followed by Tukey's post-hoc test; \*p<0.05, \*\*p<0.01.

Fig. S4



**Figure S4:** (A) Body weight ; (B) cumulative food intake and (C) drink intake of WT or KHK<sup>-/-</sup> mice receiving water or antibiotic mix and fed 20% fructose diet for 3 wks. Under fructose diet, the KHK<sup>-/-</sup> receiving water or antibiotic had similar drink intake, which was slightly higher than the intake of 2 groups of WT. (D) Microbiota composition assessed by qRT-PCR for the 4 main phyla. Cell count/gram of caecal content are represented for each probe. Values are means  $\pm$  SEMs (n=5-7/group). Means were compared with 2 way ANOVA; #p < 0.05 for AB vs water ; \$p < 0.05 for WT vs KHK<sup>-/-</sup> \*P<0.05 by one-way ANOVA followed by Tukey's post-hoc test.



**ADDITIONAL RESULTS FOR**

**ARTICLE 1**

## **INTRODUCTION**

As shown in the results of the Article 1, fructose malabsorption strikingly induced the upregulation of CCK in the distal intestine likely as an adaptative response to fructose-induced changes in gut microbiota. CCK has been reported to play an essential role in numerous physiological process (Friedrich and Gebhart, 2000, Chua and Keeling, 2006, Cao et al., 2012). Particularly, human and rodent studies reported that CCK enhanced visceral pain in healthy and IBS patients (Sabate et al., 2002, van der Schaar et al., 2013, Wang et al., 2015). This involves at least the peripheral CCK2R, the NMDA receptors of the CNS and the signaling through the vagal afferent nerves (Friedrich and Gebhart, 2000, Wang et al., 2015). Moreover, the hypersensitivity in IBS patients was also reported to be associated with elevation of CCK the plasma and the colonic tissue of those patients (Chey et al., 2001, Zhang et al., 2008). In clinical studies, fructose malabsorber patients often exhibit increased visceral hypersensitivity (Escobar et al., 2014, Waibel, 2014), however the mechanism supporting those symptoms remain unknown. Based on the data of the article 1 we hypothesize that the major upregulation of CCK in the distal intestinal region of the  $KHK^{-/-}$  mice exposed to fructose is associated with an increase in visceral pain. In IBS condition, mucosal inflammation and increased intestinal permeability have also been largely suspected to contribute to the impairment of the GI sensory function and to the abdominal pain (Camilleri et al., 2012, Piche, 2014). Thus, in order to clarify the mechanism involved in a potential change in visceral hypersensitivity in our model, we also measured the intestinal permeability and inflammatory markers expression.

The  $KHK^{-/-}$  mouse model was used to investigate the effects of fructose malabsorption on physiological visceral pain and intestinal permeability. We found that fructose malabsorption enhanced the visceral pain without increasing the intestinal permeability neither the inflammation.

## **MATERIALS AND METHODS**

### **ANIMAL EXPERIMENT**

The animal experiment protocol and feeding procedures are the same as those described in the article 1. The intestinal permeability and expression of the inflammatory markers and TJ

protein were done using the tissues of the experiment 1 of the article 1. Briefly, KHK<sup>-/-</sup> and WT (6-8 mice/group) mice were fed 20% glucose or fructose for 8 weeks. For the visceral pain, a separate experiment was conducted in Rouen. WT and KHK<sup>-/-</sup> mice (n = 7/group) received 20% fructose diet. After 3 weeks of feeding, their visceral pain response to rectal balloon distension was determined with a barostat.

## **RECTAL BAROSTAT**

Animal care and experimentation were approved by the local Committee on the Ethics of Animals Experiments (CENOMEXA n°54 and by the Ministry of Research (Ethical agreement number: n°05354.04). The assessment of visceral pain was measured using methods described previously (Larauche et al., 2009). An infinitely compliant distension bag (diameter 0.7cm) was made using a polyethylene bag attached to a PE-50 catheter (Intramedics, France) drilled in its end and taped 2 cm below the pressure sensor of a miniaturized pressure transducer catheter (SPR-524 Mikro-Tip catheter; Millar Instruments, Houston, TX, USA). Ligature points (silk 4.0) were covered with parafilm to prevent any air leak. On experimental day, mice were briefly anesthetized with isoflurane (3% in O<sub>2</sub>) and the lubricated “balloon-pressure sensor” was introduced into the colorectum and the balloon was inserted 2 cm past the anal marge into the colon. Each mouse was placed in an adjustable mouse restrainer (30 mm diameter × 90 mm length, LE5016, In vivo research instruments, USA), and left to rest for 30 min before the Colorectal distension (CRD) procedure. The balloon was then secured to the tail with tape and connected to an electronic barostat (G&J Electronics, Toronto, Canada) to perform isobaric graded Colorectal distension. At the start of each experiment, each “balloon-pressure sensor” was calibrated. The CRD protocol consisted of a set of graded phasic distensions to constant pressures of 15, 30, 45, and 60 mmHg (two times each, 20 s duration, 4 min inter-stimulus interval) (Protocole Plus Deluxe, G&J Electronics, Toronto, Canada). Voltage output was converted to pressure using CED digital analog converter (Micro 1401, Cambridge Electronic Design, Cambridge, UK) and Spike 2 software (CED, Ltd., Cambridge, UK). The intraluminal pressure changes, named the visceromotor response (VMR), caused by contraction of the abdominal muscles in response to the pain following the CDR, was also detected by the miniaturized pressure transducer catheter (SPR-524). The signal was amplified (PCU 2000, Millar Instrument, Houston, Texas) and converted to analog signal (Micro 1401 mk II A / D interface, CED, Cambridge, UK) and then recorded with Spike 2 software version 6.0. Intraluminal pressure is quantified by

measuring the area under the curve (AUC) plot during distension. The results were expressed as % change in the VMR using the VMR response to 15mmHg.

### **INTESTINAL PERMEABILITY**

The non-flushed jejunum sections of WT and KHK<sup>-/-</sup> mice were sampled and opened along the mesenteric border before to be mounted in Ussing chambers (Physiologic Instruments, USA) using 0.3cm<sup>2</sup> slider area size. The mucosal side of each tissue was exposed to oxygenated Krebs-mannitol (10 mM) buffer, while the serosal side was exposed to oxygenated Krebs-glucose (10 mM) buffer maintained at 37°C. For each section, the paracellular permeability was measured by the flux of Fluorescein isothiocyanate-Sulfuric acid (FITC-SA; Life technologies, France). FITC-SA (40µg/ml) was added to the mucosal chamber and samples were collected from the serosal chamber every 15 min for 2 h. Concentration of FITC-SA was measured via fluorescence at excitation 485 nm and emission 538 nm.

### **TISSUE RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (qRT-PCR)**

The caecum tissues of KHK-F, KHK-G, WT-F and WT-G were collected from the first animal experiment. RNA extraction, reverse transcription and qRT-PCR were done following the same protocols than those described in the article 1 and using the following primers: *Tnfa* (F-CTGTAGCCCACGTCGTAGC and R-TTGAGATCCATGCCGTTG), *Il13* (F-ACCCAGAGGATATTGCATGG and R-TGGGCTACTTCGATTTTGGT), *Ifnγ* (F-:ATCTGGAGGAACTGGCAAAA and R-TCAAGACTTCAAAGAGTCTGAGGTA), *Il10* (F-CAGAGCCACATGCTCCTAGA and R-TGTCCAGCTGGTCCTTTGTT), *ZO1* (F-GATCATTCCACGCAGTCTCC and R-GGCCCCAGGTTTAGACATTC) , *Claudin 2* (F-GTAGCCGGAGTCATCCTTTG and R-GGCCTGGTAGCCATCATAGT) and *Occludin* (F-GCGATCATAACCAGAGTCTTTC and R-TGCCTGAAGTCATCCACACT).

### **STATISTICAL ANALYSIS**

Statistical analysis was performed with GraphPad Prism software (v5; San Diego, USA), and data are expressed as means ± SEM. For means of expression data and VMR one-way and two-ways ANOVA analysis were performed respectively, followed by Tukey's post-hoc test after initial ANOVA showed significant effects. Means of permeability data were tested using a t-test.

## **RESULTS**

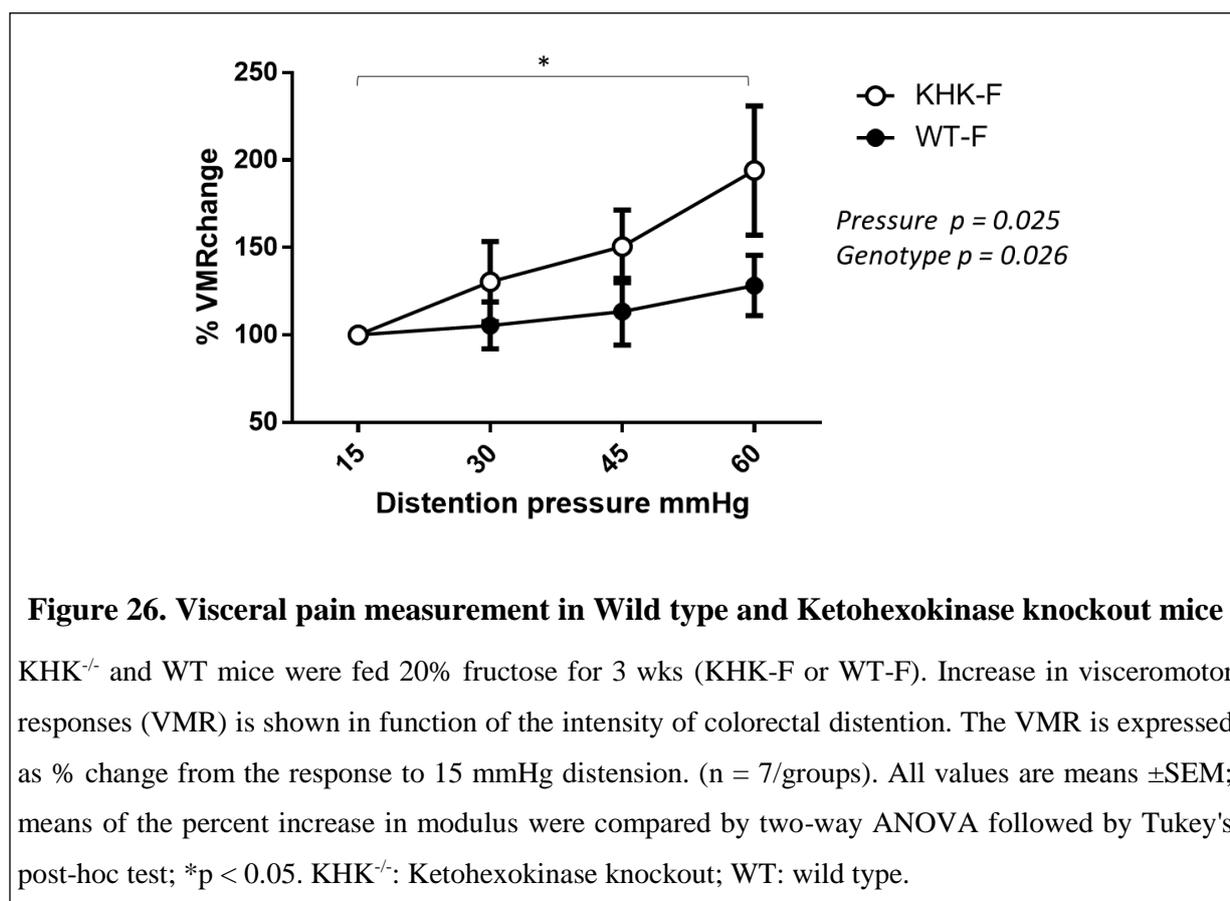
In the WT-F no changes mice in visceromotor response to distension were observed after 3 weeks of fructose feeding, whereas KHK-F displayed significant increased colorectal hypersensitivity in response to 60 mmHg distention pressure (**Figure 26**). However, although fructose malabsorption is associated with an increase in visceral pain, no significant changes in the expression of inflammatory and tight junction markers were observed in both the jejunum and caecum among the 4 groups (**Figure 27C, D, E, F**). Whereas, the paracellular permeability in the jejunum was decreased ( $P = 0.06$ ) in the KHK-F when compared to the WT-F (**Figure 27A**). The length of villi in the jejunum of KHK-F was significantly higher than WT-F (**Figure 27B**).

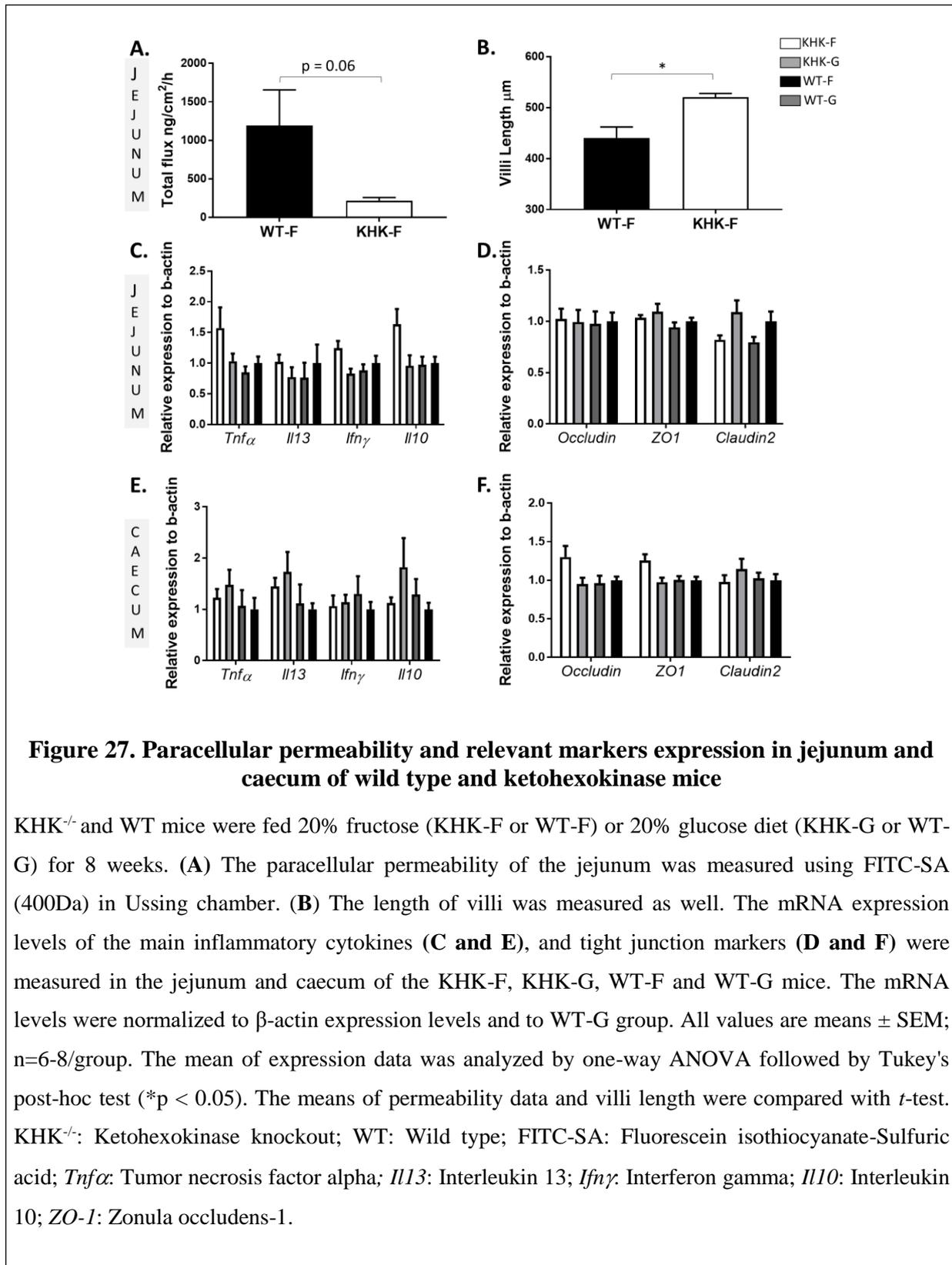
## **DISCUSSION**

In our study, fructose malabsorption is associated with an increase in visceral pain. However, this could not be explained by an increase in inflammation or permeability of the intestinal epithelium. We propose that CCK plays a key role in the visceral sensitivity associated with fructose malabsorption. Indeed, plasmatic and colonic levels of CCK are elevated in IBS patients (Zhang et al., 2008) and the role of CCK in mediating the visceral pain has been suggested in mice and humans (Harvey and Read, 1973, Chua and Keeling, 2006, Cao et al., 2012). Moreover, the two subtypes of CCK receptors (CCK1 and CCK2 receptors) regulating CCK action have both been found in colonic smooth muscles and enteric neurons indicating that they are able to regulate sensory and motor functions in the lower intestine (Dinosa et al., 1973, Harvey and Read, 1973, Morton et al., 2002, Fornai et al., 2007, Ko et al., 2011) where we measured the visceromotor response to distension. In addition, another mechanism, involving the pancreatic secretagogue properties of CCK, may explain the link between elevated CCK and visceral pain. Indeed, CCK also activates secretion of proteases (e.g amylase and trypsin) (Liddle et al., 1985, Evilevitch et al., 2004, Jaworek et al., 2010). Recently, proteases and Protease-Activated Receptors (PARs) have been described as key players in development of IBS and the increased proteolytic activity was proposed to be involved in visceral hypersensitivity of the patients (Cenac et al., 2007). Interestingly, fructose malabsorber patients also display high serum amylase concentrations (Ledochowski et al., 2001a). However, the origin of this increase remained unknown. We propose that fructose malabsorption-induced increase in CCK may stimulate pancreatic amylase secretion and thus cause visceral hypersensitivity. This study raised a potential role of CCK in visceral

hypersensitivity resulting from fructose malabsorption. This was not appreciated before because CCK was not viewed as highly expressed in the lower intestine. It would be interesting to investigate the level of CCK in patients with other GI disorders associated with visceral hypersensitivity.

Surprisingly, the jejunal permeability was decreased in the KHK-F when compared to WT-F. Previous studies reported that increase in mucosa thickness decreases the permeability (Kulkarni et al., 2009, Xue et al., 2017). We established that fructose malabsorption induced increase in villi length in the jejunum (**Figure 27B**) as well as in the ileum (**Article 1, Table S3**). This likely suggests that the mucosal thickness is increased in the KHK-F when compared to the WT-F which might explain the decreased paracellular permeability in KHK-F mice. Further studies would need to be realized to identify the factors regulating the villi length under fructose malabsorption.







## Article2

### GLUCOSE BUT NOT FRUCTOSE ALTERS THE INTESTINAL PARACELLULAR PERMEABILITY IN ASSOCIATION WITH INFLAMMATION IN MICE AND CACO-2 CELLS

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**KEY WORDS:** glucose, fructose, paracellular permeability, enteric nervous system, microbiota

**ABSTRACT:**

A causal correlation between the metabolic disorders associated with sugar intake and disruption of the gastrointestinal (GI) homeostasis (barrier dysfunction, mucosal inflammation and dysbiosis) has been suggested, but the underlying mechanisms remain largely unclear. To unravel these mechanisms, we investigated the role of physiological amounts of fructose and glucose on the barrier function of various sections of the gut.

C57BL/6 mice were fed chow diet and given water, 15% glucose or 15% fructose solution for 9 weeks. Caloric intake, body weight, glucose intolerance and adiposity were measured. In the jejunum, caecum and colon, the intestinal paracellular permeability, cytokine and tight junction protein expression were assessed. In the caecum, marker of the enteric nervous system (ENS) and gut microbiota composition were determined. The permeability of Caco-2 cell monolayers was measured in response to  $\text{IFN}\gamma + \text{TNF}\alpha$  in presence of glucose and fructose. Glucose fed mice, developed a marked increase in total adiposity, glucose intolerance, paracellular permeability in the jejunum and caecum and relative abundance of *Desulfovibrionaceae*. Fructose did not affect any of these parameters. In the caecum, both glucose and fructose intake were associated with a significant increase in mRNA level of IL13,  $\text{IFN}\gamma$  and  $\text{TNF}\alpha$  and MLCK protein level. *In vitro* when compared to glucose, fructose lessens the  $\text{IFN}\gamma + \text{TNF}\alpha$ -induced decrease in intestinal permeability.

This study provides strong evidence that physiological amount of fructose does not alter the intestinal permeability. The data also highlight the major deleterious effect of glucose on gut barrier function along the GI tract and suggest that *Desulfovibrionaceae* may play a key role in the onset of GI inflammation in response to glucose.

**ABBREVIATION:**

Ct: comparative threshold cycle; DMEM: Dulbecco's modified Eagle's medium; ECL-HRP: enhanced chemiluminescent horseradish peroxidase; EGC: Enteric glial cells; ENS: Enteric nervous system; FBS: fetal bovine serum; FITC-SA: Fluorescein isothiocyanate-Sulfuric acid; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFAP: Glial fibrillary acidic protein; GI: gastrointestinal; IFN- $\gamma$ : Interferon gamma; IL1 $\beta$ : Interleukin 1 beta; IL6: Interleukin 1; IL13: Interleukin 13; JAMs: Junctional adhesion molecules; MLC: Myosin light chain; MLCK: Myosin light chain kinase; OGTT: oral glucose tolerance test; PCoA: principal coordinates analysis; RT: Room temperature; TEER: Transepithelial resistance; TJ: Tight junction; TNF- $\alpha$ : Tumor necrosis factor alpha; ZO: Zonula occludens.

## **INTRODUCTION:**

Increase in consumption of fructose (mainly fructose-containing soft drinks) has been tightly linked to the surge in metabolic diseases such as obesity, non-alcoholic fatty liver disease, hypertriglyceridemia, metabolic syndrome and type 2 diabetes (1, 2). Chronic low-grade inflammation state (indicated by higher levels of plasmatic inflammatory markers, including C-reactive protein, interleukin 6 (IL6) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ )), characteristic of obesity and metabolic syndrome, largely contribute to the development of the related chronic metabolic diseases (3, 4). The origins of low-grade inflammation observed in metabolic disorders is still not entirely understood. However, recent work showed that the gut contributes to its establishment (4, 5). Gut barrier defects (increased permeability, mucosal inflammation and dysbiosis) favor the translocation of microbial products, which promulgate inflammation to the peripheral tissues (6, 7).

To maintain the intestinal barrier function, the preservation of the paracellular permeability is essential. The barrier formed between epithelial cells is primarily regulated apically by the tight junctions (TJ). The TJ complex is formed by the TJ proteins: occludin, claudins, junctional adhesion molecules (JAMs), and zonula occludens (ZO). The intestinal paracellular permeability depends on TJ protein expression levels, their phosphorylation status and their subcellular organization (8-10). While the events involved in the loss of the barrier function are not fully understood, cytokines such as interleukin 1 beta and 13 (IL1  $\beta$  and IL13), interferon gamma (IFN $\gamma$ ) and TNF $\alpha$ , have been shown to remodel the TJ architecture and thus increase paracellular permeability (11-13). Other factors, such as the enteric nervous system (ENS), also participate in the regulation of the intestinal permeability. The ENS is constituted of enteric neurons which are surrounded by large populations of enteric glial cells (EGCs). Several studies suggested that glial fibrillary acidic protein (GFAP)-positive EGCs participate in the regulation of the inflammatory response and in the regulation of the integrity of the gut epithelium (14).

Association of high intake of fructose with systemic inflammation has been inconsistent (15, 16). Fructose consumption has been found to lead to a decrease in occludin protein levels in the mouse small intestine (17, 18) and is associated with endotoxemia, suggesting an altered intestinal barrier function (18, 19). Furthermore, antibiotic or probiotic treatments prevent the development of nonalcoholic fatty liver disease resulting from chronic fructose intake, suggesting the role of the microbiota in the metabolic disorders linked to fructose (20, 21).

However, the direct causes of fructose-induced alteration of intestinal barrier function in the onset of metabolic outcome in response to chronic fructose intake remain unexplored. In fact, the effect of fructose on intestinal permeability had rarely been measured and often in association with high levels of dietary lipids (22) or non-physiological amounts of fructose (15). Recently, the hyperglycemia was demonstrated as a major factor in the regulation of the intestinal permeability (23); since fructose is also associated with increase in glycemia (15, 24), the direct role of luminal fructose on the gut barrier function is questionable. To the opposite, glucose was shown to have a direct acute effect on intestinal permeability *in vitro* in Caco-2 cells (25), and *in vivo* in the jejunum in rats (26, 27) and in humans (28).

In this study, using glucose intake as a positive control, we aim to clarify the impact of fructose on the intestinal permeability prior to the onset of hyperglycemia. Normally, fructose absorption takes place in the small intestine (29). However, a recent study showed that high fructose intake can overwhelm the sugar absorptive capacity of the small intestine (30), with the excess of monosaccharide reaching the lower intestine (31). Therefore, we assessed the effects of chronic intake of physiological amounts of fructose on the paracellular permeability of the small intestine, the caecum and the colon. In order to clarify the mechanism involved, we evaluated changes in gut microbiota and in expression of TJ proteins as well as inflammatory and ENS markers. Our results demonstrate that fructose does not increase the intestinal permeability in the small intestine where its luminal concentration is the highest. Moreover, while the inflammation of the caecum was similar in fructose- and glucose- fed mice, the intestinal paracellular permeability increased only in response to chronic intake of glucose, but not of fructose. Finally, we showed that in an *in vitro* Caco-2 cell model, fructose counteracts inflammation-induced permeability increase.

## **MATERIAL AND METHODS**

### **Animals and Experimental design**

This study was conducted in accordance with French guidelines on animal experimentation and validated by the Ethics Committee in Animal Experiment of INRA Jouy-en-Josas (Comethea, registration number: APAFIS#1620-2015102618572930v2). Males C57BL/6NRj (Janvier Labs, St Berthevin, France) mice aged 7 weeks were individually housed and maintained in a 12 h-light/12 h-dark cycle. Mice received, before and during the experimental procedure, a chow diet meeting their complete need (Ssniff, Soest, Germany). After 2 weeks

of adaptation, the mice received as drinking solution either water (Control), 15% glucose (Glucose) or 15% fructose (Fructose) for 9 weeks. Food and water were provided ad libitum. Body weight, drink, and food intakes were measured three times per week. At experiment completion, non-starved mice were euthanized under anesthesia. Cardiac blood was collected into EDTA tubes, centrifuged (4°C, 2000g, 15 min) and stored at -80°C for cytokine analysis. Non-flushed 2 cm sections of jejunum, caecum, and colon were sampled and stored in Krebs buffer for instant intestinal permeability measurement in Ussing chambers. Flushed sections of jejunum, caecum and colon were sampled, flash frozen in liquid nitrogen and stored at -80°C for protein and mRNA analysis. The caecal content was sampled and stored at -80°C for bacterial DNA analysis. Visceral fat was sampled and weighed.

### **Oral Glucose Tolerance Test**

Oral glucose tolerance test (OGTT) was performed after 8 weeks of exposure to 15% glucose or 15% fructose beverage. Glucose was orally administered (2g/kg body weight) to the mice after 12 h fasting. Glucose levels were measured with a glucometer (Accu-Chek®, Roche Diagnostics, France) before and 30, 60, 90, and 120 min after glucose administration.

### **Ussing Chamber Permeability measurement**

The fresh intestinal sections of jejunum, caecum, and colon were opened along the mesenteric border and mounted in Ussing chambers (Physiologic Instruments, San Diego, USA) using appropriate slider area size (0.2cm<sup>2</sup> for jejunum and caecum, 0.1cm<sup>2</sup> for colon). The mucosal side of the tissue was exposed to oxygenated Krebs-mannitol (10 mM) buffer, while the serosal side was exposed to oxygenated Krebs-glucose (10 mM) buffer. All buffers were maintained at 37°C. Fluorescein isothiocyanate-Sulfuric acid (FITC-SA; Thermo Fisher Scientific, France) was used as a marker of TJ paracellular permeation, Thus, the paracellular permeability was measured by the flux of FITC-SA. After adding FITC-SA (40µg/ml) to the mucosal chamber, 100µl in duplicate were collected from the serosal chamber every 15 min for 2 h. The concentration of FITC-SA was measured via fluorescence at excitation 485 nm and emission 538 nm.

### **RNA extraction and Quantitative real-time PCR**

The total mRNA from jejunum, caecum and colon sections were extracted using MirVana miRNA isolation kit (Thermo Fisher Scientific, France) and 2µg of mRNA was reverse

transcribed using High Capacity Complementary DNA Reverse Transcription Kit (Thermo Fisher Scientific, France) as described previously (32). Quantitative real-time PCR (qRT-PCR) was performed on the StepOnePlus real-time PCR machine (Thermo Fisher Scientific, France) using SYBR-Green® PCR 2X Master Mix (Applied-Biosystems, France) to quantify the gene expression of TJ protein and inflammatory marker expression.  $\beta$ -actin was used as housekeeping gene to normalize the mRNA abundance of each target gene (primers are listed in **Table 1**). Expression values of target genes were calculated based on the comparative threshold cycle (Ct) method to generate  $\Delta$ Ct values. The relative abundance of each mRNA in each sample was then normalized according to the equation: Relative Quantity RQ =  $2^{-\Delta\Delta Ct}$  (33).

### **Protein extraction and Western Blot**

Total protein from caecum sections was extracted using RIPA buffer (Sigma-Aldrich, France) with 4% and 10% protease inhibitor (Roche, Sigma-Aldrich, France) and phosphatase (Roche, Sigma-Aldrich, France), respectively. Proteins were quantified using the RC DC Protein Assay kit (Biorad, France). Equivalent amount (50 $\mu$ g) of proteins was analyzed by Western blot using precast gradient gels 4 to 15% Tris-HCl (BioRad, France). After transferred, membranes were blocked for 1 hour in blocking buffer (5% skim milk in TBST) and then incubated overnight at 4°C with mouse anti-MLCK (Myosin light-chain kinase) antibody (Sigma-Aldrich, France) or rabbit anti-S100b (Ab52642, Abcam, France) diluted 1:2000 and 1:5000 in blocking buffer respectively. The membranes were incubated with peroxidase labeled secondary antibodies (goat anti-mouse, 1:5000, Dako, France and anti-rabbit 1:400, Abcam, France) for 1h at room temperature (RT). The blots were revealed by enhanced chemiluminescent horseradish peroxidase substrate (ECL-HRP Thermo Fisher Scientific, France) and visualized using ChemiDoc MP imaging system (Bio-Rad, France). Subsequently, membranes were stripped and reprobed with mouse anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) antibody (Sc-365062 Santa Cruz, USA). All bands were digitally captured and densitometry analysis was performed using image Lab 5.0 software (Bio-Rad, France).

### **Immunoprecipitation of GFAP**

For immunoprecipitation 300 $\mu$ g of caecum protein extract was incubated for 2 h at RT and then overnight at 4°C with rabbit monoclonal anti-GFAP antibody (1:40, Ab68428, Abcam,

France). The immunocomplexes were captured with 50% Protein A Agarose beads (Cell Signaling, Saint Quentin Yvelines, France) on a rotator for 2h at RT. After centrifugation (4°C, 20,000g, 5mins), the precipitates were washed three times with RIPA buffer. The final pellets were suspended in 30µl of 2x loading buffer and incubate at 95 °C for 15 minutes. The entire suspension was loaded onto a SDS-PAGE gel and the western blot for GFAP (1:10000) was performed as described above and using Peroxidase-labeled secondary anti-rabbit antibody (1:400, Abcam, France).

### **Plasma analysis**

IL13, IL6 and IFN $\gamma$  levels were determined by ELISA (Peprotech, France) using 50 µl of plasma diluted 1:2.

### **Caco-2 cell culture and TEER measures in vitro**

Caco-2BBE cells (kindly donated by JR. Turner, Harvard Medical School) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, France) with 10% fetal bovine serum (FBS, Sigma-Aldrich, France) and 10ng/mL human transferrin. Caco-2BBE cells were seeded at  $2.10^5$  cells/well and differentiated as monolayers on collagen-coated polycarbonate membrane transwells with 0.3µm pores (Thermo Fisher Scientific, France) for 15-18 days after confluence. DMEM medium was changed every 3 days. Transepithelial resistance (TEER) was measured every 3 days with Voltohmmeter instrument (Millicell-ERS, Merck Millipore, France). After 15-18 days (when TEER was about  $250\Omega.cm^2$ ), the culture medium was changed to 4.5g/l glucose or fructose medium with 10% FBS and 10ng/ml transferrin in both basal and apical chambers 1-day prior cytokine treatment (D0). At D1, recombinant human IFN- $\gamma$  (Peprotech, France) was added into glucose or fructose medium (10ng/ml concentration) in the basal chamber for 24 hours. At D2, fresh medium with TNF- $\alpha$  (Peprotech, France) at 10ng/ml or 50ng/ml were changed in the basal chamber afterward. Both IFN- $\gamma$  and TNF- $\alpha$  treatments were without manipulating the apical chamber. The TEER was measured before adding TNF- $\alpha$  and every hour afterward for 6 h. Results were expressed as percent of TEER of control monolayers (cells exposed to glucose or fructose for 48h without IFN- $\gamma$  and TNF- $\alpha$  ) of the same experiment.

### **Microbiota DNA extraction and 16S DNA Sequencing**

All bacterial DNA was extracted from caecum content using GNOME DNA isolation kit (MP Biomedicals, France) (32). Quantification of bacteria DNA was performed by Real-time PCR with TaqMan® Universal PCR 2× MasterMix (Applied-Biosystems, France) on 10ng of DNA targeting and using the All bacteria primers and probes described previously (32, 34). The V3-V4 region of the 16S rRNA genes was amplified by PCR performed using MolTaq polymerase (Molzym, France) and the primers V3F: TACGGRAGGCAGCAG and V4R: ATCTTACCAGGGTATCTAATCCT. PCR products quality was control by electrophoresis and then was purified. Purified amplicons were sequenced using the Miseq® sequencing technology (Illumina) at the GeT-PLaGe platform (Toulouse) (32). qPCR for *Desulfovibrio* genus quantification were performed using the forward primer DSV691-F CCGTAGATATCTGGAGGAACATCAG and the reverse primer DSV826-R: ACATCTAGCATCCATCGTTTACAGC (35). qPCR reactions were conducted in a final volume of 25 µL with 0.2 µM final concentration of each primer and 5 µL of DNA samples.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism software (v7; San Diego, CA). All data were analyzed using Kruskal-Wallis test followed by a Dunn's multiple comparison test. For TEER data in Caco-2 cells, two-way ANOVA was performed. 16S rDNA sequencing data were analyzed using the Phyloseq package in R and custom crypts as described in the Materials and Methods of the Article 1. Effect of diet on microbiota composition was tested using a linear model with the DESeq2 analysis pipeline. Differences with p-values < 0.05 were considered as statistically significant.

## RESULTS

### Glucose and fructose effects on physiological parameters

Mice drinking glucose solution gained more weight than their fructose- and water-drinking counterparts, such that 9 weeks after the beginning of the experiment they were 15% heavier than the other two groups (**Fig. 1A**). This likely resulted from a significantly higher total daily calorie intake by the glucose-drinking mice than by mice receiving fructose or water solutions (**Fig. 1B**). Chronic intake of glucose, but not fructose, increased the glucose intolerance in the mice after 8 weeks of treatment when compared to control (**Fig. 1C and D**). Nine weeks of chronic intake of fructose was associated with a significant increase in plasmatic levels of IL6

when compared to the control group (**Fig. 1E**). The plasmatic levels of the other proinflammatory cytokines remained unchanged among the three groups (**Fig. 1F and G**). Glucose-drinking mice also displayed a significantly higher visceral adiposity ( $2.94\% \pm 0.19$ ) than the fructose-drinking ( $1.66\% \pm 0.22$ ) or control ( $1.52\% \pm 0.14$ ) groups after 9 weeks. There was no difference in liver weight among the three groups (data not shown).

### **The effects of Glucose and fructose on intestinal permeability, tight junction protein and inflammation.**

Intestinal barrier function in the small (jejunum) and large intestine (caecum and proximal colon) was evaluated *ex vivo* in Ussing chamber system. 9 weeks of glucose consumption significantly increased paracellular permeability to FITC-SA in both jejunum and caecum (**Fig.2-A1 and -A2**) when compared to the control groups, while fructose had no effect (**Fig.2-A1 and -A2**). In the colon, neither glucose nor fructose changed the paracellular permeability (**Fig.2-A3**).

In the jejunum, glucose-induced changes in permeability were not associated with changes in *Mlck* and TJ protein expression levels (**Fig.2-B1**). Of the pro-inflammatory cytokines tested, only *Il1 $\beta$*  expression was significantly upregulated in the jejunum of the mice consuming glucose when compared to the control group (**Fig.2-C1**), while fructose had no effect. Surprisingly, *Tnf $\alpha$*  expression was significantly lower in the jejunum of both, the fructose and glucose, groups when compared to the controls. In the caecum glucose-induced increase in permeability was associated with a significant increase in *Mlck* expression when compared to the control group (**Fig.2-B2**). In contrast, fructose-drinking mice displayed a significant decrease in expression of *claudin2* and *occludin* relative to the control and glucose groups respectively. In caecum, both glucose and fructose significantly increased the expression of *Ifn $\gamma$* , *Tnf $\alpha$*  and *Il13* when compared to control group (**Fig.2-C2**). Only *Il1 $\beta$*  expression remained unchanged among the three groups. In the colon, almost no differences were observed among the three groups (**Fig.2-B3 and -C3**). Western blot showed that in the caecum MLCK protein level increased in response not only to glucose (in parallel to mRNA), but also fructose (**Fig.3**).

### **Glucose and fructose effects on EGC of the ENS**

The above results showed that in the caecum both the glucose and fructose caused similar upregulation of pro-inflammatory cytokine mRNA and MLCK protein levels which normally would be associated with increased paracellular permeability. Yet, only glucose, but not fructose increased the permeability in the caecum. To clarify this surprising observation, we investigated the impact of fructose and glucose on the EGCs of the ENS which could participate in the maintenance of epithelial barrier function (36). GFAP and S100 $\beta$  protein levels were quantified in the whole caecum segment wall preparations. Glucose did not change the protein expression of GFAP when compared to control or to fructose groups (**Fig.4A**). However, GFAP protein expression level was almost significantly increased in fructose condition when compared to control ( $p = 0.056$ ) while it was very similar with glucose treatment ( $p > 0.999$ ). The two treatments did not alter the overall glial marker S100b expression (**Fig.4B**).

### **The effects of glucose and fructose on intestinal permeability Caco-2 cells in vitro**

To investigate further the differential effects that glucose and fructose and whether these sugars act directly on enterocytes, we used Caco-2 cell line. Differentiated enterocyte monolayers were cultured in presence of 4.5g/L glucose or fructose for 48h. Then the TEER was measured in response to IFN $\gamma$  priming followed by TNF $\alpha$  treatment. In absence of proinflammatory challenge 4.5g/L fructose did not affect the TEER when compared to 4.5g/L glucose (**Fig. S1**). In both, glucose and fructose culture conditions, TNF $\alpha$  caused a time-dependent decrease in Caco-2 TEER (**Fig. 5**). However, this effect was significantly lower when cells were cultured with fructose than with glucose.

### **Glucose and fructose effects on the gut microbiota composition**

Several studies have reported that diet-induced changes in gut microbiota composition contribute to the intestinal inflammation and alteration of the gut permeability (37). Therefore, we investigated the effects of glucose- and fructose-drinking on the caecal ecosystem composition using microbial 16S rRNA sequencing. The  $\alpha$ -diversity was measured at the OTU level using the richness (observed species) and inverse Simpson index. Both parameters showed no significant change in richness and  $\alpha$ -diversity of the microbiota in response glucose or fructose intake (**Fig. 6A** and **6B**). However, principal coordinates analysis (PCoA) of Bray-Curtis compositional dissimilarity between caecum samples from each group showed a clear separation between the communities in response to glucose and fructose (**Fig. 6C**).

Glucose-drinking mice demonstrated a more significant shift in caecal microbial composition than fructose when compared to the control mice. Bacteroidetes and Firmicutes dominated the microbiome of the 3 groups and their relative abundance was not affected by the sugar intake (**Fig. 6D**). Only, the relative abundance of Proteobacteria was modestly increased in the cecal community of the glucose group when compared to the fructose or control mice. At a lower taxonomic level, within Proteobacteria phylum, the relative abundance of *Desulfovibrionaceae* significantly increased in the cecal content of the glucose group when compared to fructose or control groups (**Fig. 6E**). Within the Firmicutes phylum, the fructose intake modestly increased the relative abundance of *Lactobacillaceae* when compared to the 2 other groups. No other changes were observed at the family level in response to fructose or glucose intake. At the species level, the effects of glucose on *Desulfovibrionaceae* were related to the main increase in abundance of *Desulfovibrio Vulgatus* (**Fig. 7A**) which was confirmed by qRT-PCR (**Fig. 7C**). Similarly, the fructose effect on *Lactobacillaceae* was associated at the species level with the increase in *Lactobacillus crispatus*, *Lactobacillus reuteri* and *Lactobacillus johnsonii* abundances (**Fig. 7B**).

Ten bacterial species were also positively increased in the caecal communities of both glucose and fructose group when compared to control mice. Six species belong to the Bacteroidetes phylum including *Bacteroides vulgatus*, 3 to the Firmicutes phylum, and 1 to the Proteobacteria phylum (**Fig.S2A**). 9 bacteria species decreased in abundance in both glucose and fructose group. Of those, 8 belong all to the Firmicutes phylum and only *Bacteroides eggerthii* was in the Bacteroidetes phylum (**Fig.S2B**).

## DISCUSSION

In the current study, we provide experimental evidence that chronic intake of enriched fructose beverage did not alter the paracellular permeability in the jejunum, caecum and colon and this despite a clear increase in systemic and caecal inflammation in these mice. Data using Caco-2 cells revealed that fructose may counteract the increase in inflammation-induced permeability, strengthening this lack of association between fructose and alteration of the intestinal paracellular permeability. To the opposite glucose intake was associated with a clear increase in paracellular permeability in the jejunum and the caecum.

Our investigation of the consequence of fructose on intestinal paracellular permeability was motivated by strong evidence from mice models fed high amounts of fructose that fructose

triggers the disruption of intestinal TJ protein expression, especially occludin, ZO1 and claudin2 (15, 18, 22, 38). In our current study, despite a minor decrease in occludin and claudin2 expression in the caecum in response to fructose intake, these changes were not associated with the alteration of intestinal permeability. In the caecum, while fructose consumption is associated with an increase in expression of IL13, TNF $\alpha$  and IFN $\gamma$  in a range similar to what is observed in response to glucose intake, the paracellular permeability remains unchanged in the mice receiving fructose when compared to the control group and increased only in the caecum of the glucose mice. The ability of cytokines to regulate TJ protein expression, assembly and modulate epithelial permeability had been well described (39, 40). IL13 is known to increase the paracellular permeability through claudin2 (41) while TNF $\alpha$  and IFN $\gamma$  required the activation of the MLCK pathway (42). TNF $\alpha$  and IFN $\gamma$  stimulate MLCK mRNA transcription, protein expression and activity which in turn activate the phosphorylation of the myosin light chain (MLC). The phosphorylated form of MLC results in the contraction of peri-junctional actinomyosin filaments and the TJ opening (13, 42-45). This is coherent with the change in paracellular permeability observed in the caecum of the mice consuming glucose since the increase in TNF $\alpha$  and IFN $\gamma$  was associated with a higher mRNA and protein expression of MLCK. However, even in a similar configuration, the mice consuming fructose failed to increase their intestinal epithelial paracellular permeability in the caecum. We also found *in vitro* that barrier dysfunction induced by IFN $\gamma$ /TNF $\alpha$  develops less rapidly when Caco-2 cell were cultured in presence of fructose when compared to glucose. Thus, taken together these data suggest that fructose is unable to activate the intestinal paracellular permeability and may be able to counteract inflammation-induced paracellular permeability increase.

The mechanism underlying this finding remains to be clarified. The phosphorylation of MLC (myosin light-chain) by MLCK and subsequent actomyosin contraction is an ATP-dependent process (46). Unlike glucose-metabolizing enzymes, the metabolism of fructose results in ATP depletion due to the lack of control of the first steps of the fructose metabolism by KHK and aldolase (47, 48). This depletion in ATP had been shown in fructose metabolizing tissues such as renal tubules (49, 50), liver (51) and intestine (52). Thus, in the enterocytes exposed to fructose, the diminution of ATP levels may prevent ATP-dependent actomyosin contraction process.

Other factors known to participate in the regulation of the intestinal permeability may be involved in the weakened effects of inflammation on paracellular permeability in presence of fructose *in vivo*. In the past few years, several studies have demonstrated that EGCs are involved in maintaining gut homeostasis (36). In mice most of the enteric glia don't coexpress S100 $\beta$  and GFAP (53-55). Thus, the specific increase in GFAP suggests that only a GFAP-positive subset of the enteric glial cells may be activated in response to fructose. Intestinal increase in GFAP protein expression had been reported to be a sign of reactive gliosis. Active gliosis had been observed in the gut of patients with inflammatory bowel disease (56, 57) and *in vitro* TNF $\alpha$  induced increase in GFAP level in EGC (55). Since ablation of GFAP-positive EGC resulted in severe gut inflammation (56, 58, 59), it had been proposed that increase in GFAP and reactive gliosis were preserving the intestinal epithelium integrity, even if a recent study challenged this hypothesis (60). In our study fructose-induced GFAP may be the sign of active gliosis which one deployed its protective effects on the integrity of the intestinal epithelium *in vivo*.

However, previous studies clearly showed that chronic intake of fructose was associated to significant increase in intestinal permeability (15, 22). However, in those studies mice received a high level of fructose (about 65%) (15) or fructose was combined with high fat diet (22). In both cases, the feeding procedure was associated with an increased glycemia. Recently, the hyperglycemia was demonstrated as a major factor able to trigger the increase in intestinal permeability (61). This glycemia-induced increase in intestinal permeability may explain the dissimilar effects of fructose on intestinal permeability obtained in our study and the one observed by others. More importantly, this hypothesis suggests that the increase in gut permeability does not precede the low-grade inflammation and glucose intolerance often observed in response to dietary fructose. On the contrary, the alteration of the gut permeability could be one of the consequences of the low-grade inflammation and glucose intolerance.

Interestingly glucose and fructose beverage intake led to intestinal inflammation, but only in the caecum. Recent study clearly demonstrates the link between diet and microbiota in the enhancement of the gut inflammation (37). We propose that the adaptation of the caecal ecosystem to glucose and fructose intake, shown by the change in  $\beta$  diversity, plays a role in the onset of intestinal inflammation. The more prominent family affected by glucose is the *Desulfovibrionaceae* with a marked increase in relative abundance of *Desulfovibrio Vulgaris*.

Interestingly *desulfovibrionaceae* are endotoxin producers (62) and have been linked to glucose tolerance in response to western diet intake in mice (63) and are also associated with obesity in human (64). Moreover, *Desulfovibrionaceae* are sulfate-reducing bacteria that generate hydrogen sulfide (H<sub>2</sub>S) (65) which one had been shown to be able to induce pro-inflammatory cytokines (66) and the abundance of H<sub>2</sub>S producing bacteria had been negatively correlated to intestinal permeability (67). Intake of both glucose and fructose was also associated with increase in relative abundance of *Bacteroides vulgatus* which has been reported to correlate positively with insulin resistance and type 2 diabetes in human (68, 69). Thus *Bacteroides vulgatus* and *Desulfovibrio Vulgaris* could represent intestinal microbiota species responding to glucose or fructose intake and contributing to the onset of inflammation in the distal intestine in response to sugar intake.

This study provides strong evidence that physiological amounts of fructose do not alter the intestinal paracellular permeability in any of the regions of the GI tract. Since the intestinal inflammation and the levels of IL6 were already elevated in response to fructose we believe that alteration of intestinal permeability does not precede but follows the onset of metabolic outcome (low-grade inflammation, hyperglycemia) associated with chronic fructose consumption. We have also demonstrated that despite being absorbed in the small intestine, glucose intake has major harmful effects on the intestinal paracellular permeability of the caecum where it may interact with the microbiota. In particular, glucose-induced increased abundance of bacteria of *Desulfovibrionaceae* family might participate to the deleterious outcome of high glucose consumption.

## **AUTHOR CONTRIBUTIONS**

The authors' responsibilities were as follows: XZ, CH, and VD designed the research; XZ, FD, FBC, CH and VD conducted the research; XZ, CH, MM, and VD analyzed the data; XZ and VD wrote the manuscript; XZ, CH, HB and VD contributed to the discussion. The authors have declared no conflicts of interest.

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**TABLE:****Table 1:** Primer used for SYBR green Quantitative Real Time-PCR

Primers for SYBR Green		
Name (Gene)	Gene Bank accession n°	Primers
$\beta$ Actin ( <i>beta-actin</i> )	NM_007393.4	Forward: 5'-CTAAGGCCAACCGTGAAAAG-3' Reverse: 5'-ACCAGAGGCATACAGGGACA-3'
tight junction protein 1 ( <i>Tjp1</i> or <i>ZO1</i> )	NM_009386.2	Forward: 5'- GATCATTCCACGCAGTCTCC -3' Reverse: 5'- GGCCCCAGGTTTAGACATTC -3'
Claudin2 ( <i>Cldn2</i> )	NM_016675.4	Forward: 5'- GTAGCCGGAGTCATCCTTTG -3' Reverse: 5'- GGCCTGGTAGCCATCATAGT -3'
Occludin ( <i>Ocln</i> )	NM_008756.2	Forward: 5'- GCGATCATACCCAGAGTCTTTC -3' Reverse: 5'- TGCCTGAAGTCATCCACACT -3'
myosin light polypeptide kinase ( <i>Mlck</i> )	NM_139300.3	Forward: 5'- ATAGCCTTGGCCAGGTGTC -3' Reverse: 5'- GCTGTCTGTGCCAAGTTCG -3'
interleukin 1 beta ( <i>Il1<math>\beta</math></i> )	NM_008361.4	Forward: 5'- AGTTGACGGACCCCAAAG -3' Reverse: 5'- AGCTGGATGCTCTCATCAGG -3'
tumor necrosis factor ( <i>Tnf</i> )	NM_013693.3	Forward: 5'-CTGTAGCCCACGTCGTAGC-3' Reverse: 5'-TTGAGATCCATGCCGTTG-3'
interleukin 13 ( <i>Il13</i> )	NM_008355.3	Forward: 5'-ACCCAGAGGATATTGCATGG-3' Reverse: 5'-TGGGCTACTTCGATTTTGGT-3'
interferon gamma ( <i>Ifn<math>\gamma</math></i> )	NM_008337.4	Forward: 5'-ATCTGGAGGAACTGGCAAAA-3' Reverse: 5'-TTCAAGACTTCAAAGAGTCTGAGGTA-3'

**FIGURE LEGENDS:**

**Figure 1. Effects of glucose and fructose on metabolic parameters.** (A) Body weight changes during 9 weeks. (B) Average daily caloric intake originating from solid diet or drink solutions. (C) Blood glucose levels during oral glucose tolerance tests. (D) Area under the curve (AUC) of blood glucose levels. Plasma levels of (E) IL6 (F) INF $\gamma$  and (G) IL13. All values are means  $\pm$  SEM; n=8/group; \* indicates a significant difference among groups ( $p < 0.05$ ).

**Figure 2. Paracellular permeability, tight junction protein and inflammatory marker expression levels in various region of the GI tract.** *Ex vivo* (in Ussing chamber) paracellular permeability to FITC-Sulfonic Acid (FITC-SA, 400Da) was measured in the jejunum (A1), caecum (A2) and colon. The mRNA expression levels of tight junction proteins were measured in the jejunum (B1), caecum (B2) and colon (B3). The mRNA expression levels of inflammatory cytokine markers were measured in the jejunum (C1), caecum (C2) and colon (C3). The mRNA levels data were normalized to control group. All values are means  $\pm$  SEM; n=8/group. \* indicates significant differences among groups ( $p < 0.05$ ). FITC-SA: Fluorescein isothiocyanate-Sulfuric acid; *ZO-1*: Zonula occludens-1; *Mlck*: Myosin light-chain kinase; *Il1 $\beta$* : Interleukin 1 beta; *Tnf $\alpha$* : Tumor necrosis factor alpha; *Il13*: Interleukin 13; *Ifn $\gamma$* : Interferon gamma.

**Figure 3. Protein expression level of MLCK in the caecum.** (A) Representative images of Western blots for MLCK. (B) Relative band intensity of MLCK normalized to GAPDH and expressed relative to control group. All values are means  $\pm$  SEM; n=6/group. MLCK: Myosin light-chain kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

**Figure 4. Protein expression level of GFAP and S100 $\beta$  in the caecum.** Representative images of Western blots for (A) GFAP and (B) S100 $\beta$ . Relative band intensity of (C) GFAP expressed relative to control group and (C) S100 $\beta$  normalized to GAPDH and expressed relative to control group. All values are means  $\pm$  SEM; n=4/group. GFAP: Glial fibrillary acidic protein; S100 $\beta$ : S100 calcium-binding protein B; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

**Figure 5. The effects of glucose and fructose on barrier function in response to cytokines in Caco-2 cells.** Caco-2BBE cells were differentiated on transwells for 17 days after confluence until reaching stable TEER. Cells were cultured for 24h in presence of glucose or fructose DMEM before exposure to cytokines. The cells were treated, in presence of glucose or fructose, with 10ng/ml IFN $\gamma$  in the basal chamber for 24 hours prior TNF $\alpha$  treatment. The TEER was measured for 5h following the exposure to TNF $\alpha$ . All values are means  $\pm$  SEM; n=6-8/group; <sup>a, b, c</sup> at each time point means without a common superscript letter differ ( $P < 0.05$ ) as analyzed by two-way ANOVA.

**Figure 6. Impact of glucose and fructose on richness and diversity of bacterial caecum content.** Analysis was based on 16S rDNA sequencing (region V3-V4). (A) Observed species richness and (B) Inverse Simpson Index as indicators of  $\alpha$ -diversity. (C) Principal coordinates analysis (PCoA) of Bray-Curtis compositional dissimilarity at the operational taxonomic unit (OTU) level. Each dot represents of one mice. Relative distribution of (D) phyla, (E) families and (F) genus. For figures D, E and F, each column represents bacterial community of one mice.

**Figure 7. Bacterial species abundance modified by fructose or glucose intake.** Effects of (A) glucose intake or (B) fructose intake on bacterial composition of members of Bacteroidetes, Firmicutes, and proteobacteria at the species level. The increased abundance of *Desulfovibrio* induced by glucose was confirmed by qPCR (C). For A and B plots, the Y-axis refers to number of read determined after rarefaction of the samples. For plot C Data were normalized to levels in control and universal 16S primer was used as a reference. All values are means  $\pm$  SEM; n=8/group. \* indicates significant differences among groups ( $p < 0.05$ ).

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**Fig.1**

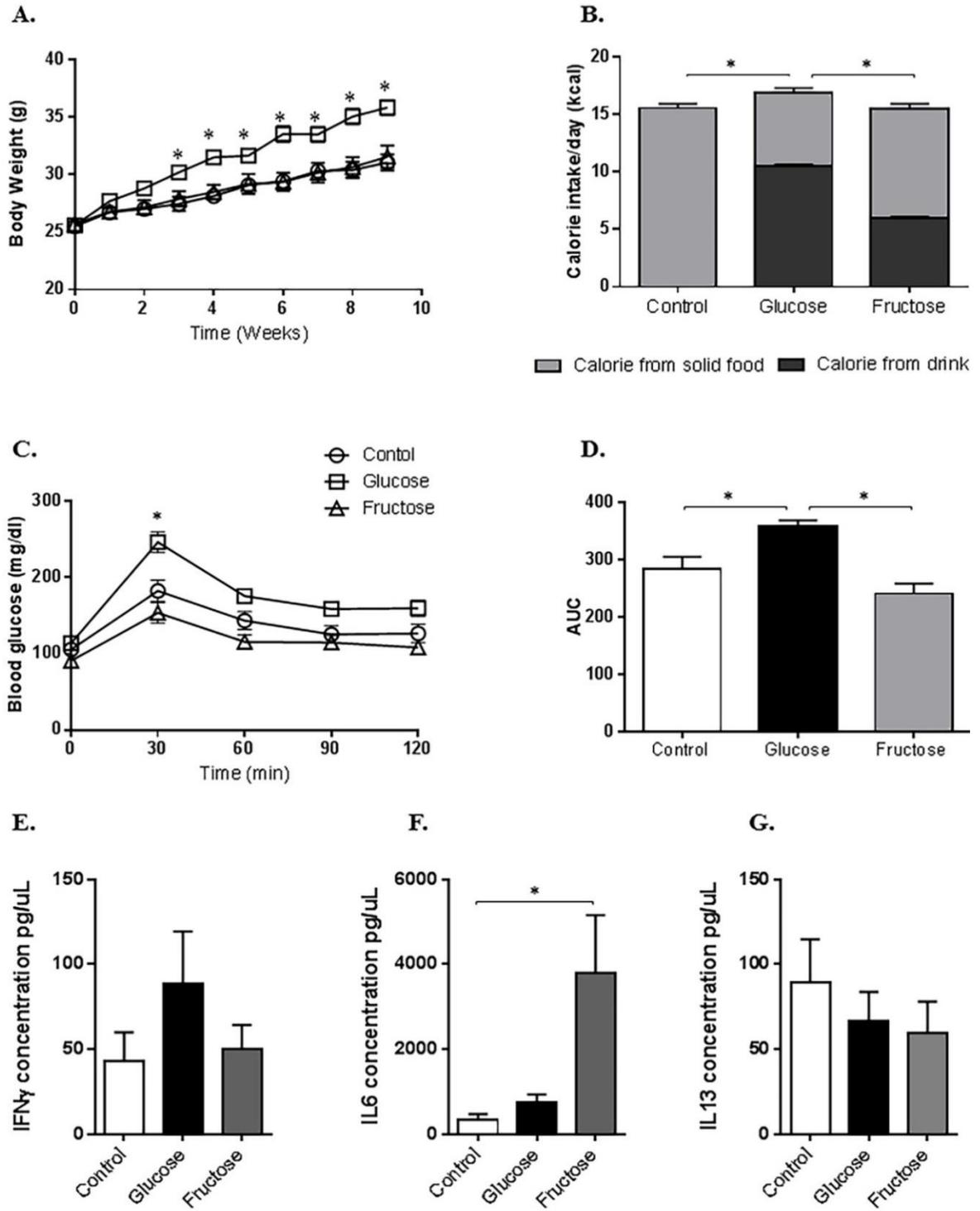
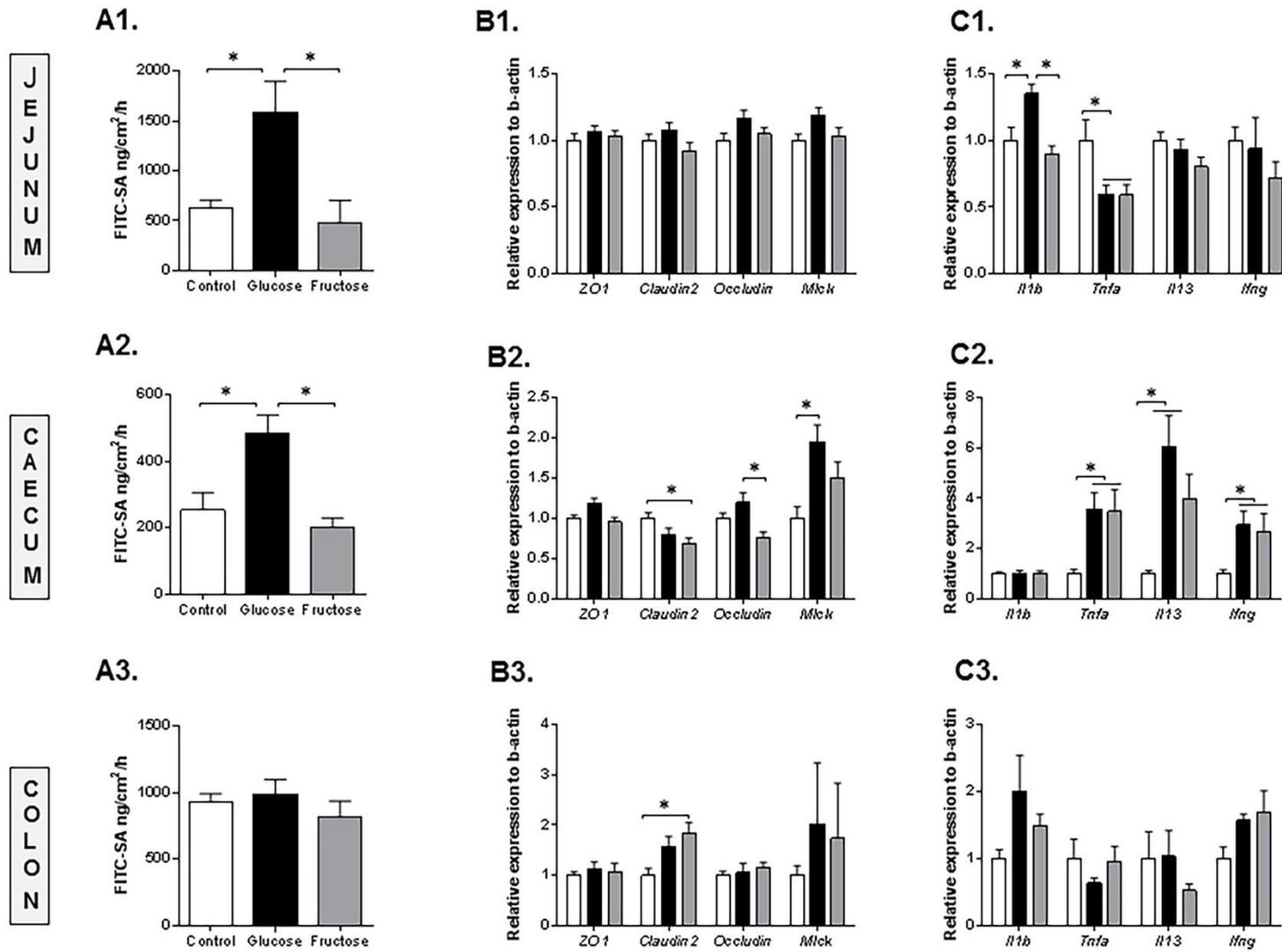


Fig.2



**Fig.3**

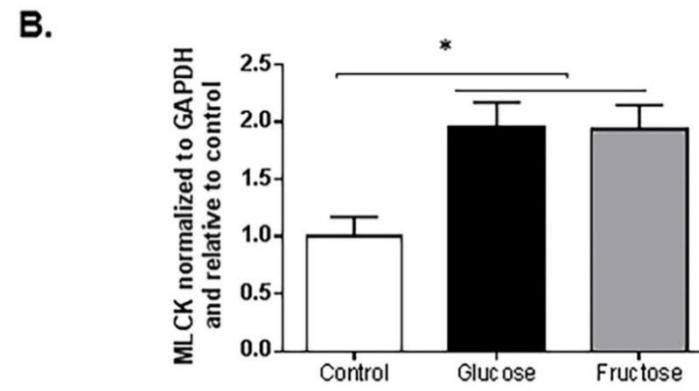
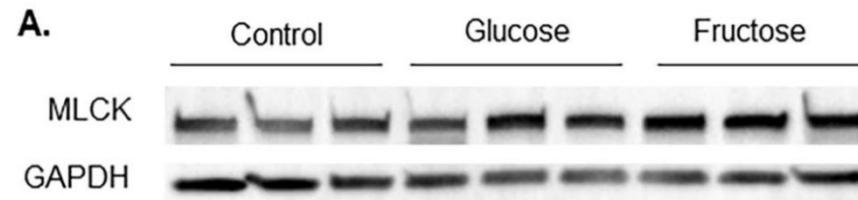
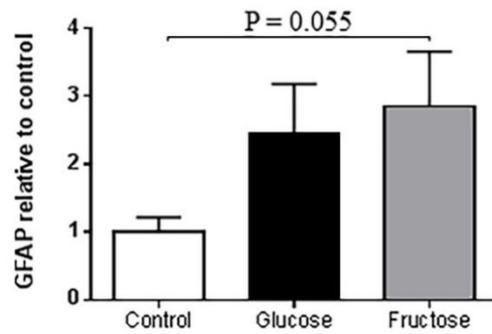
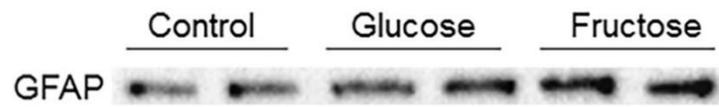


Fig.4

A.



B.

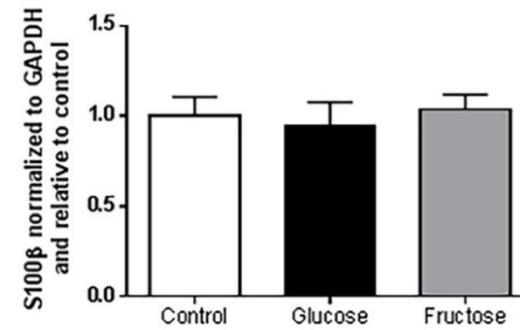
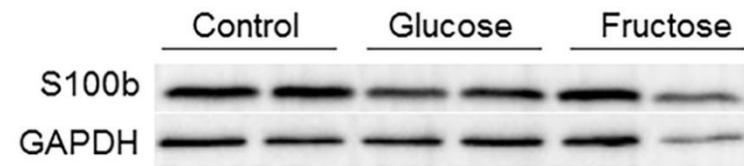


Fig.5

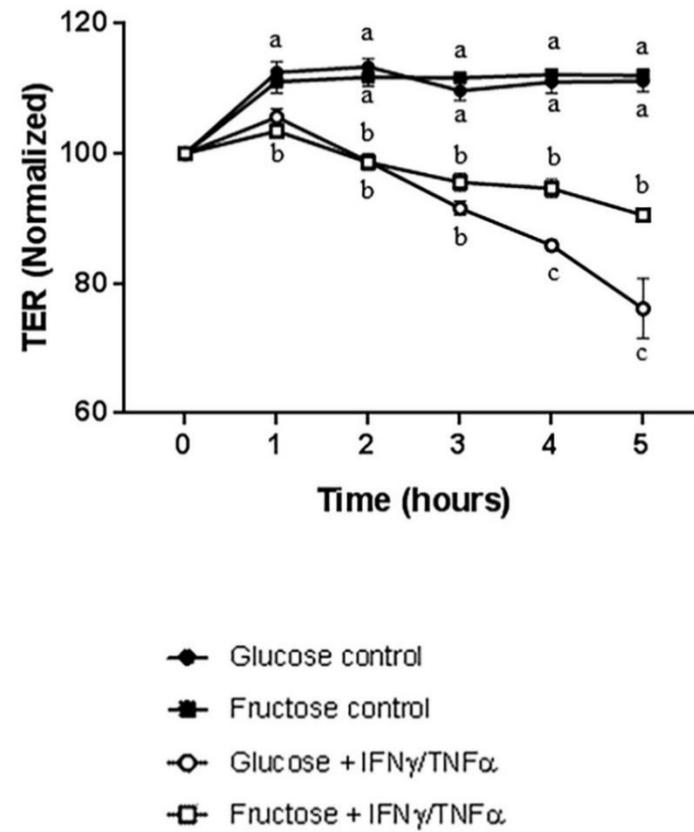


Fig.6

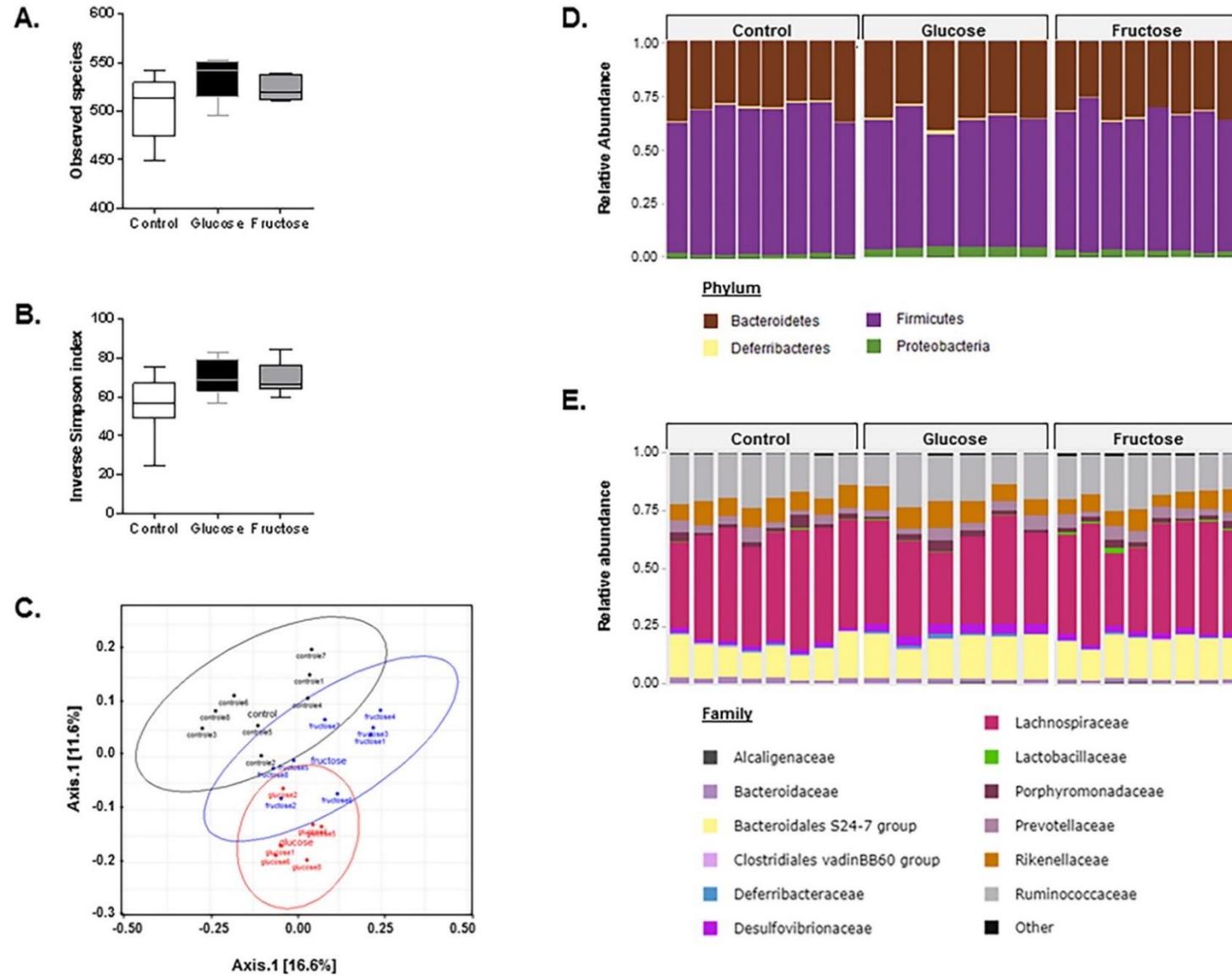
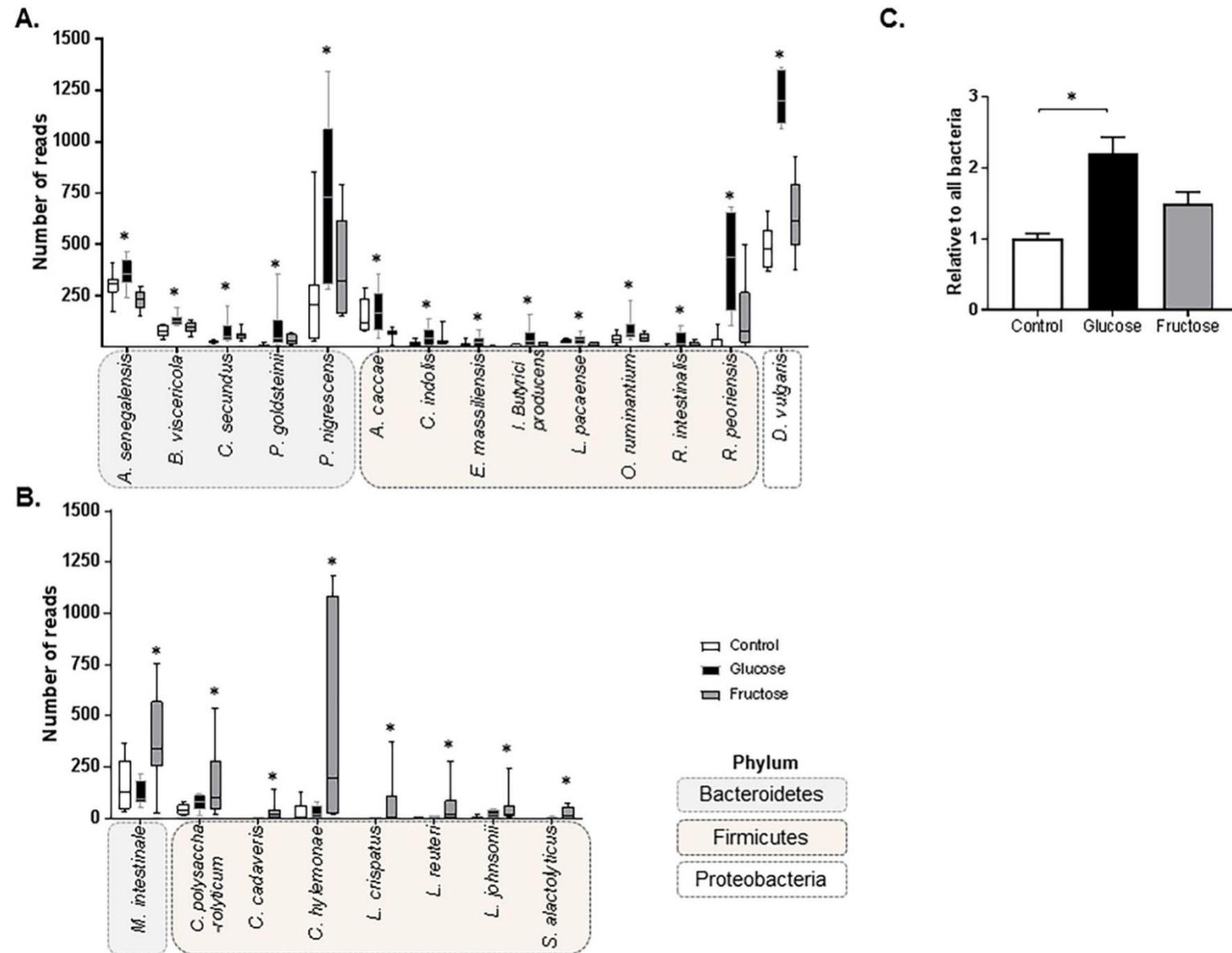
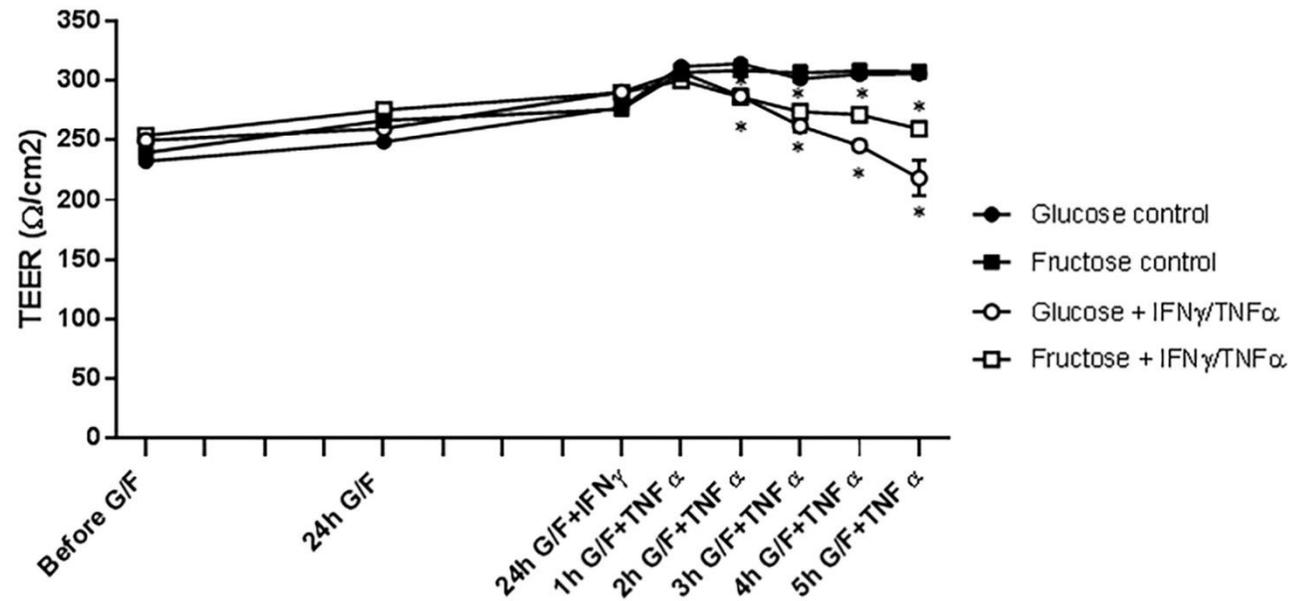


Fig.7



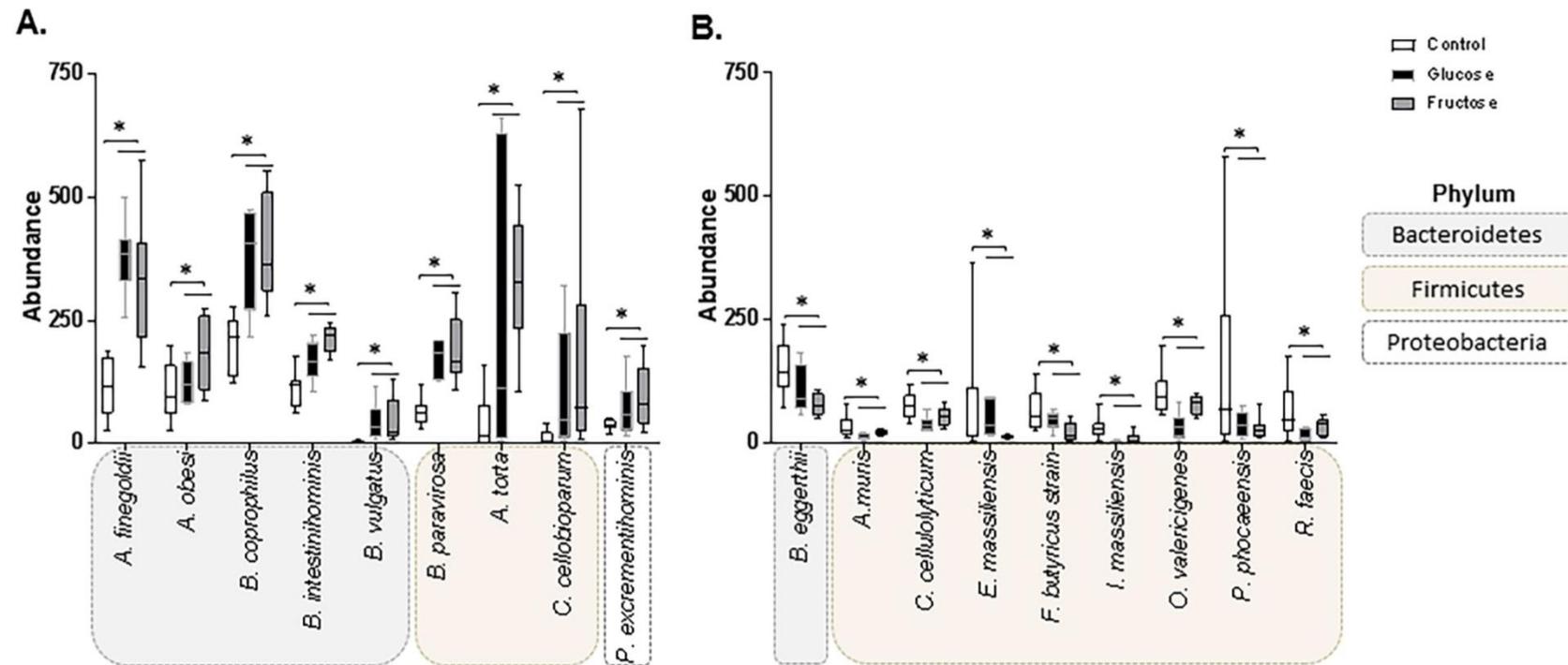
## Supplementary figures

Fig. S1



**Fig S1. The effects of glucose and fructose on barrier function.** Caco-2BBE cells were differentiated on transwells for 17 days after confluence in DMEM. Cells were cultured for 24h in presence of glucose or fructose DMEM before exposure to cytokines. No changes in TEER were detected after switching the Caco-2 cell from a glucose DMEM media to a fructose DMEM media. Then, the cells were treated, in presence of glucose (G) or fructose (F), with 10ng/ml IFN $\gamma$  in the basal chamber for 24 hours prior TNF $\alpha$  treatment for 5h. The TEER was measured during the entire procedure. All values are means  $\pm$  SEM; n=6-8/group; \*p < 0.05 compared to control glucose.

Fig. S2



**Fig S2. Bacterial species abundance modified similarly by fructose and glucose intake.** Bacteria displaying (A) an increased or (B) a decreased in their relative abundance in response to glucose and fructose intake. The Y-axis refers to number of read determined after rarefaction of the samples.



**GENERAL DISCUSSION**

**&**

**PERSPECTIVES**

## GENERAL DISCUSSION AND PERSPECTIVES

In this thesis project, the effects of dietary fructose on intestinal homeostasis were explored in two different physiological contexts: fructose malabsorption, and normal fructose absorption, using mice (KHK<sup>-/-</sup> or WT) exposed to physiological quantities of fructose (15-20% in diet or beverage). We demonstrated that gut microbiota composition and metabolism change in response to fructose malabsorption in association with a major increase in CCK in the ileum and caecum. We linked the changes in microbiota to the intestinal endocrine response by showing that the propionate, a microbial metabolite upregulated in the caecum content of the mice with fructose malabsorption, regulates CCK. We also identified specific CCK-expressing EEC subtypes that increase in number corroborating recent observations showing that EECs are plastic and that they are able to modulate the panel of peptides they expressed in response to changes in the luminal environment. Finally, we associated the raised in CCK with an increase in visceral pain. Therefore, our results provide a possible explanation for the symptoms experienced by patients with fructose malabsorption. On the other hand, glucose but not fructose stimulated the increase of intestinal permeability in the jejunum and caecum. In the caecum, the glucose-induced increase in permeability is likely regulated by MLCK pathway in response to an increase in pro-inflammatory cytokines. Surprisingly, despite being associated with increases in intestinal inflammation and MLCK protein level, fructose intake did not modify the intestinal permeability. Based on the data of my PhD project and on the literature, we will discuss: (1) the possible physiological consequences of the increase in CCK in response to fructose malabsorption, (2) the role of fructose-induced CCK in the development of the symptoms associated with the fructose malabsorption, (3) the role of the microbiota and microbial metabolites in the regulation of CCK, (4) the impact of luminal fructose and intestinal fructose metabolism on gut permeability and (5) the unlikely central role of the gut barrier dysfunction in mediating the deleterious effects associated with chronic fructose intake.

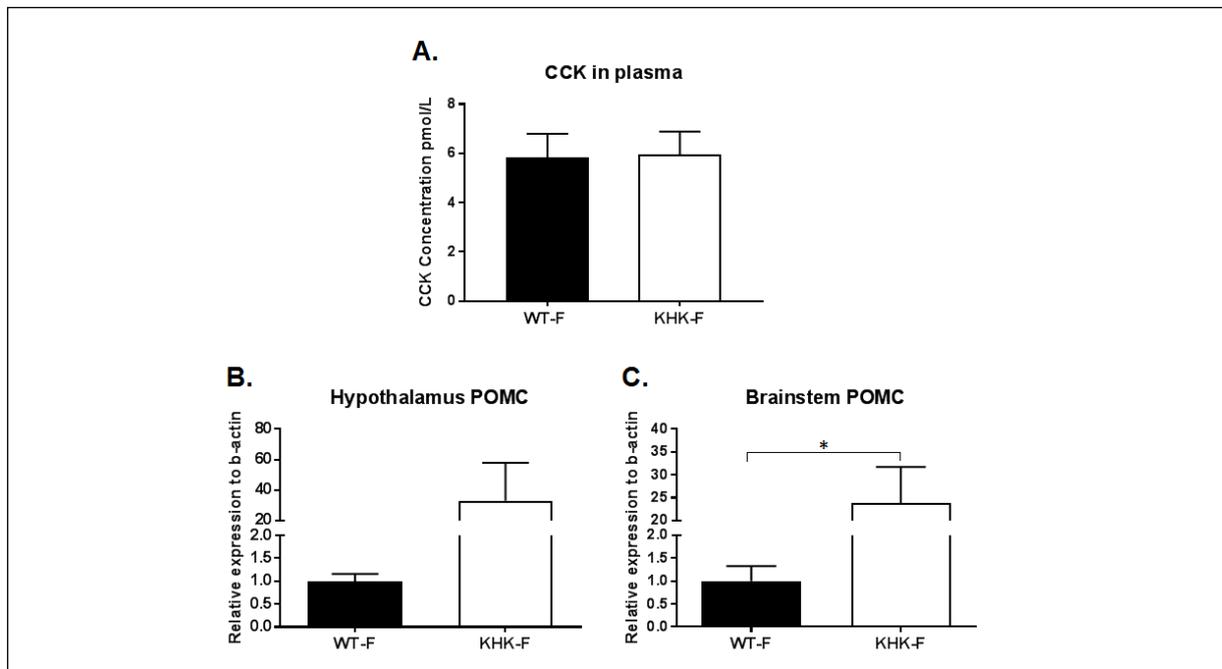
### **I-The potential physiological consequence of the increase in CCK in the lower intestine**

In this project, we discovered that fructose malabsorption induced a major increase in CCK expression and secretion in the caecum (**Article 1, Figs. 1C and 2D**). For a longtime the I-cells from the duodenum were believed to be the only cells supporting the gastrointestinal secretion of CCK. This unusual expression pattern had been discussed above (see the discussion of the article 1) in the context of the recent literature about the plasticity of the

EECs. Nevertheless, our results also invite a question whether ileal/caecal CCK regulates the same physiological functions that the CCK secreted in the duodenum. In the present work, no change in portal levels of CCK was observed (**Figure 28A**). This could be explained by the lack of synchronization in term of “time after feeding” of the mice before euthanasia since the release in CCK in response to meal happens in a 10 to 60 min window (Liddle et al., 1985). However, the responses to intestinal CCK are mainly supported by the activation of the afferent vagal nerve which is connected to the EECs through paracrine transmission (Raybould and Tache, 1988) or even, as demonstrated recently, by a direct EECs-neuron interaction (Bohorquez et al., 2015, Kaelberer et al., 2018). Thus, even in the absence of increased levels of plasmatic CCK, CCK could support a variety of physiological responses. The action of CCK is mediated by different receptors. For instance, CCK1R receptor is involved in the pancreatic exocrine secretion (Takiguchi et al., 2002) and gallbladder contractions (Xu et al., 2008a) in response to protein and fat digestion. Those parameters were not measured in our study. Still, the NMR analysis of the caecum content clearly showed changes in the bile acid composition in the KHK-F (**Article 1, Figs. 5C and 7C**), but this could also result from the drastic changes observed in the microbial composition. In order to determine whether fructose malabsorption affects the GI functions regulated by CCK, it would be interesting to determine the profile of the bile acids, the gut motility and gallbladder contraction in the KHK-F and their counterpart controls.

CCK also regulates food intake through the activation of CCK1R of the vagal nerve (Moran et al., 1997) which subsequently relay the information to specific brain sites that regulate food intake, including the nucleus tractus solitarius (localized in the brainstem) and the hypothalamus (Moran et al., 1998, Bellissimo and Anderson, 2003, Clerc et al., 2007). In the nucleus tractus solitarius, CCK activates POMC neurons (Wang et al., 1998, Appleyard et al., 2005) which contribute to the satiety effects of CCK (Fan et al., 2004). In agreement with these data, we found that the POMC expression level was significantly upregulated in the brainstem of the KHK-F when compared to the WT-F, but not in the hypothalamus (**Figure 28B and C**). However, no significant changes in food intake and body weight were observed during the feeding period in the KHK-F when compared to the WT-F (**Article 1, Fig. S4**). Chronic CCK treatments resulted in the reduction of meal size but in the increase in meal frequency (Crawley and Beinfeld, 1983, West et al., 1987). These effects of CCK on food intake pattern may explain the lack of effect of KHK-F-induced CCK on body weight and global food intake. Therefore, it would be interesting to investigate precisely the feeding

pattern in the KHK-F in order to determine whether CCK secreted by the ileum and caecum could also play a role in regulating the satiety in response to a meal.



**Figure 28. Plasmatic CCK and Central POMC gene expression in fructose fed wild type and Ketoheokinase knockout mice**

Wild type (WT) and ketoheokinase knockout (KHK<sup>-/-</sup>) mice were fed with fructose (WT-F and KHK-F respectively) for 3 or 8 weeks (n=5-6/group). CCK levels in plasma were measured by RIA after 8 weeks of feeding (A). Gene expression levels of POMC were measured in the (B) hypothalamus and (C) brainstem after 3 weeks of feeding. All values are means  $\pm$  SEM; means were compared by Mann-Whitney test; \*p<0.05. CCK: Cholecystokinin; RIA: Radioimmunoassay; POMC: Proopiomelanocortin.

## II-The potential link between the increase in intestinal CCK and the symptoms associated with fructose malabsorption

In humans, fructose malabsorption is associated with recurrent abdominal pain (Wintermeyer et al., 2012, Escobar et al., 2014). In this thesis project, we showed that when the KHK<sup>-/-</sup> mice were exposed to fructose they also developed visceral hypersensitivity (Figure 26). We proposed that the visceral pain associated with the fructose malabsorption is the result of the upregulation of CCK in the lower intestine. Interestingly, IBS patients display a high level of plasmatic CCK (Zhang et al., 2008) which had been suspected to participate in the visceral pain symptoms associated with that condition (van der Schaar et al., 2013). Furthermore, as described above, the CCK receptors are present in colonic smooth muscles and enteric neurons. Therefore, they are able to regulate sensory and motor functions in the lower

intestine in response to an increase in CCK secretion (Morton et al., 2002, Fornai et al., 2007, Ko et al., 2011). In addition, CCK also activates secretion of proteases (e.g. amylase and trypsin) (Liddle et al., 1985, Evilevitch et al., 2004, Jaworek et al., 2010) which were proposed to be involved in visceral hypersensitivity of the IBS patients (Cenac et al., 2007). Interestingly, patients with fructose malabsorption also display high serum amylase concentrations (Ledochowski et al., 2001a) whose origin remains unknown. This suggests that fructose malabsorption-induced increase in CCK may either stimulate pancreatic amylase secretion thereby causing visceral hypersensitivity in patients with fructose malabsorption, or that CCK directly triggers the visceral pain via the CCK receptors of the GI wall. To test these hypotheses, it would be of high interest to measure the levels of CCK in the plasma or in intestinal biopsies of distal intestine in patients with fructose malabsorption. Evaluation of proteases in the plasma and in the intestinal tissues of the *KHK<sup>-/-</sup>* mice fed fructose would explore more thoroughly the hypothesis of the protease in the onset of the visceral pain. Thus, our results provide a possible explanation for the symptoms experienced by patients with fructose malabsorption. Confirming the role of CCK and clarifying the mechanisms involved would allow us to identify a number of possible therapeutic entry points to alleviate this condition.

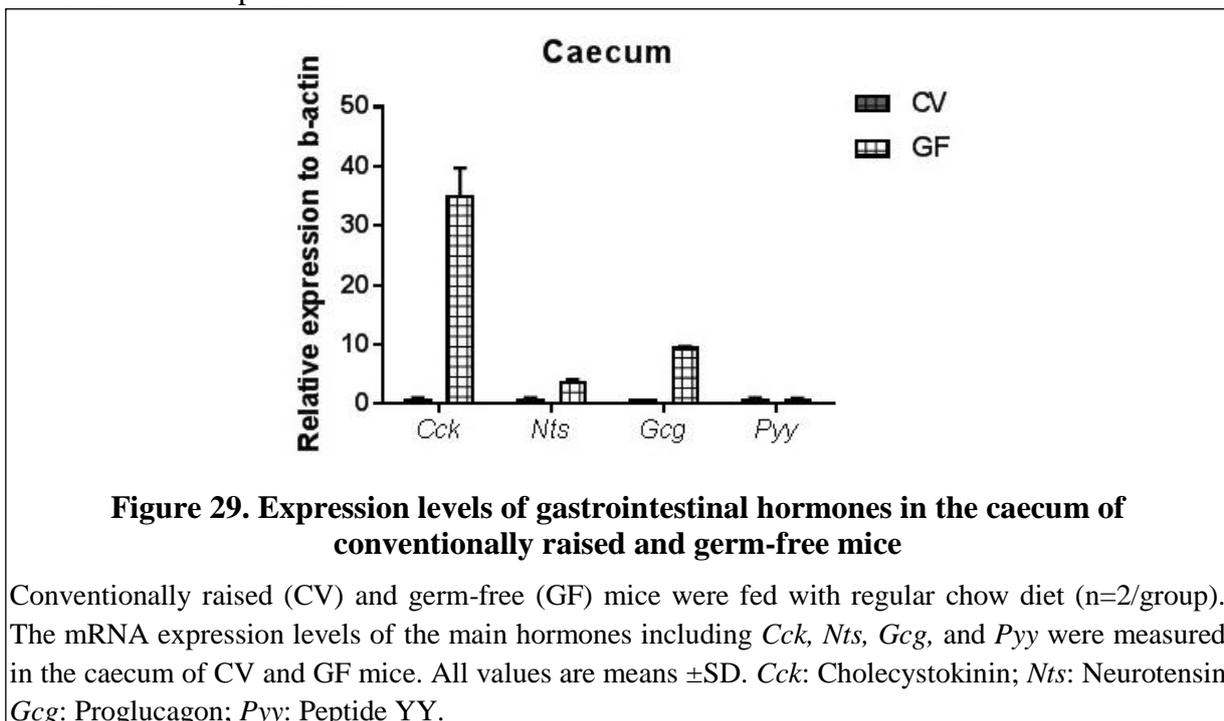
A higher incidence of early signs of depression has been reported in patients with fructose malabsorption than those with other gastrointestinal disorders (Ledochowski et al., 1998, Ledochowski et al., 1999, Ledochowski et al., 2000, Ledochowski et al., 2001b, Enko et al., 2018). Chronic intake of high-fructose (65%) diet was also associated with hyper-anxiety (Reddy et al., 2016). The activation of CCK-ergic system had been demonstrated to be involved in the development of anxiety and depression behaviors (Dauge and Lena, 1998). This mainly involves the CCK2R expressed in numerous brain structures such as the cortex and the limbic system (including the hippocampus, the amygdala and the nucleus accumbens) (Moran et al., 1986, Del Boca et al., 2012, Desai et al., 2014). Whether the CCK secreted in the GI tract (especially in the lower intestine) could regulate mood behaviors remains unclear. However, peripheral administrations of CCK, CCK antagonists or agonists can modulate or induce anxiety or depression behaviors in humans and rodents (Dauge and Lena, 1998). It would be interesting to explore dependence of these behaviors on fructose consumption using our *KHK<sup>-/-</sup>* mouse model and to investigate the precise role of the CCK-ergic system by using specific agonist of CCK2R or CCK1R.

### III-Nature of the stimuli able to activate CCK in the lower intestine

As mentioned above, in the discussion of the article 1, it is unlikely that fructose itself changes the gut peptide expression in the ileum and caecum since no increase in *Cck* or other peptide expressions was detected in the duodenum and jejunum, where the concentrations of luminal fructose are expected to be the highest. Moreover, the drastic changes in gut microbiota composition in KHK-F mice and the effect of antibiotic treatment suggest that the microbiota, likely via the compounds that it generates from fructose metabolism, is involved in the EEC response to fructose malabsorption. Until now, the regulation of CCK in the distal intestine has not been studied. Thus, the nature of the stimuli able to activate CCK expression and secretion in the ileum and caecum of the KHK-F remains to be uncovered. Since the effect of fructose malabsorption was restricted to the lower intestine, we first examined the possible role of bacterial metabolites, such as SCFAs, already known to regulate the expression of other GI hormones. Our NMR data revealed a significant and specific increase in the propionate in the caecal content of the KHK-F mice, and we confirmed the ability of propionate to activate the expression of CCK *in vitro* in the GLUTag cells. The role of propionate in the regulation of PYY and/or GLP-1 had been described *in vivo* and *in vitro* (Chambers et al., 2015, Psichas et al., 2015, Larraufie et al., 2018). However, to our knowledge the role of propionate, and through it indirectly the role of the gut microbiota, in the regulation of CCK had never been described before. Interestingly, a drastic diminution of the gut ecosystem (after antibiotic treatment) or the total absence of bacteria in the GI tract (GF mice) induce a major increase: (1) in GLP-1 cells density in proximal colon, (2) in *Gcg* expression level in caecum and (3) in the plasmatic level of GLP-1 (Wichmann et al., 2013, Selwyn et al., 2015). We observed in our data an increase in *Gcg*, *Nts* and *Cck* expression levels in the caecum of the mice treated with antibiotics (**Article 1, Fig. 6**) which unfortunately interferes with the interpretation of our results. In order to strengthen the potential role of gut microbiota in the modifications of the GI hormone expression profile observed in the KHK<sup>-/-</sup> mice, we determined whether NTS and CCK also respond to the absence of microbiota. In the caecum of GF mice, *Cck* expression was 30 times higher than in the conventionally raised mice (**Figure 29**) and *Nts* and *Gcg* expression was 5 and 10 times higher respectively. Thus, the microbiota seems to be able to play a major role in the regulation of CCK in the lower intestine, similarly to what had been described until now for GLP-1. Despite not being explored further in our project, it seems also that *Nts* expression is regulated by microbial metabolites. Until now we showed that at least lactate is able to

regulate its expression (**Article 1, Fig. 8**). Further exploitation of our NMR data needs to be done to determine if other bacterial metabolites (*e.g.* the bile acids) may be involved.

Another possible pathway related to the role of microbiota in the regulation of CCK is the ability of the bacteria to synthesize *de novo* most of the amino acids (Gill et al., 2006). Indeed, among the nutrients known to regulate the expression and secretion of CCK in the small intestine, the amino acids had been vastly studied (Wang et al., 2011). As mentioned in the introduction, phenylalanine, leucine, tryptophan, and glutamate had been shown to activate the release of CCK (Wang et al., 2011, Daly et al., 2013, Shirazi-Beechey et al., 2014). Phenylalanine, leucine, and tryptophan derived from intermediates of glycolysis, such as pyruvate or phosphoenolpyruvate. Because of the accumulation of glucose as well as lactate and fumarate in the caecum of the KHK-F (**Article 1, Fig. S5**), those glycolytic intermediates are likely available for the bacteria and may contribute to the *de novo* synthesis of amino acids. To explore furthermore this pathway, experiments using KHK<sup>-/-</sup> mice fed labeled fructose (C<sup>-13</sup>) in association with analysis dedicated to caecal amino acids identification would need to be performed.



#### IV-The role of the fructose-induced changes in the microbiota composition in the alteration of gastrointestinal endocrine and barrier functions

As shown in our results section, the relative abundance of *Lactobacillus*, particularly *Lactobacillus johnsonii*, was strongly increased in the caecum of the KHK-F mice (**Article 1, Fig. 4J**). The abundance of *Lactobacillus johnsonii* and other bacteria belonging to

*Lactobacillus* genus (*Lactobacillus Crispatus*, *Lactobacillus reuteri*) was also modestly increased in the caecum of the mice that received 15% fructose in the drinking solution (**Article 2, Fig. 7b**). *Lactobacilli* belong to a family of bacteria able to use carbohydrates, including sucrose and fructose, as energy source for growth (Anwar et al., 2008). Thus, increase in *Lactobacillus* abundance in the caecum of the KHK-F mice is likely to indicate that fructose had reached this GI section in the KHK<sup>-/-</sup> mice fed fructose. Our inability to detect fructose by NMR may result from its rapid use by the microbiota of the KHK-F mice after it adapted to the presence of fructose to optimize its metabolism. *Lactobacillus johnsonii* is known as a probiotic and had been reported to have immunomodulation properties (Ashraf and Shah, 2014) and protective effects on intestinal barrier integrity (Liu et al., 2015). Those protective characteristics of *Lactobacillus johnsonii* might explain the lack of inflammation and the reinforcement of the intestinal barrier functions observed in the intestine of the KHK-F. The abundance of other bacteria belonging to the Actinobacteria phylum (*Bifidobacteriaceae*, the *Coriobacteriaceae* and the *Corynebacteriaceae*) also strongly increases in response to fructose malabsorption. From now on, the potential ability of those bacteria to regulate the expression of *Cck* or other GI hormones in response to fructose malabsorption needs to be investigated. The consequences of the fructose availability in the caecum may be compared to the effects of inulin and oligofructose on the gut microbiota composition. Inulin and oligofructose are both indigestible polymers composed mainly of fructose units linked by  $\beta$ -(1 $\rightarrow$ 2) d-fructosyl-fructose bonds and that bypass the digestion and absorption in the small intestine, but are digestible by bacteria. Inulin is composed of 2 to 60 fructose units and the oligofructose of 2-10 units. Numerous studies reported the selective increase in *Bifidobacterium* and to a lesser extent in *Lactobacillus* in response to dietary supplementation in inulin or oligofructose (Meyer and Stasse-Wolthuis, 2009, Weitkunat et al., 2017). Interestingly, a very recent paper showed a dose-response effect of inulin on the induction of *Cck* expression level in the caecum and colon in rats (Singh et al., 2018). A similar effect was observed for *Pyy* and *Gcg* mRNA levels. In this paper, the authors also reported a positive correlation between caecal content in *Bifidobacterium* spp. or *Lactobacillus* and mRNA abundance of *Cck*, *Pyy* and *Gcg*, suggesting a major role of *Bifidobacterium* spp. and *Lactobacillus* in the regulation of the peptide expression. These data corroborate ours, and reinforce the idea that Actinobacteria or *Lactobacillus johnsonii* are potential activators of the *Cck* and *Gcg* expression. Furthermore, fructose or other sugars must be available in the lower intestine. A network of bacteria probably participates in the

metabolic cascade leading to the production of the active metabolites (SCFAs such as propionate or others that remain to be uncovered) from fructose metabolism (**Article 1, Fig. S5**). For example, in addition to *Lactobacillus*, *Lachnospiraceae* family abundance also significantly increased in KHK-F. Member of the *Lachnospiraceae* family such as *Coprococcus* are able to produce propionate from numerous hexoses (Reichardt et al., 2014) and therefore could be relevant for the changes in peptides expression observed in response to fructose malabsorption. Interestingly, some bacteria belonging to the *Ruminococcus* family showed the strongest increase in abundance in response to fructose malabsorption. *Ruminococcus spp.* are able to produce propionate but from fucose (a major component of host-derived glycan) but not from fructose or other hexoses (Reichardt et al., 2014). This suggests that mucin degradation could be implicated in the production of propionate observed in the KHK in response to fructose malabsorption.

Besides the Firmicutes, bacteria belonging to the Proteobacteria phylum, such as *Desulfovibrio*, were also strongly impacted by the dietary sugar. On one hand, *Desulfovibrio vulgaris* was strongly increased in the WT mice receiving 15% glucose (**Article 2, Fig. 7A**). In the caecum of the KHK-F, *Desulfovibrio simplex* abundance was strongly decreased while the one of *Desulfovibrio vulgaris* increased markedly (**Article 1, Fig. 4J**). *Desulfovibrio* is a sulfate-reducing bacteria that generate hydrogen sulfide (H<sub>2</sub>S) (Montgomery et al., 1990). Usually, H<sub>2</sub>S results from the fermentation of sulfur-containing amino acids and it had been shown to induce the activation of pro-inflammatory cytokines (Bhatia, 2015). *Desulfovibrionaceae* are also endotoxin producers (Zhang-Sun et al., 2015) associated with increased gut permeability (Panasevich et al., 2018) and glucose intolerance (Zhang et al., 2010). And like H<sub>2</sub>S, *Desulfovibrio*-derived LPS is able to activate the epithelial inflammatory response (Weglarz et al., 2003). Consequently, the increased abundance of *Desulfovibrio vulgaris* in response to glucose, but not fructose, may contribute to the increase in inflammation and permeability associated with glucose intake. Recently, H<sub>2</sub>S was discovered to stimulate the GLP-1 secretion in the gut epithelium (Pichette et al., 2017) emphasizing the potential role of *Desulfovibrio* in endocrine functions of the gut. Thus, the increased abundance of *Desulfovibrio desulfuricans* observed in KHK-F mice may also contribute to the increase in *Gcg* expression levels in response to fructose malabsorption. However, since H<sub>2</sub>S is also associated with anti-inflammatory properties as well as pro- and anti-nociceptive effects (Linden, 2014), further investigations are needed to clarify the role the sulfate-reducing bacteria in our different models.

Despite a clear characterization of the sugar-induced changes in gut microbiota composition, the specific role of single or a few bacteria in the regulation of either enteroendocrine hormone expression and/or intestinal barrier function still needs to be addressed. To examine the role of distinct bacteria in the intestinal endocrine functions, single bacteria supernatant or inactive bacteria could be added to GLUTag cell cultures to determine their ability to activate specific peptide expression/secretion. A similar approach could be done with Caco-2 cells to investigate the role of specific bacteria on intestinal barrier functions. The colonization of GF mice with candidate bacteria (e.g. *Lactobacillus johnsonii*) would allow us to investigate the ability of sole or few bacteria to modulate the secretory activity of EECs in the lower intestine. The requirement of fructose to turn on the EECs response could also be tested in such experimental mice models.

### **V-Impact of fructose on intestinal permeability in normo- and mal-absorptive fructose conditions**

According to our results, intestinal permeability was modified differently in normo- (WT exposed to 15% fructose) and mal-absorptive (KHK<sup>-/-</sup> exposed to 20% fructose) fructose situations. In WT mice, chronic intake of 15% fructose did not affect intestinal permeability in the small intestine, the caecum and the colon (**Article 2, Fig. 2**). In contrast, glucose induced a significant increase in jejunal and caecal permeability in association with an increase in inflammatory cytokines in the caecum (**Article 2, Fig. 2**). TNF $\alpha$  and IFN $\gamma$  are known to activate MLCK pathway (Ye et al., 2006) while IL13-induced paracellular permeability through a claudin 2-dependent pathway (Weber et al., 2010). One of the hypotheses is that glucose-induced intestinal inflammation results in the phosphorylation of MLC thereby inducing actomyosin contractility, reorganizing the TJ protein architecture and leading to the increase in paracellular permeability (Graham et al., 2006, Shen et al., 2006, Shen et al., 2011, Turner et al., 2014). *In vivo* or *in vitro*, when intestinal epithelium or intestinal cell monolayers are exposed to a pro-inflammation environment, fructose appears to counteract inflammation-induced permeability when compared to glucose (**Article 2, Figs. 2 and 5**) and (**Figure 30**). The phosphorylation of MLC by MLCK and subsequent actomyosin contraction is an ATP-dependent process (Shen et al., 2010). Unlike glucose-metabolizing enzymes, the metabolism of fructose results in ATP depletion due to the lack of control of the first steps of the fructose metabolism by KHK and aldolase (Mayes, 1993, Feinman and Fine, 2013). This depletion in ATP has been demonstrated in fructose metabolizing tissues such as

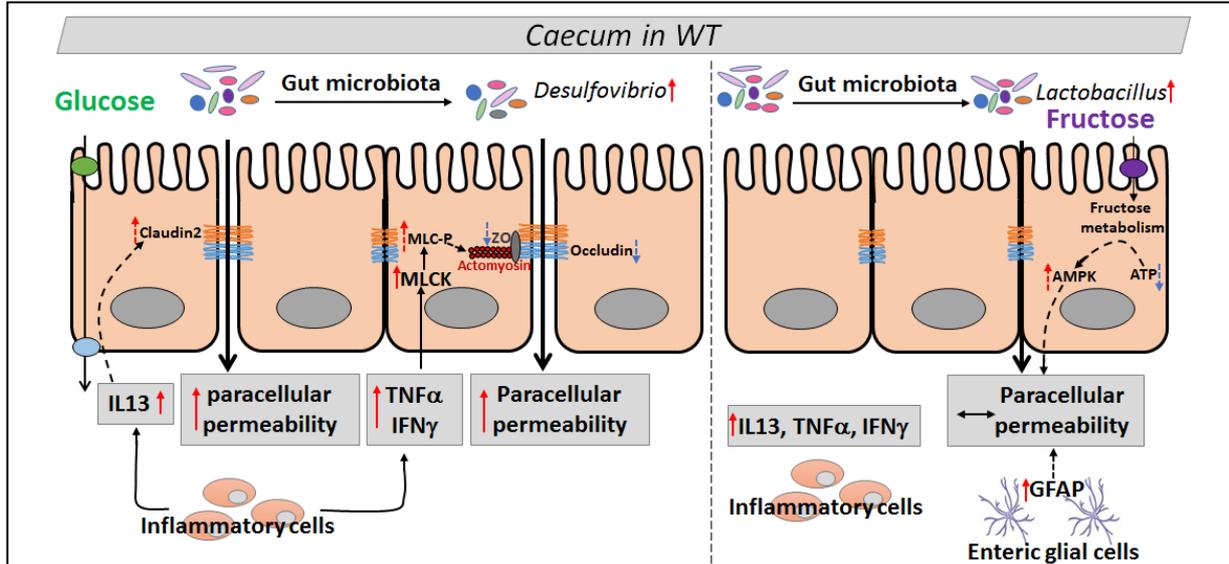
renal tubules (Burch et al., 1980, Lanaspá et al., 2014), liver (Lanaspá et al., 2018) and intestine (Tharabenjasin et al., 2014). Thus, in the enterocytes exposed to fructose, the diminution of ATP levels may prevent ATP-dependent actomyosin contraction process. Moreover, the AMP-activated protein kinase (AMPK) is a cellular energy sensor that is activated in response to intracellular ATP depletion and intracellular increase in the AMP/ATP ratio (Carling, 2004). The uncontrolled metabolism of fructose could lead to the activation of AMPK (Cha et al., 2008, Kinote et al., 2012). Recent studies demonstrated that regulation of TJ protein architecture assembly requires AMPK activation (Zhang et al., 2006, Zheng and Cantley, 2007, Peng et al., 2009). Thus, the activation of AMPK may also participate in the restrained decrease in permeability (or TEER) in the intestinal cells exposed to fructose in presence of pro-inflammatory cytokines. This hypothesis would need to be further investigated using Caco-2 cells cultured with fructose and in presence of either activator (*e.g.* 5-aminoimidazole-4-carboxamide 1 $\beta$ -D-ribo nucleoside (AICAR)) or inhibitor (*e.g.* compound C) of AMPK. This way the effect of the regulation of AMPK activity on the TEER or permeability to small molecules would be evaluated.

However, contrary to our results, a recent study clearly showed that 12 weeks of chronic diet of 65% fructose induced a significant increase in intestinal permeability similar to that in 65% glucose-fed rats (Do et al., 2018). Interestingly, the glycemia of both groups, 65% fructose and 65% glucose-fed rats, was increased significantly after 12 weeks when compared to control rats. Recently, the hyperglycemia was demonstrated as a major factor able to trigger the increase in intestinal permeability (Thaiss et al., 2018). This glycemia-induced increase in intestinal permeability may explain the controversial and non-consistent effects of fructose on intestinal permeability. Indeed, depending on the duration of feeding and the amount of fructose in the experimental diets, the mice or rats may or may not become hyperglycemic and therefore may or may not develop or not impaired intestinal permeability. This hypothesis suggests that the increase in gut permeability does not precede the low-grade inflammation and glucose intolerance often observed in response to dietary fructose. Thus, the increase in gut permeability would not be the cause but rather one of the consequences of the low-grade inflammation and glucose intolerance. In order to clarify the chronology of these events, it would be interesting to determine the time-course of intestinal permeability changes related to the onset of blood glucose levels in response to fructose intake.

The hypothesis of a major role of the ATP level in controlling the intestinal permeability in intestinal cells exposed to fructose, suggests that in absence of fructose metabolism, such as in  $KHK^{-/-}$  mice, the intestinal permeability should increase. However, our results (**Figure 27**) showed the opposite: when malabsorbed, the fructose-induced a decrease in permeability in the jejunum of the  $KHK^{-/-}$  mice. However, in contrast to the WT mice receiving 15% fructose in their drinking solution, the KHK-F did not show any increase in the expression levels of the pro-inflammatory cytokines known to trigger the increase in intestinal permeability.  $KHK^{-/-}$  mice fed fructose also developed a thicker mucosal layer than the WT-F mice (**Figure 27B** and **Article 1, Table S5**). Interestingly, increased mucosal thickness had been associated with a decrease in permeability (Kulkarni et al., 2009, Xue et al., 2017) providing a potential explanation for the strong maintenance in gut barrier functions observed in KHK-F. However, the mechanism by which fructose malabsorption regulates the mucosal thickness remains to be determined. Microbiota adaptation, gastrointestinal hormone secretion or the ENS had been identified as potential regulators of intestinal hyperplasia in response to nutritional challenges (Rubin and Levin, 2016). Interestingly, GLP-2 is a gastrointestinal peptide known for its trophic properties on the GI tract (Shin et al., 2005, Dube and Brubaker, 2007). The elevation of GLP-2 level in response to the nutrient malabsorption had been associated with crypt cell proliferation in the intestine (Martin et al., 2005). *Gcg* is the precursor of GLP-1 and GLP-2 and its expression is strongly upregulated in the ileum and caecum of the KHK-F. To determine whether GLP-2 is involved in the intestinal morphological adaptations observed in the KHK-F, it would be interesting to measure the GLP-2 concentration in the plasma and in the various section GI tract of KHK-F and WT-F mice.

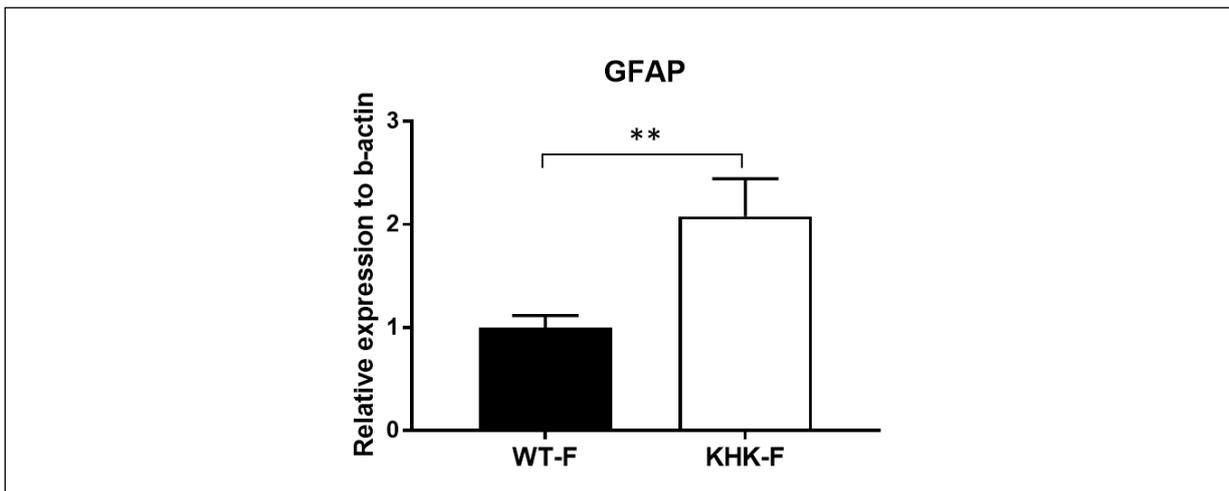
In addition to GLP-2, the ENS may also be involved in the regulation of intestinal permeability by fructose. Our findings indicated that fructose upregulates GFAP gene expression and protein levels in the caecum of KHK-F (**Figure 31**) and WT mice receiving fructose (**Article 2, Fig. 4A**) respectively. GFAP is considered as the specific marker for the EGCs (Jessen and Mirsky, 1980) and increase in GFAP could be used as an indication of gliosis. The disruption of EGCs had been associated with the increase in intestinal paracellular permeability (Cornet et al., 2001, Aube et al., 2006). In contrast, EGCs stimulation had been shown to protect the intestinal barrier functions (Savidge et al., 2007, Van Landeghem et al., 2009, Flamant et al., 2011). In both, KHK-F and WT mice exposed to 15% fructose in the drinking solution, GFAP is increased, suggesting an activation of the EGC which may participate in the protection of the GI barrier function. How EGCs respond

to fructose remain unexplored. The expression of GLUT5 or KHK in EGCs had not been yet investigated. It would be interesting to determine by immunohistochemistry whether the EGCs express GLUT5 and KHK and to determine in primary cell culture if fructose has the ability to activate EGCs.



**Figure 30. Pathways involved in the modulation of paracellular permeability in response to glucose or fructose intake**

Solid arrows represent findings of the current project; Dashed arrows represent hypothetical pathway discuss in this project.



**Figure 31. Caecal gene expression level of glial fibrillary acidic protein in wild type and Ketoheokinase knockout mice fed fructose**

Wild type (WT) and ketoheokinase knockout (KHK<sup>-/-</sup>) mice were fed with 20% fructose diet (WT-F and KHK-F respectively) for 8 weeks (n=7/group). GFAP gene expression levels were measured by qRT-PCR. All values are means ± SEM; means were compared by Mann-Whitney test; \*\*p < 0.01. GFAP: Glial fibrillary acidic protein.

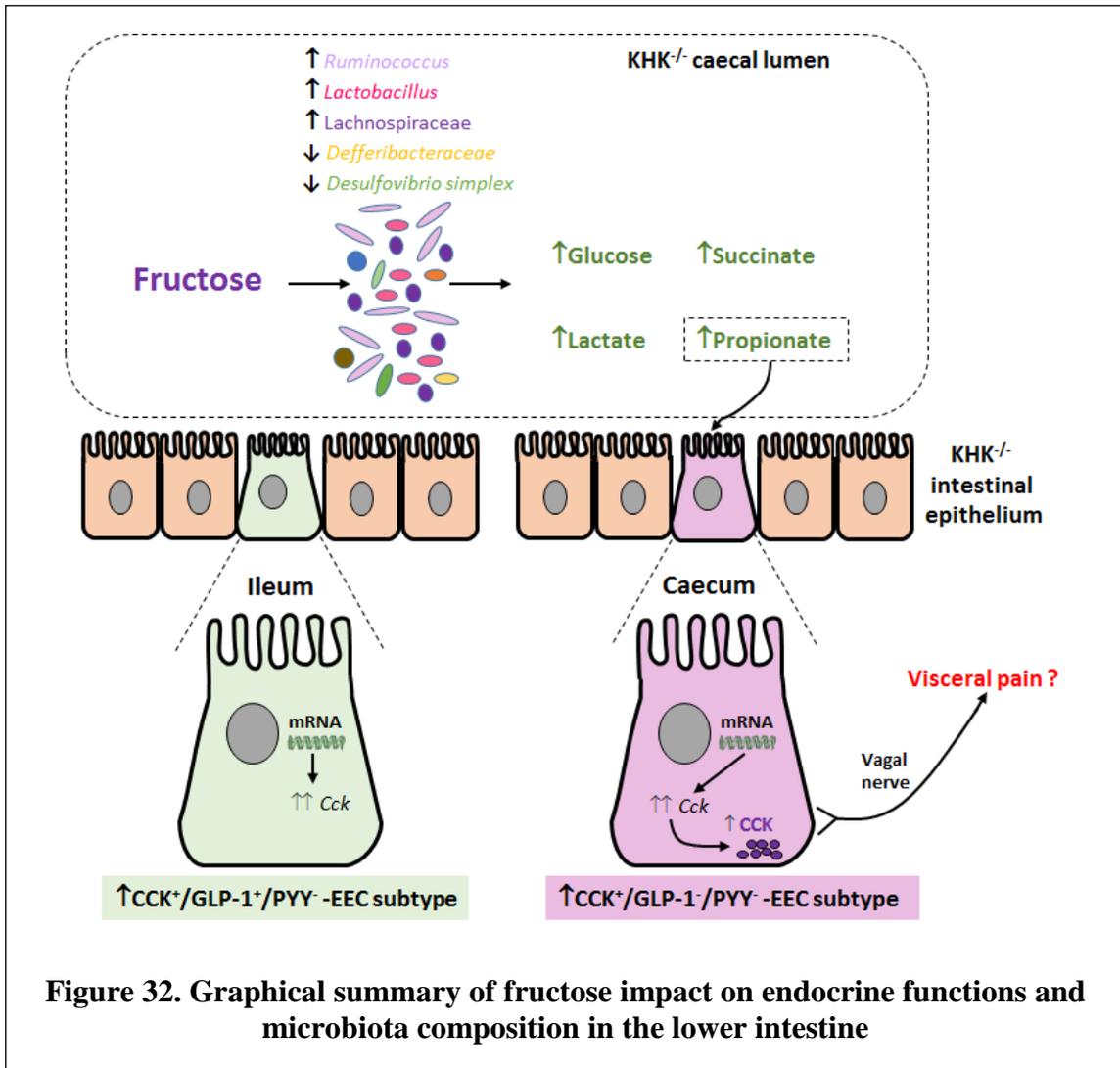


Figure 32. Graphical summary of fructose impact on endocrine functions and microbiota composition in the lower intestine

## GENERAL CONCLUSION

In my thesis project, we showed that the main monosaccharides present in our diet, fructose and glucose, undermined the intestinal homeostasis. Glucose intake challenges the intestinal barrier integrity along the GI tract from the small intestine to the caecum, and in the latter, despite its effective absorption in the proximal regions of the small intestine. Under malabsorptive conditions, fructose disrupts the endocrine function of the lower intestine (**Figure 32**). In both, normo- and mal-absorptive conditions sugar-induced changes in microbiota composition and function likely contribute to the changes of the above-mentioned GI functions. While luminal fructose itself does not seem to be able to disrupt the intestinal permeability, its accumulation in the lower intestine has dramatic consequences on GI hormone expressions, especially of *Cck*. Further research needs to be done to clarify the role and the mechanism by which CCK could be associated with the development of visceral pain that is associated with the fructose malabsorption. Nevertheless, we showed that CCK is a strong candidate to be involved in this process, and it would be interesting to investigate the level of CCK in patients with other GI disorders associated with visceral hypersensitivity. The broad co-expression of the gut peptides demonstrated previously (Egerod et al., 2012, Svendsen et al., 2015) and confirmed in the present study suggested a large adaptability of the EECs to the luminal environment. Our study is one of the first ones demonstrating this adaptability. Importantly, our results show that response to the changes in the luminal environment can be EEC-subtype specific. In our system, only some CCK<sup>+</sup>-EEC subtypes, as defined by peptide combinations they express, increased in their density in response to fructose malabsorption. Whether the CCK-containing cell populations that are GLP-1 positive or GLP-1 negative differ markedly in their functional responsiveness and physiological role will be interesting topics for future studies. Thus, the nature of the EEC in response to specific luminal environment changes may be one of the defining characteristics of EEC subtypes with distinct functional identities.



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## ABSTRACT

50% of the adult population cannot fully absorb a load of 25g of fructose, while the average daily intake is ~50g. Thus, changes in the food intake patterns created a condition of fructose malabsorption leading likely to fructose overspill into the distal part of the intestine. Recent insight emphasized the central role of the intestinal homeostasis on the host physiology of and the importance of the interactions between nutrients and microbiota in this process. The overall objective of my PhD was to decipher the role of luminal fructose-induced changes in GI homeostasis in two conditions: fructose malabsorption or fructose normal absorption. Two mouse models were used: a mouse model of fructose malabsorption (ketoheokinase-knockout,  $KHK^{-/-}$ ) fed 20% fructose and mice model of normal fructose absorption exposed to 15% fructose from drinking solution. In  $KHK^{-/-}$ , fructose intake was associated with visceral hyper-sensitivity and changes in microbiota composition and metabolism. These changes in the intestinal ecosystem were able to regulate the pattern of peptide secreted by the enteroendocrine cells of the ileum and caecum by stimulating the secretion of CCK in L cells normally dedicated to the secretion of PYY and GLP1. However, in  $KHK^{-/-}$  and WT fructose did not alter the intestinal permeability, even in intestinal regions that developed inflammation in response to fructose intake. This study highlighted the critical role of luminal fructose on intestinal endocrine functions, but not barrier functions within malabsorption and normal absorption conditions, respectively. Potential links between increase in CCK secretion and host physiology need to be further explored.

Keywords: KHK; fructose; malabsorption; CCK; microbiota; paracellular permeability

## RESUME

50% de la population adulte ne peut pas absorber complètement une charge de 25g de fructose, tandis que l'apport quotidien moyen est ~50g. Chez les individus consommant de grandes quantités de fructose, l'intestin grêle mais également distal est certainement exposé au fructose alimentaire. Bien que de nombreuses études aient mis en évidence le rôle primordial de la physiologie de l'intestin et de son microbiote intestinal dans la santé de l'hôte, les interactions entre fructose et bactéries intestinales sont peu étudiées. Mon projet de thèse avait pour but de tester l'hypothèse selon laquelle le fructose alimentaire peut modifier la composition en bactéries de l'intestin et que ce déséquilibre de la flore intestinale influence certaines fonctions endocrines et de barrière de l'intestin chez des souris présentant ou pas une malabsorption du fructose (souris transgéniques pour la ketoheokinase ( $KHK^{-/-}$ ) ou souris recevant 15% de fructose via l'eau de boisson). Chez les souris  $KHK^{-/-}$  recevant du fructose la composition et les fonctions du microbiote sont modifiées. Ces changements affectent les fonctions sécrétrices des cellules entéroendocrines de l'iléon et du caecum en stimulant notamment la sécrétion de la CCK. Mais en conditions normo- ou mal-absorptives le fructose ne modifie pas la perméabilité intestinale, même dans les régions du tube digestif développant une inflammation. Cette étude a mis en évidence le rôle essentiel du fructose luminal sur les fonctions endocrines de l'intestin distal, mais minimise l'importance de la perméabilité intestinale. Les liens potentiels entre l'augmentation de la sécrétion de CCK et la physiologie de l'hôte devront être approfondis.

Keywords: KHK; fructose; malabsorption; CCK; microbiote; perméabilité paracellulaire