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Jérémy Ranfaing

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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**Etude des activités anti-adhérentielles et anti-  
bactériennes de la canneberge (*Vaccinium  
macrocarpon*) et de la propolis**

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Le 8 Novembre 2017

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# LISTE DES ABREVIATIONS

## Généralités

UPEC : *Escherichia coli* uropathogène  
PAC-A : Proanthocyanidine de type A  
BLSE : Béta-lactamase à spectre étendu  
IU : Infection(s) urinaire(s)  
CMI : Concentration minimale inhibitrice  
InPEC : *Escherichia coli* pathogènes intestinaux  
ExPEC : *Escherichia coli* pathogènes extra-intestinaux  
ATP : Adénosine triphosphate  
NK : Natural Killer  
LC-MS : Chromatographie liquide avec spectrométrie de masse  
DMAC : 4-diméthylaminocinnamaldehyde

## Gènes et protéines

Fim: gènes liés à la synthèse des fimbriae de type 1  
Pap : gènes liés à la synthèse fimbriae de type P  
Fli : gènes liés à la synthèse du flagelle  
motAB : Composants du moteur du flagelle  
tsr : *serine sensor receptor*  
fur : *ferric iron uptake regulon transcriptional repressor*  
feoB : *ferrous iron transporter*  
ftnA : *ferritin iron storage*  
fecB : *ferric citrate ABC transporter periplasmic binding protein*  
rpoS : *RNA polymerase sigma S factor*  
cpxR : *response regulator in two-component regulatory system*  
csiD : *carbon starvation protein*

# **INTRODUCTION GENERALE**

## Problématique générale

L'infection urinaire (IU) est définie par la présence d'un microorganisme pathogène dans l'urine en présence d'une symptomatologie compatible. Elle constitue un problème de Santé publique majeur car plus de 150 millions de personnes sont touchées par cette pathologie dans le Monde chaque année. Cela représente un énorme coût en matière de prise en charge [1].

Les IU sont réparties en cystites et en pyélonéphrites chez la femme. Chez l'homme, en raison d'une possible difficulté dans le diagnostic de la localisation de l'IU, la terminologie d'infections urinaires masculines a été récemment adoptée. Les IU sont plus fréquentes chez les femmes, quel que soit leur âge, avec deux pics de fréquence, l'un au début de la vie sexuelle et l'autre après la ménopause [2]. Même si ces infections sont pour la plupart sans gravité elles provoquent tout de même un fort handicap social.

Une des particularités des cystites est leur propension à récidiver. En effet, une importante proportion de femmes qui contracte une cystite va développer des cystites récidivantes [3]. Cette récurrence est définie par au moins 4 épisodes de cystite en l'espace d'une année. Le traitement préconisé pour traiter une cystite est une prise d'antibiotiques. Pour la cystite récidivante, la prise d'antibiotiques peut-être répétée sur des périodes de plusieurs mois induisant le risque d'émergence de souches bactériennes résistantes aux antibiotiques.

Jusqu'à présent, l'émergence de souches résistantes aux antibiotiques était plutôt liée aux infections nosocomiales. En effet, les hôpitaux représentent un environnement propice à l'émergence de résistance bactérienne que ce soit en termes de consommation d'antibiotiques ou en termes de transmissions croisées. Cependant, ces dernières années, des études épidémiologiques concernant les IU ont montré l'émergence de souches bactériennes communautaires résistantes aux antibiotiques [4].

La bactérie la plus souvent isolée, quel que soit le type d'IU, est *Escherichia coli*. Il s'agit d'une bactérie commensale à Gram négatif présente principalement dans notre appareil digestif. Cette bactérie peut devenir pathogène lorsqu'elle se trouve dans un autre environnement comme le système urinaire. Les *E. coli* uropathogènes (UPEC) possèdent de nombreux facteurs de virulence tel un large panel de fimbriae, de toxines et sont suffisamment mobiles pour pouvoir atteindre les reins [5]. De plus, les UPEC développent de plus en plus des mécanismes de multirésistance contre des antibiotiques utilisés couramment lors d'IU. Les plus fréquemment isolées sont les souches



sécrétrices de bêta-lactamases à spectre étendu (BLSE) qui, ces dernières années, se sont répandues dans le milieu communautaire [6].

Ainsi, dans certaines situations l'antibiothérapie a atteint ses limites d'autant plus que la synthèse de nouveaux antibiotiques a été très fortement ralentie ces dernières années. Il est donc nécessaire de développer une alternative à la classique antibiothérapie. Parmi les potentielles options, l'utilisation de la canneberge (*Vaccinium macrocarpon*) est très souvent citée. Utilisée depuis de nombreuses années afin de lutter contre les IU, ses mécanismes d'actions anti-bactériens demeurent partiellement décrits bien que de nombreux auteurs aient évoquées son efficacité [7][8][9]. D'autre part, peu d'études se sont intéressées à l'association de la canneberge avec d'autres composés, en particulier des composés naturels tels que la propolis. En effet, la propolis dispose également de propriétés anti-bactériennes intéressantes et connues depuis de nombreuses années [10]. Une étude préalable de notre équipe a mis en évidence que l'ajout de propolis à une solution de canneberge augmentait les propriétés anti-adhérentielles de cette dernière et diminuait la virulence des souches d'UPEC par rapport à un traitement composé de canneberge seule [11].

Ce travail de thèse s'est donc intéressé à l'étude du mécanisme d'action de la canneberge seule ou en association à la propolis sur la pathogénicité des UPEC.

Le premier objectif de ce travail a été d'étudier l'impact de la canneberge, de la propolis et de l'association de ces deux composés sur le transcriptome d'une souche d'UPEC, le mode d'action de ces deux composés n'étant pas complètement connu à l'heure actuelle. Des tests phénotypiques ont également été effectués sur plusieurs souches d'UPEC afin de confirmer les résultats obtenus en transcriptomique.

Le deuxième objectif a été de développer un outil permettant d'évaluer l'efficacité de compléments alimentaires à base de canneberge. Une des difficultés actuelle reste la mise au point d'une mesure exacte et standardisée du principe actif de la canneberge : la proanthocyanidine (PAC). Sur la base de l'étude de l'impact de la canneberge sur le transcriptome d'*E. coli*, une technique mesurant l'activité de la canneberge sur l'expression de gènes cibles d'UPEC a été développée.

Le troisième objectif a été d'évaluer l'effet potentialisateur de la propolis sur les antibiotiques habituellement utilisés contre les pathogènes urinaires. Les souches résistantes aux antibiotiques se développant, il est primordial de trouver une solution pour potentialiser l'effet d'antibiotiques déjà présent sur le marché. Pour cela, l'évolution des courbes de croissances d'une souche d'UPEC a été mesurée en présence d'antibiotiques, de propolis et de la combinaison des deux composés.

**Canneberge, propolis et infections urinaires**

L'évolution des Concentrations minimales inhibitrices (CMI) des antibiotiques en présence de propolis a également été évaluée.

## **L'infection urinaire communautaire bactérienne de l'adulte**

### **I) Définitions**

Les infections urinaires regroupent toutes les infections portant atteintes au système urinaire (vessie, prostate et reins). Les femmes sont davantage touchées que les hommes, principalement à cause de la moindre longueur de leur urètre [12]. Chez les femmes ces infections peuvent se déclarer à n'importe quel moment de leur vie avec deux pics de fréquence, l'un au début de la vie sexuelle et l'autre après la ménopause. Chez les hommes se sont principalement les jeunes enfants, à cause de malformations du système urinaire, et les hommes de plus de 50 ans, en raison de l'hypertrophie de la prostate qui sont le plus souvent atteints. Une grande majorité de ces infections sont sans gravité mais représentent un handicap social important. Les signes cliniques vont varier en fonction du site de l'infection.

Les recommandations de la Société de pathologies infectieuses de langue française (SPILF) de 2014, réactualisées en 2015 ont introduit la notion d'infection urinaire à risque de complication et le concept d'infection urinaire chez l'homme [13].

Il est possible de distinguer :

- les infections urinaires simples, survenant chez des personnes sans facteur de risque de complication ;
- les infections urinaires à risque de complication, survenant chez des personnes présentant au moins un des facteurs de risque de complication : uropathie, immunodépression grave, insuffisance rénale sévère (clairance < 30ml/min), sujet âgé >75 ans ou 65 ans et au moins 3 critères de Fried (perte de poids involontaire au cours de la dernière année ; vitesse de marche lente ; faible endurance, fatigue ; activité physique réduite) ;
- les infections urinaires graves correspondant aux pyélonéphrites aiguës et aux infections urinaires masculines associées à un sepsis grave ou un choc septique ou une indication de drainage chirurgicale ou interventionnel ;

- les cystites récidivantes correspondant à la survenue d'au moins 4 épisodes pendant 12 mois consécutifs ;
- la colonisation bactérienne (bactériurie asymptomatique) correspondant à la présence d'un microorganisme dans les urines sans manifestations cliniques associées [14][15] ;

## **1) Infections urinaires simples**

### **a) Cystite aiguë**

Une cystite aiguë est caractérisée par des brûlures mictionnelles, une pollakiurie (fréquente envie d'uriner mais en faible quantité), une impériosité urinaire (envie d'uriner très pressante même en l'absence d'urines) et parfois d'une hématurie (présence de sang dans les urines). Une caractéristique importante de la cystite est l'absence de fièvre.

### **b) Pyélonéphrite aiguë**

Lorsque l'infection atteint les reins via les uretères, le terme de pyélonéphrite est employé. Les symptômes sont caractérisés par une douleur de la fosse lombaire associée à de la fièvre. Une translocation de bactéries des voies urinaires vers le secteur sanguin constituant une bactériémie est possible.

## **2) Infections urinaires à risque de complications**

Les infections urinaires à risque de complications regroupent les situations où il existe des facteurs qui compromettent le système urinaire (anomalie fonctionnelle ou organique de l'appareil urinaire, immunodépression, défaillance rénale, personne âgée comme définie plus haut) [16][17].

Les infections urinaires masculines sont systématiquement considérées comme à risque de complications au même titre que l'infection urinaire pendant la grossesse.

## **3) Cystites récidivantes**

Après un épisode de cystite, environ 25% des femmes vont développer un autre épisode dans un laps de temps relativement court [3]. Il s'agit d'infections urinaires récidivantes lorsqu'il existe au moins 4 épisodes de cystite aiguë dans l'année qui précède. Sa physiopathologie n'est pas univoque : recontamination à partir du tractus gastro-intestinal ou résurgence à partir de réservoirs bactériens vésicaux. Le traitement de ces récurrences implique un traitement antibiotique pouvant perdurer plusieurs mois, voire années [1].

#### 4) Colonisation urinaire

La colonisation urinaire (ou appelée encore bactériurie asymptomatique chez la femme enceinte) est définie par la présence de microorganismes dans les urines sans manifestations cliniques. En dehors de la femme enceinte, il n'existe pas de seuil de bactériurie.

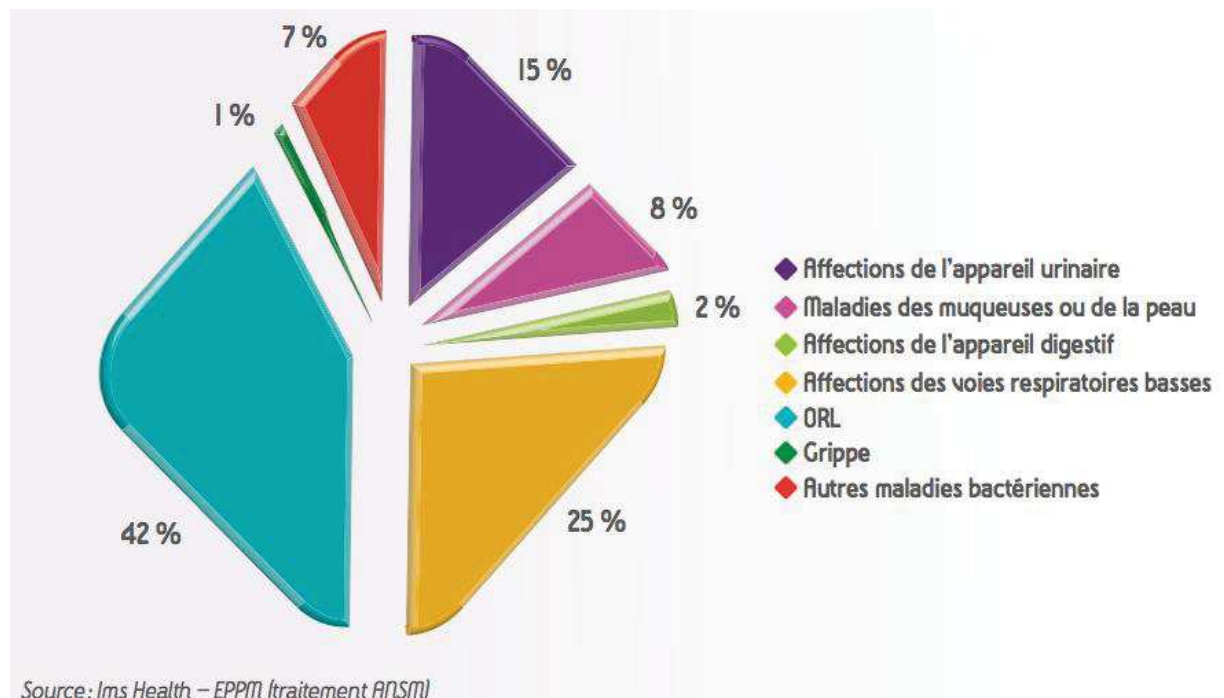
## II) Epidémiologie de l'infection urinaire communautaire

### 1) Epidémiologie mondiale

Les infections urinaires font partie des maladies les plus communes, avec plus de 150 millions de cas dans le monde [18][19] chaque année, ce qui fait qu'elles ont un impact économique très important [1]. Aux Etats-Unis, avec en moyenne 11 millions de cas par an, le coût est estimé à 3,5 milliards de dollars [20].

### 2) Epidémiologie nationale

En France, on estime que ces infections touchent près de 6 à 8 millions de patientes et le coût associé est quant à lui estimé à environ 58 millions d'euros [21]. En 2015, les prescriptions d'antibiotiques liés aux infections urinaires extra-hospitalières atteignaient la 3<sup>ème</sup> place (Figure 1).



**Figure 1** : Principaux diagnostics associés aux prescriptions d'antibiotiques en 2015 (données ANSM, 2017).

### **III) Physiopathologie des infections urinaires.**

#### **1) Les modes de contamination.**

L'arbre urinaire est physiologiquement stérile. Seul l'urètre distal est colonisé par le microbiote périnéal. En principe, chez la femme c'est ce microbiote qui va être le point de départ de l'infection urinaire via une contamination ascendante. Chez l'homme les causes sont plus complexes, cependant elles sont favorisées par l'existence d'un obstacle sous-vésicale qui va induire une mauvaise vidange vésicale.

#### **2) Les mécanismes de défense de l'hôte.**

Le système urinaire dispose de plusieurs mécanismes physiques afin de se prémunir des infections bactériennes ascendantes.

##### **a) Le passage des reins jusqu'à la vessie.**

L'urine est produite par les reins et va être transportée jusqu'à la vessie via les uretères. Ce flux va être un obstacle pour les bactéries ne disposant pas de protéines d'adhésion adaptées à ces tubes fibro-musculaires. La connexion avec la vessie va ensuite se faire par la jonction urétéro-vésicale. Lorsque le volume de la vessie augmente cette jonction va se fermer afin d'empêcher un reflux vésico-urétéral ainsi que le passage de bactéries [22].

##### **b) Les propriétés de l'urine.**

L'urine elle-même est une défense pour l'hôte. Sa composition est très faible en nutriments facilement accessibles ce qui freine la croissance bactérienne. De plus, son osmolarité, son pH et sa teneur en acides organiques vont induire un stress important pour les bactéries. Enfin, si jamais une croissance bactérienne a lieu, la miction suivante va éliminer la quasi-totalité des bactéries n'ayant pas réussi à adhérer à la surface de la vessie.

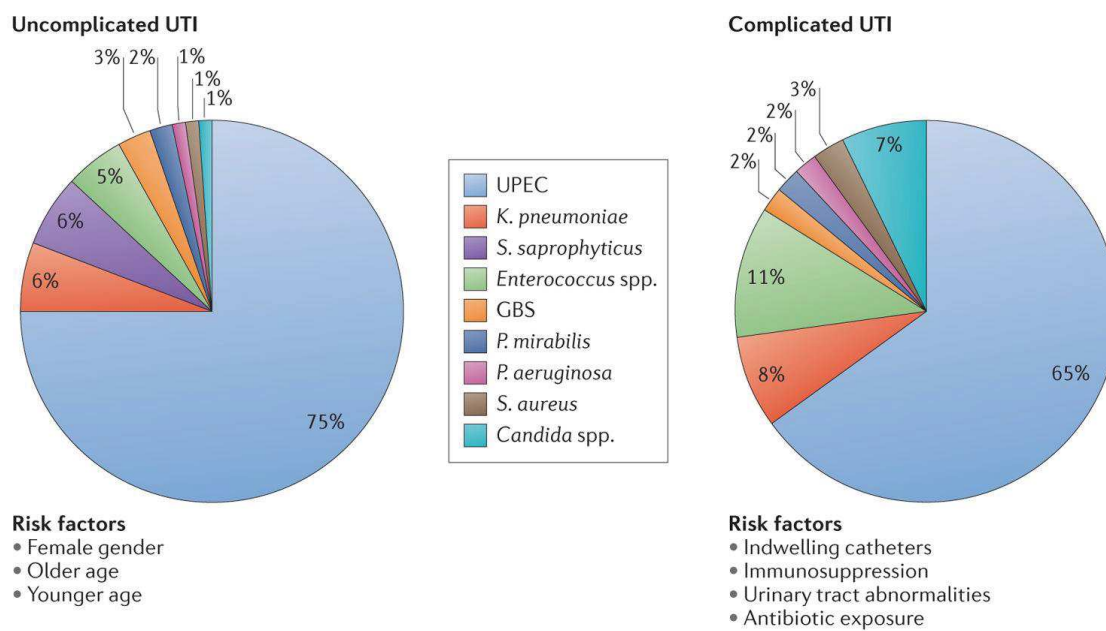
##### **c) De la vessie au milieu extérieur**

La longueur de l'urètre est la principale raison expliquant la faible incidence des infections urinaires chez les hommes. Il va mesurer 13 à 20 cm chez les hommes alors qu'il est beaucoup plus court chez la femme, entre 4 et 5 cm. C'est également lors de cette étape que les capacités d'adhésion et de mobilité des bactéries vont être mises à l'épreuve [22].

## IV) Microbiologie

### 1) Les pathogènes urinaires

La bactérie la plus fréquemment isolées lors des infections est *E. coli*. Ensuite, viennent par ordre de prévalence : *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, Streptocoque du groupe B, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* et *Candida* spp. [12][15][23][24] (Figure 2).



**Figure 2** : Epidémiologie des infections urinaires

Répartition des différents pathogènes urinaires isolés après une infection urinaire compliquée ou non [24].

### 2) *Escherichia coli* uropathogène

#### a) Généralités

*E. coli* est une bactérie appartenant à la classe des  $\gamma$ -protéobactéries et à la famille des *Enterobacteriaceae*. Cette famille comprend des bacilles droits à Gram-négatif mesurant 0,3 à 1  $\mu\text{m}$  de diamètre sur 0,5 à 6  $\mu\text{m}$  de long en fonction du stade de croissance, non sporulés, mobiles et aéro-anaérobies facultatifs.

*E. coli* est une bactérie du microbiote commensal intestinal de l'homme et de la plupart des animaux. Elle va coloniser de manière asymptomatique le système digestif de l'Homme dans les premières

heures suivant la naissance et constitue à partir de là l'espèce bactérienne dominante du microbiote du colon humain.

Cependant, *E. coli* peut également devenir un pathogène opportuniste suite à l'expression de facteurs de virulence spécifiques. On distingue deux groupes : les *E. coli* pathogènes intestinaux (InPEC) responsables de gastro-entérites et les *E. coli* pathogènes extra-intestinaux (ExPEC) responsables d'infections variant suivant le site de contamination [5].

Les *E. coli* uropathogènes (UPEC) entrent dans la seconde catégorie. Ils possèdent un large panel de facteurs de virulence membranaires et excrétés qui vont intervenir dans de nombreuses voies métaboliques impliquées dans la physiopathogénèse des infections urinaires (adhésion, mobilité, métabolisme du fer, sécrétion de toxines). Une variabilité existe dans la prévalence de ces gènes de virulence selon les souches [25]. Ces gènes de virulence acquis par les UPEC sont localisés dans des îlots de pathogénicité qui sont intégrés au génome bactérien [26]. Ces gènes peuvent également être localisés au niveau de plasmides mais ce phénomène est beaucoup plus rare [27].

### **b) Adhésion**

La première voie métabolique impliquée est l'adhésion. Pour remonter l'urètre jusqu'à la vessie la bactérie doit adhérer à la paroi de l'urètre. Pour cela, la bactérie va déployer un ensemble de fimbriae variés (type 1 et type P pour les fimbriae majoritaires). Une fois dans la vessie la bactérie va également devoir adhérer aux cellules épithéliales afin de pouvoir initier la synthèse de toxines. Elle peut également « remonter » jusqu'au rein via l'uretère. Cette étape est cruciale car si la bactérie n'adhère pas correctement, elle va se faire éliminer naturellement par le flux urinaire.

Chez les bactéries à Gram négatif (dont *E. coli*), différents systèmes de synthèse des protéines responsables de l'adhésion existent. Le système de sécrétion de type IV mais également le système de « Chaperone-Usher » [28]. Ce système est responsable de la synthèse d'une grande partie des fimbriae chez les UPEC, dont les fimbriae de type 1 et les fimbriae de type P [29]. Ce système est impliqué dans la synthèse de sous-unités du fimbriae qui va traverser la membrane cytoplasmique, des protéines chaperones périplasmiques vont ensuite les assembler et les faire passer dans le milieu extracellulaire [30].

- **Fimbriae de type 1 (Figure 3)**

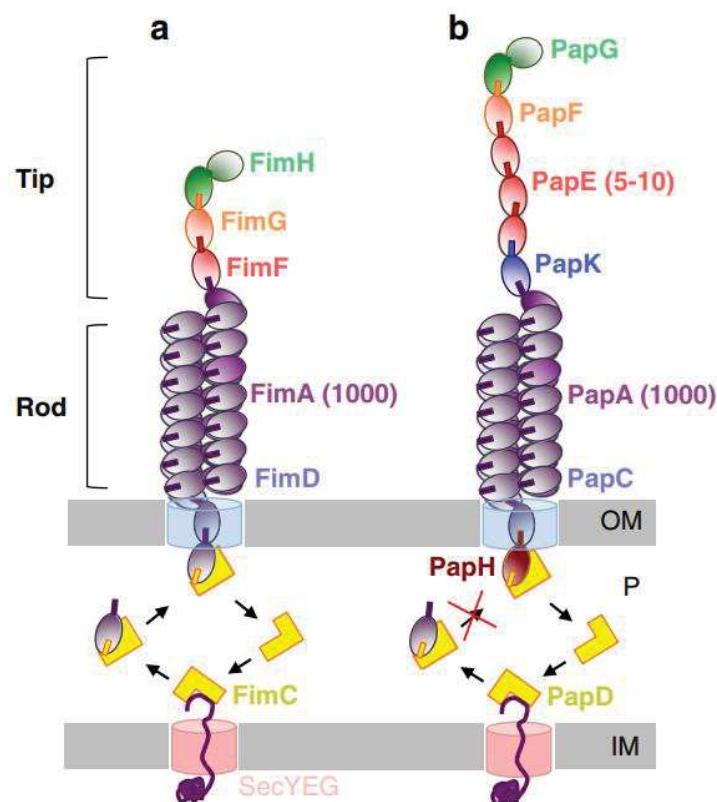
Le fimbriae de type 1 est essentiel pour l'adhésion et la colonisation chez la souris mais également chez l'humain [31][32]. Les souches d'*E. coli* ne possédant pas les gènes codant pour les fimbriae de type 1 induisent des infections moins sévères que chez celles les exprimant. C'est FimH, la protéine à

**Canneberge, propolis et infections urinaires**

l'extrémité du fimbriae qui va adhérer aux cellules de l'épithélium. C'est cette interaction avec la cellule-hôte qui va également initier l'internalisation de la bactérie et la formation de colonies bactériennes intracellulaires [33]. C'est cette capacité accrue à s'internaliser qui va conférer aux UPEC une plus grande persistance.

- Fimbriae de type P (Figure 3)

Les fimbriae de type P sont fréquemment associés aux pyélonéphrites. Contrairement aux fimbriae de type 1 qui se fixent sur les récepteurs mannosides, PapG (protéine responsable de l'adhésion chez les fimbriae de type P) va se fixer sur les glycolipides présents à la surface des cellules hôtes. Il existe différents variants de PapG [34]. Le variant PapGIII va induire principalement des cystites chez l'humain mais des infections du rein chez le chien. Cela s'explique par sa liaison préférentielle avec le globopentaosylceramide, récepteur prédominant chez les reins du chien mais très minoritaire chez l'homme [35][36]. Le variant PapGII quant à lui va se lier au globotetraosylceramide qui va se trouver principalement dans les reins humains et il est associé aux pyélonéphrites ainsi qu'aux bactériémies [36].



**Figure 3** : Structure des fimbriae de type 1 (figure 2.A) et de type P (figure 2.B) [37].



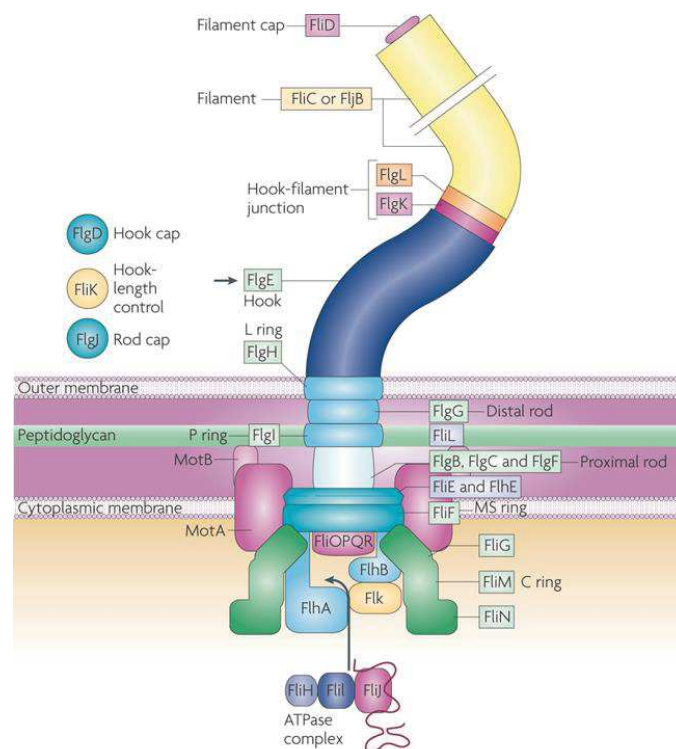
- Curli fimbriae

Les curli fimbriae sont composés de plusieurs sous-unités majeures (CsgA) et d'une autre sous-unité (CsgB) qui va ancrer les protéines CsgA à la membrane. Les curli fimbriae ne sont pas seulement impliqués dans l'adhésion avec des cellules, ils sont aussi impliqués dans l'adhésion sur des surfaces abiotiques ainsi qu'à la formation de biofilm [38]. De plus, il a été montré récemment que les curli fimbriae étaient nécessaires pour l'infection de la vessie [39].

### c) Mobilité

La mobilité a un rôle clef dans la physiopathologie de l'infection urinaire. En effet, les infections sont ascendantes allant de l'urètre à la vessie et dans certains cas allant de l'uretère aux reins [40].

Chez *E. coli*, la mobilité est portée par le flagelle (Figure 4). Elle est portée par un long filament composé majoritairement de FlcC (corps), de FlcD (tête) et de FlgE qui va lier l'ensemble au moteur du flagelle. Ce moteur est situé dans la membrane et va, en échange d'ATP, fournir l'énergie nécessaire pour initier le mouvement [41].



Nature Reviews | Microbiology

**Figure 4** : Schéma du flagelle d'*Escherichia coli* (Nature Reviews Microbiology).

Le swimming est un mode de déplacement individuel utilisé principalement par la bactérie pour se déplacer en milieu liquide.

Il existe un autre type de déplacement pour la bactérie : le swarming [42]. Il s'agit d'un mode de déplacement en milieu semi-liquide qui, à l'inverse du swimming, est collectif [43]. Pour effectuer le swarming, la bactérie va se différencier. Elle va s'allonger, augmenter le nombre de flagelles et produire du matériel extra-cellulaire (exopolysaccharides) afin de se lier à d'autres bactéries dans le même état afin de faciliter le mouvement [44][45].

#### **d) Synthèse de toxines**

Une fois que la bactérie a adhéré aux cellules épithéliales, elle va commencer à synthétiser et sécréter des toxines. L'environnement de la vessie étant très pauvre en nutriment, la bactérie va devoir chercher ses ressources dans les cellules de l'hôte. Parmi les nutriments primordiaux qu'elle doit récupérer, il y a le fer (qui se trouve dans une configuration assimilable dans les cellules).

- $\alpha$ -Hémolysine

Une des stratégies que *E. coli* va déployer est la sécrétion d' $\alpha$ -hémolysine. Cette toxine va former des pores à la surface des cellules et déclencher leur lyse ainsi que leur exfoliation (qui sera importante lors de la formation de biofilm) [46][34][24]. Cette exfoliation va également permettre aux bactéries de former un réservoir de bactéries quiescentes qui est une explication aux infections urinaires récurrentes. Récemment il a été montré que les UPEC pouvaient limiter la réaction pro-inflammatoire en ciblant les cellules NK (Natural Killer) via cette toxine après son adhésion avec ses fimbriae de type 1 [47].

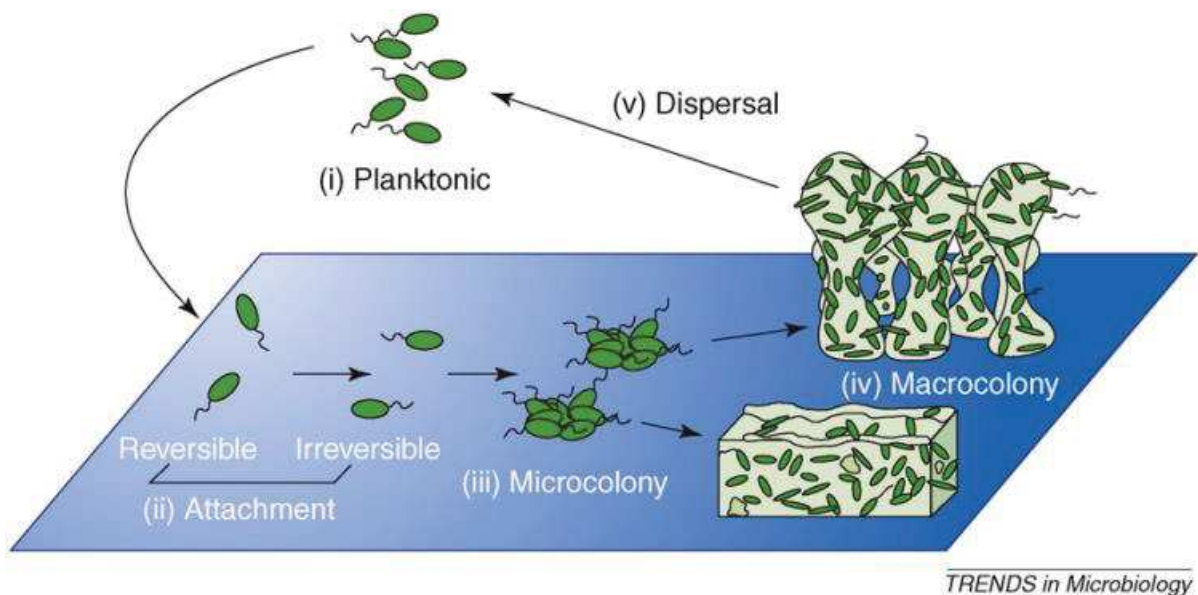
- Toxine cytotoxique nécrosante de type 1

*E. coli* peut également sécréter la toxine cytotoxique nécrosante de type 1 [24]. Cette toxine va favoriser l'internalisation de la bactérie dans la cellule épithéliale via un réarrangement du cytosquelette de la cellule [48]. Cela va donner un avantage à la bactérie dans les phases précoces de l'infection urinaire principalement en permettant à *E. coli* d'échapper aux polynucléaires neutrophiles [49].

#### **e) Formation de biofilm (Figure 5)**

Le biofilm est une structure complexe composée de nombreuses bactéries liées entre elles par une matrice extra-cellulaire. Cette structure permet aux bactéries d'échapper au système immunitaire de l'hôte et de mieux résister à l'action d'antibiotiques ou tout autre traitement anti-bactérien [50][51]. La première étape de la formation du biofilm est une adhésion réversible à une structure (cellules-

hôtes ou surface abiotique). L'adhésion devient secondairement irréversible et les bactéries vont commencer à se multiplier tout en produisant des exopolysaccharides qui vont former une matrice extra-cellulaire pour les bactéries. La structure va ensuite se complexifier, les bactéries au centre du complexe devenant de moins en moins actives et pouvant servir de nutriments aux bactéries des couches supérieures. La dernière phase étant le relargage de bactéries dans le milieu [52].



**Figure 5 :** Différentes étapes de la formation du biofilm [53].

La première est l'adhésion réversible d'une bactérie planctonique à une surface (i et ii). L'adhésion devient secondairement irréversible, des microcolonies vont alors se former (iii). Pour finir la structure se complexifie et devient une macrocolonie (iv). Après un temps variable, un détachement d'une partie de cette macrocolonie se réalisera et ces bactéries deviendront à nouveau planctoniques.

La formation de biofilm a deux impacts majeurs dans le cas d'une infection urinaire. Premièrement, la formation de biofilm à l'intérieur de la vessie a un rôle majeur dans le cadre des infections urinaires récurrentes [54]. Ce biofilm peut être initié à partir de l'exfoliation des cellules superficielles de l'épithélium de la vessie par la production d' $\alpha$ -hémolysine. Deuxièmement, la formation de biofilm est systématique lors de la pose d'un cathéter urinaire. Les infections liées à la pose d'un cathéter urinaire sont les plus communes des infections nosocomiales [55]. Le traitement de ces infections est alors difficile, le biofilm conférant une résistance accrue aux antibiotiques [56].

### **3) Autres uropathogènes**

*Escherichia coli* uropathogène est le pathogène urinaire le plus fréquemment isolé quel que soit le type d'infection urinaire. Cependant d'autres espèces bactériennes sont capables de coloniser le tractus urinaire.

#### **(1) *Klebsiella pneumoniae***

Comme pour *E. coli*, *K. pneumoniae* va utiliser des fimbriae de type 1 pour coloniser la vessie. De façon surprenante, malgré le fait que FimH possède une grande homogénéité avec celle d'*E. coli*, elle ne va pas se fixer sur les mêmes récepteurs cellulaires [57]. Cela a pour conséquence une adhésion plus faible à l'uroépithélium. Pour compenser, *Klebsiella pneumoniae* va exprimer des pili de type 3 qui vont être impliqués dans l'adhésion et la formation de biofilm [58].

#### **(2) *Proteus mirabilis***

*Proteus mirabilis* va produire des fimbriae mannose-résistants facilitant l'adhésion et la formation de biofilm dans la vessie et dans les reins. De plus, *P. mirabilis* va exprimer des protéines d'adhésion spécifiques des infections de la vessie (TaaP) et des infections des reins (AipA) [59]. Cette bactérie va également être très impliquée dans les infections urinaires après la pose d'un cathéter urinaire. *Proteus mirabilis* peut sécréter de l'uréase, ce qui va avoir pour effet d'augmenter le pH urinaire et ainsi la formation de cristaux. A partir de ces cristaux, cette bactérie va pouvoir créer un biofilm cristallin autour du cathéter qui est totalement imperméable au système immunitaire de l'hôte [24].

## **V) Traitements des infections urinaires**

Les principaux antibiotiques utilisés au cours des infections urinaires sont indiqués ci-dessous.

### **1) Nitrofurantoine**

Cet antibiotique est utilisé en première intention dans le traitement probabiliste des cystites aiguës à risque de complication [13]. La résistance d'*E. coli* à cet antibiotique est faible et stable (0.9% dans la période 2003-2012). Il demeure très efficace pour traiter les infections à des souches sécrétrices de BLSE [2][60][61].

### **2) Fosfomycine**

Cet antibiotique est utilisé en première ligne dans le traitement des cystites aiguës simples [2][13]. Une très grande majorité des souches d'*E. coli* isolées est sensible à cet antibiotique (99,4%) [62]. En revanche, même si le taux de résistance à la fosfomycine reste bas, certaines souches de *K. pneumoniae* et d'UPEC voient leur taux de résistance augmenté ces dernières années. 19.9% dans la

**Canneberge, propolis et infections urinaires**

période de 2010 à 2013 sur l'ensemble des souches étudiés (46% pour *Klebsiella* et 4% pour *E. coli*) [63].

### **3) Triméthoprim - sulfaméthoxazole**

A cause du taux de résistance en hausse depuis plusieurs années, cet antibiotique n'est plus utilisé en traitement probabiliste [64]. En revanche, si la bactérie isolée est sensible, il peut être utilisé pour traiter les cystites, les pyélonéphrites et les infections urinaires masculines [13][65].

### **4) Fluoroquinolones**

Cette classe d'antibiotiques n'est pas recommandée en première ligne du traitement probabiliste des cystites. Elle est préconisée pour le traitement probabiliste des pyélonéphrites en alternative aux céphalosporines de 3<sup>ème</sup> génération. Le taux de résistance à cet antibiotique a augmenté de 3,6% à 11,8% durant la période 2003 à 2012 [60]. De plus, seule une petite proportion de souches productrices de BLSE est sensible aux fluoroquinolones [66].

### **5) Céphalosporines**

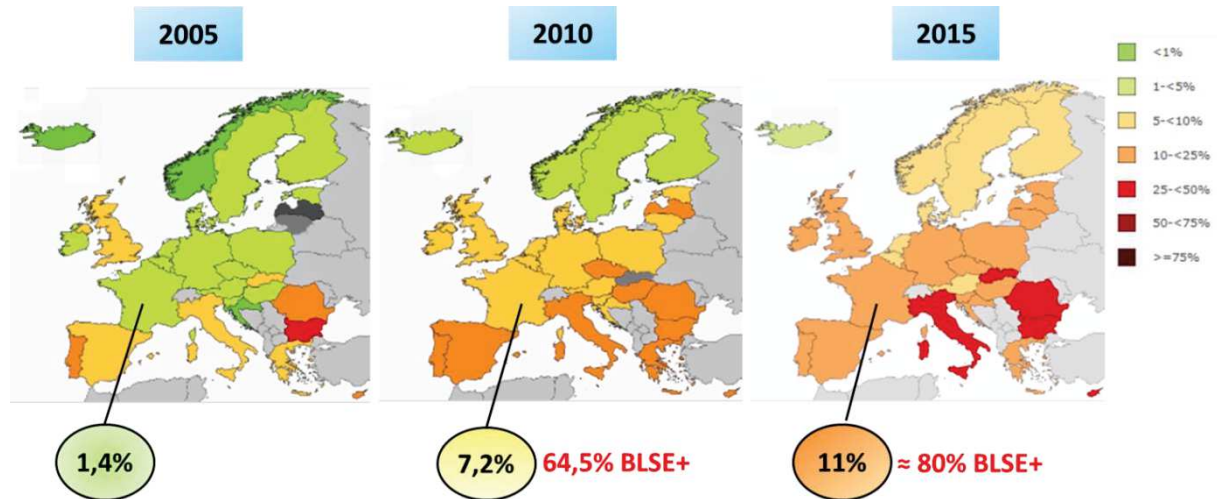
Pour les infections urinaires, seuls les céphalosporines de 3<sup>ème</sup> génération sont recommandées [13][55].

Le cefotaxime ou la ceftriaxone sont recommandés dans le traitement probabiliste des pyélonéphrites et des infections urinaires masculines [13][67]. En effet, une grande majorité des souches isolées reste sensible. Cependant leur indication doit rester limitée en raison de l'émergence de souches sécrétrices de BLSE [68].

## **VI) Résistance bactérienne**

### **1) Evolution de la résistance.**

Ces dernières années la proportion de souches résistantes aux antibiotiques a augmenté de façon dramatique. Les souches productrices de bêta-lactamase à spectre étendu (BLSE) étant très largement majoritaires.



**Figure 6 :** Evolution de la résistance bactérienne en Europe (Source : European Antimicrobial Resistance Surveillance Network (EARS-Net)).

La figure 6 montre une très nette progression des souches résistantes en Europe (dont la France) avec une forte proportion de souches sécrétrices de BLSE. Cette progression est en lien avec le mésusage des antibiotiques.

#### a) Béta-lactamase à spectre étendu

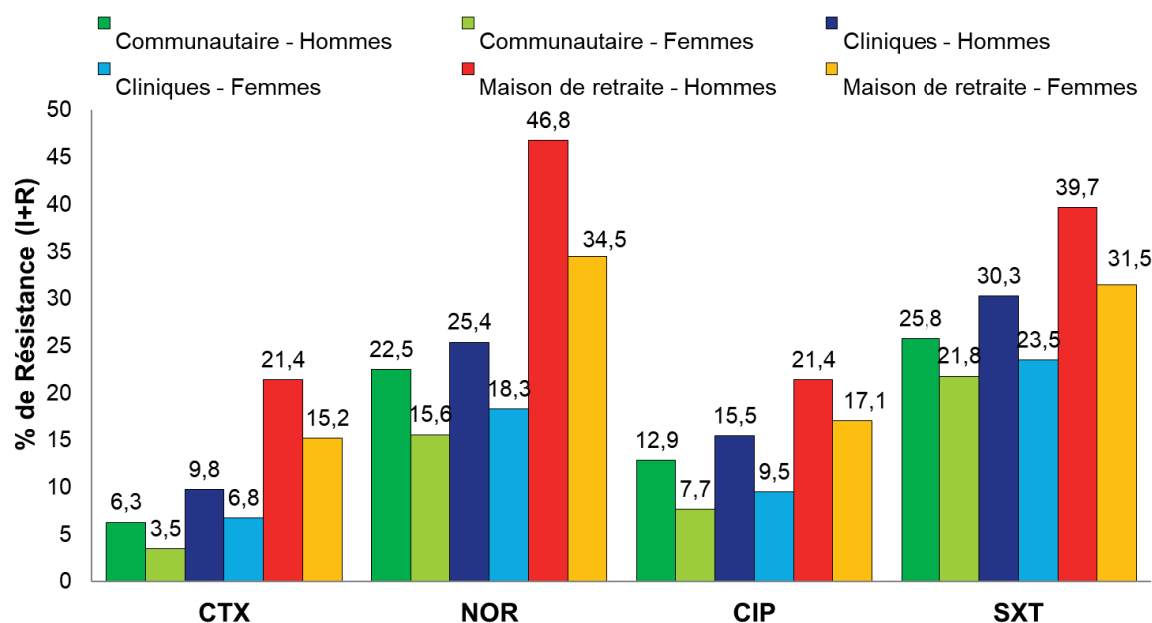
Les BLSE sont une famille d'enzyme permettant à certaines bactéries de résister aux antibiotiques possédant un cœur  $\beta$ -lactame (pénicillines, céphalosporines ...) [69]. Ces antibiotiques agissant sur la synthèse du peptidoglycane, ces enzymes sont sécrétés à l'extérieur de la membrane. Cette résistance est portée par un plasmide [70]. Le nombre de bactéries uropathogènes produisant des béta-lactamase à spectre étendu est en nette augmentation ces dernières années [71].

#### b) Céphalosporinase

Les céphalosporinases sont codées par le chromosome [72]. Lorsque l'enzyme est codée par le chromosome sa production résulte d'une sur-expression du gène *ampC* [73]. Les souches produisant cette enzyme montrent une résistance accrue aux céphalosporines et aux inhibiteurs de  $\beta$ -lactamases [74]. Cette enzyme est présente principalement chez *E. coli* et *K. pneumoniae* [75][76][77].

## 2) Résistance bactérienne et infections urinaires

Le problème de la résistance aux antibiotiques des souches provoquant des infections urinaires n'est pas qu'un problème hospitalier, de plus en plus de souches communautaires présentent ces résistances (plus de 2 millions de cas en France par an – données ONERBA). Dans ces cas, *E. coli* représente 70-95% des souches.



**Figure 7 :** Répartition de la résistance aux antibiotiques utilisés au cours d'infections urinaires (CTX : céfotaxime ; NOR : norfloxacine ; CIP : ciprofloxacine ; SXT : co-trimoxazole) de souches d'*E. coli*. Elles sont classées en fonction de leur provenance (communautaire, clinique, maison de retraite) et du sexe des patients.

Sur des données de 2013 (Figure 7) on peut constater que le pourcentage de résistance est fonction du sexe. Les hommes semblent plus atteints par des bactéries résistantes. Il en va de même pour l'âge. Quel que soit l'antibiotique on constate que le pourcentage de résistance est nettement plus important dans les maisons de retraite par rapport aux autres cas. De plus, on remarque que le pourcentage de résistance est très proche entre les souches communautaires et les souches hospitalières.



## VII) Alternatives thérapeutiques aux antibiotiques

### 1) Canneberge (*Vaccinium macrocarpon*)

#### a) Généralités

La canneberge est un petit fruit rouge poussant sur un arbuste, principalement en Amérique du Nord. C'est un fruit à la chair ferme avec une saveur fruitée légèrement acide et astringente du fait de sa haute teneur en tanin (polyphénol).



Aujourd'hui elle est cultivée de manière industrielle dans des cannebergières, des terrains aménagés de manière à être inondés de la même façon que les rizières. Il existe de nombreuses variétés différentes mais celle qui est utilisée en biologie est *Vaccinium macrocarpon*.

La canneberge est composée de 80% d'eau 10% de carbohydrate [78]. Parmi les autres constituants, il existe des flavonoïdes, des anthocyanidines (pigment naturel du fruit), des catéchines, des triterpénoïdes, des acides organiques et un faible taux d'acide ascorbique. Les produits à base de canneberge ont été déclinés sous de nombreuses formes (boisson, compléments alimentaires, sauces, concentrés et poudres sous forme de gélules).

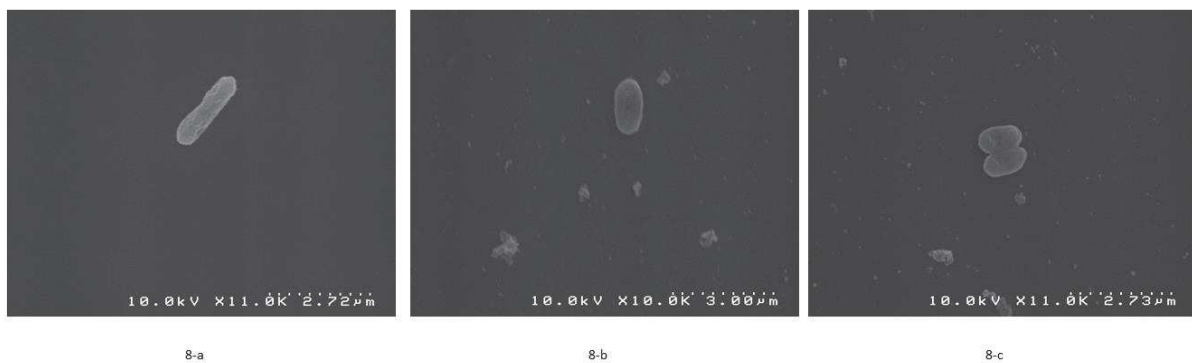
#### b) Effet anti-adhésion de la canneberge

L'adhérence de la bactérie à l'uroépithélium est la première étape dans la physiopathologie des infections urinaires. Cette étape est suivie par une multiplication et une colonisation du tractus urinaire [79]. L'adhésion va permettre à la bactérie de remonter le tractus urinaire en évitant son élimination par le flux urinaire. Une des premières études à mettre en évidence l'effet anti-adhérentiel de *Vaccinium macrocarpon* a été menée par Sobota en 1984 [80]. Cet auteur a montré



qu'après absorption de 450 ml de jus de canneberge, une diminution de près de 75% de l'adhésion des souches d'*E. coli* isolées chez des patients ayant eu une infection urinaire était constatée. Cet effet a été visualisé 1 à 3h après l'absorption du jus de canneberge [81].

On a longtemps pensé que l'effet anti-adhérentiel de la canneberge était dû à l'acidité du fruit qui aurait un effet bactériostatique sur la bactérie. Par la suite l'action d'un composé qui aurait un effet analogue au récepteur du fimbriae a été évoquée, empêchant par compétition l'adhésion de la bactérie à l'épithélium. Plus récemment, il a été montré que les mécanismes d'action de la canneberge comprenaient : une inhibition de la synthèse des fimbriae de type P, une déformation du corps cellulaire de la bactérie (réduisant sa surface d'adhésion à la paroi vésicale) [82] et un changement des propriétés électrostatiques de la membrane (provoquant un décalage dans la distribution des potentiels électriques).



**Figure 8 :** Illustration de l'impact de la canneberge sur *E. coli* uropathogène

Photo de microscopie à balayage. La figure 8.a représente une bactérie après incubation dans un milieu standard (Lurio Broth). Les figures 8.b et 8.c représentent des bactéries après incubation dans un milieu standard complémenté avec une solution de canneberge. La figure 8.b montre bien l'arrondissement de la forme de la bactérie après un traitement à la canneberge. La figure 8.c montre deux bactéries collées l'une à l'autre, montrant l'effet « magnétique » que peut avoir la modification des propriétés électriques de la membrane.

Une étude menée par Zafriri a identifié les deux composants qui pouvaient porter le potentiel anti-adhésion de la canneberge : le fructose et la proanthocyanidine de type A [83].

### c) Proanthocyanidine de type A (PAC-A)

Le fructose étant présent dans tous les fruits, ce sont les PAC-A qui portent le potentiel anti-adhérentiel de la canneberge. Les PAC sont des composants produits par les plantes en réponse à un stress environnemental ou lors d'infections bactériennes [84]. Une analyse par spectrométrie de masse a démontré que l'activité biologique anti-adhésive des PAC était due à la fraction oligomérique de l'épicatéchol possédant une double liaison interflavanique de type A [85]. Il a été montré que les PAC agissaient sur l'ensemble des souches d'*E. coli* quelle que soit leur résistance aux antibiotiques. Cela en fait un candidat alternatif idéal à l'heure de la dissémination des souches multirésistantes [86][87].

L'impact de la canneberge sur l'adhésion est déjà connu, cependant plus récemment une étude a voulu comparer l'efficacité de différents compléments alimentaires à base de canneberge sur l'effet anti-adhérentiel [88]. Il ressort de cette étude que tous les compléments alimentaires n'ont pas la même activité anti-adhérentielle. Cela s'explique par des concentrations en PAC-A variables selon le complément alimentaire étudié.

La quantification des PAC est difficile à cause de la forte teneur en pigments. Cela rend les méthodes de dosage par chromatographie et spectrophotométrie moins efficace. Il existe deux méthodes actuellement validées : la chromatographie liquide avec spectrométrie de masse (LC-MS) ou la méthode colorimétrique modifiée 4-diméthylaminocinnamaldehyde (DMAC). Les difficultés de quantification amènent des disparités entre différents produits commerciaux et peuvent expliquer la différence d'efficacité observées entre différentes études [89].

### d) Etudes cliniques

La première étude clinique évaluant les effets de *Vaccinium macrocarpon* a été publiée en 1966 [90]. Dans cette étude les auteurs ont montré l'effet positif de la canneberge chez des patients ayant des bactériuries récidivantes. Depuis de nombreuses études cliniques ont été réalisées sur des population plus variées (femmes en activité génitale, personnes âgées, enfant ayant des malformations ou non et personnes ayant des infections urinaires récidivantes) [91][92][93]. Une grande partie de ces études ont évoqué les effets bénéfiques de la canneberge sur la prévention des infections urinaires quel que soit l'âge des patients [94].

La base de données Cochrane indique que l'utilisation de *Vaccinium macrocarpon* semble être une alternative intéressante dans la prévention des infections urinaires mais qu'aucune étude concluante

n'a été menée à ce jour [95]. Cependant, aucune étude n'a encore démontré le bénéfice d'un traitement à base de canneberge sur une longue période (>3 mois).

## **2) Propolis**

### **a) Généralités**

La propolis désigne toute une série de substances résineuses, gommeuses et balsamiques recueillies sur certaines parties de végétaux (bourgeons et écorces principalement) par les abeilles. Elles vont ensuite les ramener à la ruche et vont les transformer en ajoutant leurs sécrétions salivaires et de la cire [96]. En France, la principale essence d'arbre productrice de propolis est le peuplier mais on en trouve d'autre comme les conifères, l'aulne, le bouleau, le chêne ... La composition de la propolis est très variable selon la source végétale visitée par les abeilles mais également par la provenance géographique. Cependant certains composants sont relativement stables : résines et baumes (50-55%), cires (30%), huiles volatiles et essentielles (10%), matières organiques et minérales (5%) et pollen (5%).

Avant d'être utilisée en vue d'une utilisation commerciale, la propolis brute va ensuite être lavée et solubilisée dans l'éthanol à 95% afin d'éliminer les éléments indésirables (cire, pollen et autres impuretés). Le produit final va avoir une haute concentration en flavonoïdes, flavones et polyphénols qui sont reconnus pour leur action bénéfique depuis l'Antiquité.

### **b) Propriétés anti-bactériennes de la propolis**

Les activités anti-bactériennes de la propolis ont été rapportées il y a déjà de nombreuses années. Elles ont été démontrées sur des bactéries à Gram positif tel que *Staphylococcus* spp., *Streptococcus* spp. et *Bacillus* spp. [97][98][99] mais également sur deux bacilles à Gram négatif : *P. aeruginosa* et *E. coli* [10]. De plus la propolis a une action anti-bactérienne contre les bactéries anaérobies [100]. Le mécanisme d'action de la propolis serait multifactoriel. Il comprendrait une synergie entre les flavonoïdes, les hydroxyacides et les sesquiterpènes [101][97]. De plus, certains extraits de propolis potentialiseraient l'effet de certains antibiotiques [101]

### **c) Propolis et infections urinaires**

Les effets antibactériens de la propolis sont connus depuis de nombreuses années. Cependant son usage dans le cadre des infections urinaires est récent. La première étude à avoir été menée dans cette optique fut conduite par notre équipe en 2011 [11]. Cette étude s'est focalisée sur l'association de la propolis avec la canneberge et son impact sur l'adhésion et la virulence de souches d'*E. coli*

uropathogènes. Les résultats de cette étude ont montré une potentialisation de l'effet de la canneberge après ajout de propolis.

L'idée d'associer la propolis à un autre composant naturel a été à nouveau étudiée cette année. En effet, deux études se sont intéressées à l'association de la propolis avec de l'hibiscus. La première a étudié l'impact de l'association hibiscus/propolis sur la quantité d'*E. coli* que l'on peut retrouver dans les fèces [102]. Les auteurs ont formulé l'hypothèse que les souches provoquant des infections urinaires sont d'origine fécale, et qu'une réduction du nombre de bactéries que l'on va retrouver à la sortie du système digestif induira une diminution du risque de développer une infection urinaire. Pour démontrer cela, des rats ont ingéré des capsules de cette association. Les résultats ont montré une diminution du nombre d'*E. coli* et d'*Enterococcus* spp. dans les urines. La seconde étude a étudié l'impact de la propolis, de l'hibiscus et de l'association de ces produits sur l'adhésion de 2 souches d'*E. coli* sur des cellules RWPE-1 (cellules de références quand il s'agit d'étudier l'adhésion à un tissu épithélial) [103]. Après un traitement avec cette association, il a été constaté une diminution de l'adhésion des bactéries à ces cellules en microscopie électronique.

Ces études montrent que l'utilisation de la propolis, dans le cadre du traitement et de la prévention des infections urinaires, pourrait être prometteuse. Cependant, ces études restent préliminaires.

Nous venons donc de voir que l'utilisation de composés naturels de type canneberge ou propolis est une stratégie intéressante qui permettrait de diminuer l'utilisation d'antibiotiques dans le traitement et la prévention des UI. D'autres stratégies ont cependant été envisagées et sont en cours d'évaluation. La revue de la littérature qui suit dresse un bilan des différentes approches proposées.

# Travail n°1

## **Alternative therapeutic options to antibiotics for the treatment of urinary tract infections**

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*Review*

## **Alternative therapeutic options to antibiotics for the treatment of urinary tract infections.**

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## **Abstract**

Urinary tract infections (UTIs) are a global health problem because of the number of people affected by these infections and the cost of treating them. Moreover, these infections may become recurrent in some people. In this case, UTIs are treated with long-term antibiotics, which have the consequence of favoring the emergence of antibiotic-resistant strains. Solutions other than antibiotics should be developed to prevent and treat recurrent UTIS. In this review, we investigate the case of vaccines, small compounds, nutraceuticals, immunomodulating agents and probiotics.

**Keywords:** Urinary Tract Infection, alternative therapeutic

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Urinary Tract Infections (UTIs) are the most common bacterial infections [1]. Millions of people, mainly women are affected by this infection and it represents a high healthcare preoccupation [2]. The problem of this infection is not so much due to its gravity but especially to the recurrence [3], and to the increase of the resistance of the strains involved [4]. The strain the most frequently isolated is Uropathogenic *Escherichia coli* (UPEC) [5] and over these last years it was one the best example of bacteria which developed mechanisms of resistance. The other pathogens commonly involved in UTIs are: *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, Group B *Streptococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp [5].

In the case of recurrent UTIs, preventive treatment may be based on long-term antibiotic prophylaxis. It is this kind of therapeutic option that may promote the development of resistance mechanisms against antibiotics and increase the cost of health care. In this context the development of new therapeutic options to prevent and treat UTIs and most particularly recurrent UTIs have to be developed. In this review, we are going to investigate all the existing approaches.

## 1) Vaccines

We do not completely understand the reasons of the recurrence of UTIs. One of the hypothesis could be a flow in the adapt immune system which could be caused by the bacteria by masking themselves or attenuate the immune response. It is in this context that vaccine targeting virulence factor of uropathogenic bacteria was born. Cystitis accounts for 90% of UTIs and 20% to 30% of them are recurrent. These are the specific virulence factors of the bacteria involved in this pathology that are mainly targeted.

### 1.1) Vaccines targeting adhesion

The major pathogen isolated in UTIs is *E. coli*. It is why a lot of studies were focused on its pathogen. The most exposed protein of the bacteria could be a good target for a vaccine but it is very difficult to target proteins of the bacterial membrane. Even if it is the most exposed proteins, a huge heterogeneity of these proteins exists in *E. coli*.

We know the importance of bacterial adhesion to urothelium in the pathogenesis of UTIs, so targeting pili with vaccines could be a good strategy. Pili are important organelles to the colonization of mucosal surfaces. They are promising candidates to a vaccine because of the importance of adhesion in the pathogenesis of UTIs and also because antibodies could block the adhesin-host cell receptor and then decrease the bacterial colonization [6].



Type 1 pilus adhesin FimH is a good candidate for a vaccine because of its critical role in UTI pathogenesis. A FimCH vaccine was designed and protected cynomolgus monkeys from cystitis [7]. Even if there were few inclusions, it is a promising result to an application to humans. Another study on mice with an intranasal and intramuscularly administration showed also a protection against cystitis, but the intranasal way produced a stronger immune response [8]. In a more recent study a recombinant protein MrpH.FimH was injected with a transurethral instillation and they demonstrated a high immune response and a protection against UTIs caused by UPEC and *Proteus mirabilis* [9].

Type P pilus is more involved in pyelonephritis than in cystitis. A vaccine which can block the renal colonization has been tested in mice [10]. No more work in this domain has been developed since the 1980s because of the antibodies produced thanks to these vaccines weren't able to block the subunit responsible of the adhesion [11]. These results could be explained by the high degree of variation of the major subunit of P pili: PapA. Then, after a better comprehension of the type P pili, a new vaccine based on purified PapDG protein demonstrated a good efficiency to prevent colonization of kidney on cynomolgus monkeys [12].

### 1.2) Vaccines targeting toxins

Target toxins produced by UPEC have been tested to protect the bladder or kidney during an UTI. The alpha-hemolysin is the most known toxin of UPEC and some studies have worked on a mice model of pyelonephritis. A co-infection with the bacteria and the vaccine have been performed simultaneously but, despite to a reduction of the renal injury, they did not show a complete protection of the kidney with their vaccine [13]. Moreover, a long term protection has not been demonstrated for this vaccine. This strategy do not seems to be designed for a human treatment.

### 1.3) Vaccines targeting iron metabolism

Another metabolic pathway which could be a good candidate for the vaccination is the iron metabolism. Like all the pathogens, the acquisition of iron is primordial for the bacteria. So some studies have targeted siderophore, heme receptors and other functional molecules which are involved in iron acquisition. In the first study, the authors tested 6 iron receptors and found that the antibodies against the yersiniabactin receptor, FyuA, conferred kidney protection in mice [14]. In another study, instead of targeting iron receptors the same team targeted molecules involved in the iron metabolism. On the 6 candidates, two conferred a protection to the bladder (IreA and IutA) and one protected the kidneys (Hma) of mice after an intranasal immunization [15].

## 2) Small compounds

Some molecules can interact with the products of the bacteria, instead of the bacteria itself, and have an impact on the pathogenicity. Moreover, it is harder to the bacteria to develop a resistance against molecule which target specific compound.

### 2.1) Small compounds targeting adhesion

The adhesion is a critical metabolic pathway for the pathogenesis of the uropathogenic bacteria. So the development of strategies targeting this pathway (or some part of this pathway) is crucial. The very conserved structure of the adhesive organelles makes them good candidates to develop antibacterial agents. [16]. The small molecules targeting adhesion can be classified in two categories: the molecules which inhibit the capacity of adhesion of the fimbriae, and the molecules which target the assembly of the fimbriae.

#### 2.1.1) Pillicide

Pilicides are compounds with small molecular weight. The main action of these molecules is to prevent the formation of UPEC pili [17]. They affect the mechanism of the chaperone by binding the hydrophobic patch of the N-terminal domain (domain conserved in chaperones) [18][19]. Different types of pilicides exist. The pilicide ec240 decreases the expression of genes related to type 1 fimbriae and decreases also the piliation. It was also demonstrated that it has an impact on the motility by modulating the expression of genes involved in the flagella [20]. *In vitro* studies which test the potential of this kind of molecules have promising results. These compounds reduced the adhesion of UPEC strains on cells by a strong reduction of the fimbriae density (70-80% of reduction) [16]. Moreover, this kind of compound has been tested on a mouse model [21]. In this study, the pilicide has a strong impact on the adhesion, the biofilm formation and also reduced the virulence *in vivo*. It is also interesting to note that it reduced the biofilm formation on abiotic surface.

#### 2.1.2) Mannoside

Mannoside are FimH receptor analogue. They are going to block the adhesion by binding, thanks to a high affinity, at the mannosylated receptors. The studies which test these compounds in a mouse model demonstrated a diminution of the bladder colonization even after an oral administration [22][23].

## 2.2) Small compounds targeting urease

Urease is an enzyme which catalyzes the hydrolysis of urea and which is very important in the pathogenesis of several uropathogen like *P. mirabilis*, *Klebsiella* sp., *Pseudomonas* sp. and *Staphylococcus* sp. [24]. This enzyme leads to the alkalization of the urine and the production of struvite and carbonate apatite which compose for the major part the urinary stones [25]. These conditions lead to an inflammation of the bladder, an increase of the risk of catheter-associated biofilm formation and could also contribute to a pyelonephritis [26][27].

The most studied inhibitors of urease are the hydroxamic acids [28]. This molecule has a high inhibitory activity against urease which is carried by the nickel ions [29]. In a first time this molecule treated UTIs by preventing urine alkalization [30][31] but more and more side effects have been found [32][33]. The worst side effect provoked by hydroxamic acid is its mutagenic power [34].

The highest activity of inhibition is carried by the phenyl phosphoramidates [35]. Some studies have tested this of molecules in a physical model [36] and in a rat model [37] and they found promising results but no more *in vivo* studies or clinical trial have been developed for many years. It could be explained by the main disadvantage: a poor hydrolytic stability which leads to a very short half-life [38].

Other molecules exist which carry an inhibitory activity against bacterial urease but they are not fully adapted to treat UTIs. One of them are the quinones, a class of active compounds which carry an high oxidizing potency [39]. The main problem to test these molecules *in vivo* is their cytotoxic and carcinogenic properties.

## 2.3) Small compounds targeting bacterial capsule

The polysaccharide capsule biogenesis is an important part of the virulence for the UPEC strains. Like other pathogens, the capsule is used as a defense against opsonophagocytosis and complement mediated killing [40][41]. The capsule is also involved in the formation of biofilm and the formation of intracellular bacterial communities [42]. Moreover, it could be interesting to target this way of the virulence of bacteria which developed mechanisms of antibiotic resistance. Indeed, without its capsule, bacteria become more sensitive to the immune system. For now, the utilization of these compounds is complicated in humans because of their antigenicity and their poor bioavailability [43]. Nevertheless, the studies in mice showed an acute sensitivity towards the immune system with a significant increase of the bacterial death after an exposure with human serum. Furthermore a better survivability of the mice have been observed [44].

### 3) Nutraceuticals

Nutraceuticals is a pharmaceutical-grade which gathers all the foods or food products which can provide medical benefits and be delivering under medical form. They have to provide health benefits in addition to the basic nutritional value of the foods.

#### 3.1) Cranberry

Cranberry (*Vaccinium macrocarpon* Ait.) is a berry which growth in North America. For years the cranberry is more and more used in prevention against UTI [45]. The main efficacy is related to the antiadherence properties of cranberry [46][47]. The A-type proanthocyanidin (PAC-A) in cranberry have been implicated as important inhibitors of Type-I fimbriae *E. coli* adhesion to uroepithelial cells.

Some studies *in vitro* and *in vivo* demonstrate the capacity of the cranberry to reduce the adhesion of bacteria to the cells [48][49]. This pathway is crucial for the pathogenicity of UTIs. Moreover, *E. coli*, the pathogen mostly isolated, is affected by the cranberry.

However the adherence is not the only key metabolic pathway involved in the pathogenesis of UTIs, motility and biofilm formation are also important. The motility is important for the ascending infection (urethra to bladder and bladder to kidney) [50]. Moreover, the biofilm formation is involved in the recurrence of UTIs and in catheters associated UTIs (CAUTI) [51][52]. Some studies investigated the impact of the cranberry on the motility and the biofilm formation. Cranberry has a negative impact on the swarming of *P. aeruginosa* and *P. mirabilis* [53][54] and on the biofilm formation of *E. faecalis*, *P. aeruginosa* and *E. coli* [55][56][57]. These results demonstrated the diversity of target that the cranberry can affect.

As see previously, the cranberry does not have an effect only against *E. coli*. Others pathogens involved in UTIs are also impacted by the cranberry [58][55][59][60]. In these studies, it was demonstrated that the cranberry has an impact on the virulence factors, the growth, the enzyme activity, the adhesion, the motility and the biofilm formation.

Nevertheless, all studies did not demonstrate that cranberry has a strong impact on the bacteria. Some studies or clinical trials showed no convincing results of cranberry on the recurrence of UTIs or the virulence factor of bacteria [61][62]. These different results could be explained by the difference between cranberry extracts. We know that PAC-A carry the anti-microbial effect of cranberry and the assay of its concentration in an extract is dependent on the methods used. Moreover, some clinical trials used cranberry juice, but it is less effective than food supplement composed of dried cranberry

with a known concentration of PAC-A. So, these contradictory results could be explained by a cranberry extract with a too small concentration of PAC-A.

In order to enhance the effectiveness of the cranberry, combinations of cranberry and other natural products with antimicrobial properties could be used. Propolis is a good candidate for an association. Propolis is a resinous material collected by bees from plants then mixed with wax and bee enzymes [63]. Propolis has antimicrobial, anti-inflammatory, anti-tumour, immune-modulatory and anti-oxidant activities. It has been used for several year to treat gastrointestinal disorder (food supplement) [64], oral health (mouthwash) [65] and dermatological care (creams and ointments) [66][67][68]. A study showed the effect of the combination of cranberry and propolis on the adhesion and the virulence. After addition of propolis, an improve of the anti-adhesion and anti-virulence effect of cranberry has been showed [69].

### 3.2) *Hyaluronic acid*

The urinary bladder epithelium is composed of the urothelial cells which carry specific sensors and properties in addition to form the first barrier to pathogens [70]. To maintain this capacity to fight the infections, these cells produced sulphated polysaccharide glycosaminoglycan (GAG). It is going to cover the epithelium and form a nonspecific antiadherence factor. A major part of the GAG layer of the bladder is composed by hyaluronic acid (HA) and chondroitin sulfate (CS). Virulence factors (secreted by *E. coli* for example) damage the GAG layer to prepare its adhesion [71].

A study investigated the impact of HA and CS on recurrent UTIs on 276 women (aged 18-75 years). The intravesical administration of HA and CS was given to 181 women and the standard treatment against recurrent UTIs was given to 95 women. A reduction of the recurrence of UTIs (62.1% with the standard treatment and 55.7% with the HA+CS treatment) has been demonstrated. The monitoring of patients has been done after 1 month, and each 6 months up to 24 months. After 241-365 days, there was a significant reduction of recurrent UTIs [72]. Another study was conducted with the same protocol with 157 inclusions. Authors also observed a significant reduction on recurrence and an augmentation on time-to-recurrence between each UTIs [73].

The possible impact of a synergy between HA+CS and estrogen on the recurrence of UTIs in postmenopausal women has been explored. A total of 145 postmenopausal women was selected and divided in three groups: treated with estrogen, treated with HA and treated with HA+CS and estrogen. A slight effect on the HA alone but also a significant impact of the estrogen and the HA+CS on the recurrence of UTIs in postmenopausal women have been observed. [74]

So the current studies showed the importance of the GAG layer in UTIs. Moreover, treatments based on HA+CS (with a hypothetical supplementation with estrogen) could be promising to find antibiotics-free methods to cure UTIs.

### 3.3) D-mannose

D-mannose is a monosaccharide closely related to glucose. The absorption, after an oral administration is fast (30 minutes to reach the organs) and it is eliminated via the urinary tract [75]. The adhesion of bacteria to the host cell is crucial, and the surface of cell is covered by mannosilable proteins which interact directly with the bacterial fimbriae [76]. So an exogenous intake of D-mannose can block the liaison between the bacterial fimbriae by competition [22]. A recent clinical trial compared the recurrence of UTIs with a daily antibiotic treatment to a daily administration of a solution of D-mannose. This study showed a strong reduction of the side effects (only diarrhea for 8% of people who have taken the D-mannose) and the same impact on the recurrence of UTIs [77].

### 3.4) Galabiose

If the type I fimbriae are able to adhere to the mannosilable receptor (via FimH) so the type P fimbriae are able to adhere (via PapG) to the galabiose-like receptor [78]. This kind of fimbriae is essential for the pathogenesis of UTIs by helping the bacteria to reach the kidneys [79]. It is also a strategy to understand the structure of the different variants of PapG (PapG II and PapG III for the most important) to design a drug able to disturb this specific adhesion [80]. In vivo studies have not been done yet to observe the impact of this sugar on UTIs. Nevertheless, an in vitro study on *Streptococcus suis* have been conducted. A very efficient inhibition of the hemagglutination with an MIC of 0.3nM has been observed [81].

## 4) Immunomodulant agents

Rapidly after the beginning of the UTI, we observed an activation of the innate immune system by secretion of cytokines and the recruitment of macrophages and neutrophils [82]. Even in absence of an effective antibiotic treatment this could be enough to counter the infection. But the persistence of several strains (like UPEC) in the bladder is the cause of an acute immune response and an inflammation of tissues. Moreover, multiple infections lead to chronic inflammation and it increase the risk to develop recurrent UTIs [83][84].

We know that the expression of cyclooxygenase (COX)-2 is increased after an infection of the bladder epithelial cells by UPEC. Moreover, there is a correlation between the severity of the inflammation and the augmentation of the expression of COX-2 [85]. Studies investigated the impact of COX-2

inhibitor on a mouse model and observed an incidence on the recurrence of cystitis. Secondly, this inhibitor did not only treat the symptoms of the infection, it also decreased the related inflammation (which improves the clinical outcome) [86]. A clinical trial has shown a similar improvement [87].

Synthetic immunomodulant agent is not the only alternative, immunomodulant coming from plants are also interesting. The Green Tea extract (GTE) is a good example [88]. Among all its components, it found a large composition of polyphenolic compound, and especially catechins. The biological properties of catechins are antioxidant, antiangiogenesis, antiproliferative activity and antineoplastic [89][90]. Some *in vitro* studies showed its antibacterial impact against UPEC and in a rat model of cystitis it has been observed a significant decrease of the inflammation and a decrease of the edema [91]. These results are promising even if the mechanisms of action are still unclear.

## 5) Probiotics

A probiotic is a micro-organism which provides benefits to the health after administration in addition to nutritional supply.

### 5.1) Vaginal lactobacilli

Vaginal lactobacilli could be used as prevention against the recurrent UTIs. They produced antimicrobial compounds and biosurfactant which disturb the adhesion between uropathogens and host cells [92]. A pilot study has been conducted and shown the efficacy of a treatment composed of vaginal suppositories with *Lactobacillus crispatus* GAI 98322. A strong reduction of the recurrence of UTIs without seeing any side effects has been observed [93].

### 5.2) Escherichia coli strain 83972

Another strategy, which can be used, is to colonize the bladder with an avirulent strain. A study used the avirulent *E. coli* 83972 isolated from an asymptomatic bacteriuria diagnosed in a woman [94]. This strain has lost its capacity to develop Type 1 and Type P fimbriae. In a mouse model of UTI it was demonstrated that this strain have a better fitness than a virulent strain of UPEC. In a poor environment, like the bladder, this difference of fitness is a crucial advantage for the competition between bacteria. So the strain 83972 could reduce the impact of UTIs by a monopolization of the resources and the space [95]. A randomized trial including patient suffering from recurrent UTIs has been conducted. Patients have been inoculated with *E. coli* 83972 or saline. In this group with *E. coli* 83972, patients developed the first UTIs long after the control group, and they have a recurrence of UTI, 3 time less than the control group [96].



### 5.3) Predatory bacteria

Another strategy to fight against multi-drug resistant strains is the utilization of predatory bacteria. The species *Bdellovibrio* and *Micavibrio* are the most studied [97].

These bacteria are small, motile and prey other Gram-negative bacteria (like *E. coli*). *Bdellovibrio bacteriovorus* is going to use its type IV pilus to adhere and penetrate in the outer membrane (bdeopast) of its prey. Inside the bacteria *B. bacteriovorus* modifies the membrane of its prey to allow its growth until it uses all the nutrients. This entire process takes only 2-3h [98].

Among the bacteria that hunt *B. bacteriovorus* and which can be involved in UTIs, there are *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. (studies developed *in vitro*) [99]. Even more interesting, it was shown *in vitro* that *B. bacteriovorus* reduced significantly the quantity of biofilm [100]. The impact cell line [101] *in vitro* did not show any negative impact and the *in vivo* test on animals seemed to have no side effect [102]. Nevertheless, on some animal models, it has not been observed any improvement on the symptoms of diseases.

Most of healthy people have *B. bacteriovorus* in their intestinal system and it was shown that people with chronic diseases (Inflammatory bowel diseases, celiac disease and cystic fibrosis) have a reduced concentration of this bacteria [103]. So the presence of these bacteria seems to have an impact on the frequency of chronic diseases. This characteristic is interesting concerning the recurrence of UTIs.

For now, no studies investigated *B. bacteriovorus* to treat UTIs but it could be a good candidate (Gram-negative bacteria resistant to antibiotic, problem of recurrence linked to biofilm formation) to fight multi-drug resistant Gram-negative bacteria.

## 6) Conclusion

The progress of the understanding of UTIs has been remarkable these last decades. Almost all the alternative therapeutic options that we present here target the adhesion. Targeting this crucial step has a lot of advantages. First, the mechanisms are well known for UPEC and the other uropathogens, so it is easier to design vaccines or small molecules to disturb the adhesion of bacteria. Then, bacteria which cannot adhere efficiently will be eliminated naturally by the urine flow. The design of chemical products is not the only solution. More and more, natural products like cranberry (*Vaccinium macrocarpon*), are used in medical practice. Another mechanism targeted by the alternative therapeutic option is the secretion of toxins. The aim of these strategies is to attenuate the symptoms of the infection and facilitate the task for the immune system. The last strategy used is to bring into opposition avirulent bacteria against uropathogens. This strategy is the most risky for the host because



the response of the immune system cannot be fully predicted or the real impact of the new strain on the pathogens could be counterproductive. However, the first clinical trial observed very encouraging results.

All these strategies have one objective: to improve the treatment of UTIs and especially the recurrent UTIs. Antibiotics are more and more used to cure these types of infections but the rise of antibiotic resistance is becoming a worldwide problem. UPEC is a very good example of this problem. These alternative treatments can improve the efficacy of antibiotic treatment by targeting another metabolic pathway of the bacteria and bypassed the resistance.

To conclude, a lot of alternative therapeutic options have been developed these last decades, in parallel with the comprehension of the most common infection: the urinary tract infection. The results of clinical trials are promising but some treatments have to be further tested to have soon an application in medicine.

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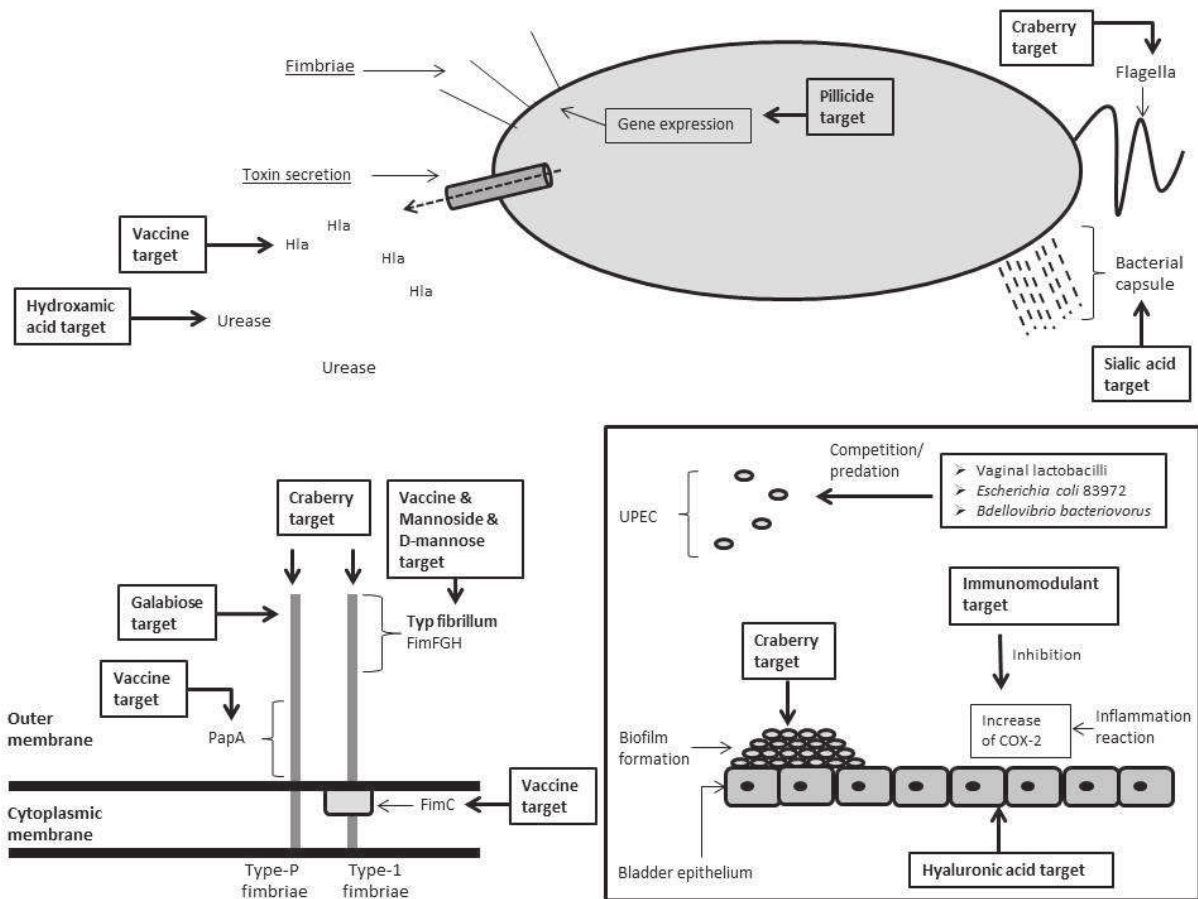


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**Figure legends**

**Figure 1:** Recapitulative scheme of the alternative therapeutic options against UPEC.

**Figure 1**



## Travail n°2

**Potentialisation of the effect of cranberry (*Vaccinium macrocarpon*)  
by propolis on the virulence of uropathogenic *Escherichia coli***

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## Résumé :

La plus connue des thérapies alternatives prescrite dans la prévention des cystites récidivantes, est la canneberge (*Vaccinium macrocarpon*). Elle va être principalement utilisée en poudre sous forme de complément alimentaire. Depuis de très nombreuses années, il est connu que son impact principal sur les bactéries, en particulier sur *E. coli*, concerne l'adhésion via un impact direct sur les fimbriae, la forme de la bactérie et sur les propriétés électriques de la membrane. Cependant son mécanisme d'action reste encore mal compris et peu d'études ont décrit les autres mécanismes d'action de la canneberge en dehors de l'effet anti-adhérentiel. D'autre part, une étude menée par notre équipe associant une solution composée de poudre de canneberge à de la propolis améliorait l'impact de la canneberge sur la diminution de l'adhésion et de la virulence de souches d'*E. coli* uropathogènes (UPEC).

L'objectif de ce travail était d'observer l'impact de la canneberge, de la propolis et de l'association des deux composés sur une souche d'UPEC isolée d'un patient ayant développé une cystite récidivante. Cette étude s'est particulièrement focalisée sur différentes voies métaboliques d'*E. coli* qui sont impliquées dans la physiopathologie de l'infection urinaire (IU) : l'adhésion, la mobilité, la réponse aux stress, le métabolisme du fer et la formation de biofilm.

Une analyse du transcriptome de la souche d'UPEC après une nuit d'incubation avec les trois différents régimes a été réalisée par microarray. Les résultats obtenus par microarray ont ensuite été confirmés par qRT-PCR. Ces résultats ont également été confirmés par des tests phénotypiques axés sur la mobilité et sur la capacité à former du biofilm de la souche testée.

Cette étude a permis de mettre en évidence l'impact de la canneberge, de la propolis et de l'association des deux sur le transcriptome d'une souche d'UPEC. En effet, la canneberge diminuait l'expression de nombreux gènes codant pour des protéines impliqués dans l'adhésion, notamment ceux qui codent pour les fimbriae de type 1 et P. Ces résultats étaient attendus au vu de l'effet anti-adhérentiel de la canneberge précédemment décrit. D'autres gènes codant pour des protéines indirectement impliquées dans l'adhésion étaient négativement impactés montrant l'effet de la canneberge sur de nombreuses voies métaboliques ayant un rôle dans l'adhésion. Il a également été montré que la canneberge entraînait la sous-expression de gènes codant pour des protéines impliquées dans la mobilité (dont ceux codant pour des composants importants du flagelle). Cet impact a été confirmé par deux tests phénotypiques mesurant deux types de déplacements de la bactérie: le « swimming » et le « swarming ». La canneberge avait de plus un rôle dans l'augmentation de l'expression des gènes codant pour des protéines liées à la réponse au stress et au

métabolisme du fer. D'autre part, il a été montré une diminution de l'expression des gènes codant pour des protéines liées à la formation de biofilm en présence de canneberge. Ces résultats ont été confirmés par deux tests phénotypiques axés sur la formation de biofilm précoce et la formation de biofilm complet. Des résultats similaires ont été obtenus après un traitement de la souche d'UPEC avec la propolis seule (hormis pour le métabolisme du fer). L'analyse transcriptomique réalisée après le traitement de la souche d'UPEC par l'association de propolis/canneberge a mis en évidence un effet potentialisateur de la propolis sur l'ensemble des voies métaboliques impactées par la canneberge seule. Cet effet synergique a également été constaté par les tests phénotypiques.

Cette étude a permis de montrer que l'association canneberge et propolis avait un effet significatif sur l'expression de gènes codant pour des protéines liées à des voies métaboliques impliquées dans la physiopathologie des infections urinaires chez une souche d'UPEC. De plus, ces résultats transcriptomiques ont été confirmés par des tests phénotypiques axés sur la mobilité et la formation de biofilm. Ce travail a donc montré l'intérêt que pourrait avoir l'utilisation de l'association canneberge/propolis dans la prévention voire le traitement des IU.

*Scientific Report*

**Propolis potentiates the effect of cranberry (*Vaccinium macrocarpon*) against the virulence of uropathogenic *Escherichia coli***

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**ABSTRACT**

Uropathogenic *Escherichia coli* (UPEC), the most prevalent bacteria isolated in urinary tract infections (UTI), is now frequently resistant to antibiotics used to treat this pathology. The antibacterial properties of cranberry and propolis could reduce the frequency of UTIs and thus the use of antibiotics, helping in the fight against the emergence of antibiotic resistance.

Transcriptomic profiles of a clinical UPEC strain exposed to cranberry proanthocyanidins alone (190 µg/L), propolis alone (102.4 µg/L) and a combination of both were determined.

Cranberry alone, but more so cranberry + propolis combined, modified the expression of genes involved in different essential pathways: down-expression of genes involved in adhesion, motility, and biofilm formation, and up-regulation of genes involved in iron metabolism and stress response. Phenotypic assays confirmed the decrease of motility (swarming and swimming) and biofilm formation (early formation and formed biofilm). This study showed for the first time that propolis potentiated the effect of cranberry proanthocyanidins on adhesion, motility, biofilm formation, iron metabolism and stress response of UPEC. Cranberry + propolis treatment could represent an interesting new strategy to prevent recurrent UTI.

**Keywords:** Adhesion, Biofilm, Cranberry, *Escherichia coli*, Motility, Propolis, Urinary tract infection, Virulence

## Introduction

Urinary Tract Infections (UTIs) are the most common bacterial infections,<sup>1</sup> affecting nine million women in America and 150 million people worldwide each year.<sup>2,3</sup> Women are predominantly affected by UTIs, with 50% of women presenting an UTI during their life, of which 25% will develop a recurrent UTI.<sup>2,4</sup> Uropathogenic *Escherichia coli* (UPEC) are the major pathogen involved in UTIs.<sup>5</sup> Over recent years, these bacteria have developed mechanisms of resistance against  $\beta$ -lactams, cotrimoxazole and fluoroquinolones, the antimicrobial agents usually used in these infections.<sup>4,6</sup> The incidence of multidrug resistant *E. coli* has dramatically increased since the beginning of the century.<sup>7</sup> In this context, it is essential to develop new strategies to prevent or treat UTIs.

Recent evidence suggests that cranberry is effective at preventing UTIs,<sup>8-10</sup> largely due to its anti-adherence properties. The A-type proanthocyanidins (PAC-A) in cranberry have been shown to be important inhibitors of Type-I fimbriae *E. coli* adhesion to uroepithelial cells by modifying the bacteria form, inducing cell rounding and thus reducing its surface of adherence.<sup>11,12</sup> Propolis, a resinous material produced by bees from mixing plant materials with wax and bee enzymes,<sup>13</sup> has been long utilized for its antiseptic and local anesthetic properties. It also displays antimicrobial, anti-inflammatory, anti-tumour, immune modulatory and anti-oxidant activities, among others.<sup>14</sup> A previous study showed that propolis could amplify the impact of PACs, offering some protection against UPEC anti-adhesion activity, bacterial multiplication and virulence.<sup>15</sup> The objective of this study was to evaluate the impact of cranberry PAC, propolis and a combination of these two components on the transcriptome of UPEC.



## Results

### Cranberry and propolis affect UPEC virulence

Expression levels of the entire genome of a clinical *E. coli* strain were measured in the presence of the cranberry and propolis products separately and compared to those of the untreated isolate. The expression level of 5,379 open reading frames was determined. Their log relative transcription levels are shown in Table S1. Overall, 1,245 and 94 genes were found to be up-regulated and 2,190 and 1,384 were found to be down-regulated by cranberry and propolis alone, respectively (Figure 1).

- **Genes involved in adhesion:** As previously observed,<sup>15-19</sup> cranberry inhibited UPEC adhesion. Thus, genes encoding Type-1 (*fimBFGHI*) and Type-P fimbriae (*papCDEGHIK*) were significantly down-expressed. Moreover similar down-regulation was observed with genes encoding chaperone usher fimbriae (*yad*, *ycbQ*) and biogenesis of type IV pili (*hofBCQ*). Interestingly, propolis alone also acts on other genes involved in UPEC adhesion: a significant down-expression of genes involved in fimbriae synthesis (*fimZ*, *papA*) and a conserved adhesin (*ecpD*) was noted.

- **Genes involved in motility pathway:** Cranberry exposure caused significant down regulation of genes involved in flagellar biosynthesis (*fliHJPTGLSMOFS*, *flgAHK*), components of motor (*motAB*) and in flagellar assembly (*div*). Moreover, cranberry treatment also resulted in over-expression of a repressor gene linked to motility (*nsrR*). Similarly, propolis exposure caused down-regulation of a gene involved in filament structure (*fliC*).

- **Genes involved in iron metabolism:** As previously observed,<sup>20,21</sup> cranberry treatment caused an up-regulation of genes encoding iron metabolism regulators (*fur*), transport protein (*feoAB*), genes involved in biogenesis pathway of Fe-S cluster (*iscAS*) and iron storage (*ftnA*, *acnA*, *sodB*). Propolis treatment alone had no significant effect on this pathway.

- **Genes involved in stress response:** Cranberry treatment caused significant over expression of genes involved in stress response (*arcA*, *rpoS*, *cpxR*), periplasmic stress (*rseAC*) and oxidative stress (*oxyR*). Moreover, propolis resulted in overexpression of a gene involved in membrane stress (*pspA*) and in oxidative stress (*csiD*).
- **Genes involved in biofilm formation:** Cranberry treatment resulted in significant down-expression of genes involved in the production of exopolysaccharide (*yjbHF*) and chemotaxis (*tsr*). Propolis also caused down-regulation of a gene important for biofilm maintenance (*emrE*).

### **Propolis potentiates the effect of cranberry**

Microarrays were then performed for the same clinical *E. coli* strain in the presence of both products. Their log relative transcription levels of the combined treatment are shown in Table S1. Upon exposure to cranberry + propolis, 2,950 genes were found to be up-regulated and 2,150 were found to be down-regulated (Figure 1). In large part, the same genes expression results were obtained for the combination as for cranberry or propolis alone, however some other modifications were observed in the combined treatment, with a notably increased number of up-regulated genes, suggesting a potentialisation of cranberry effect by propolis.

- **Genes involved in adhesion:** The combination of cranberry + propolis had an impact on genes with anti-adhesion activity. Thus, genes encoding Type-1 and Type-P fimbriae (*fimC*, *papF*), and curli filament (*csgB*) were significantly down-expressed.
- **Genes involved in motility pathway:** The combination of cranberry + propolis caused a down-expression of genes involved in flagellar biosynthesis (*fliAR*) and up-regulation of a key regulator (*lrhA*), thus increasing the loss of UPEC motility.
- **Genes involved in iron metabolism:** The iron metabolism induced by cranberry alone was also increased in the combined treatment with a significant up-expression of genes encoding

iron transport (*efeB*, *fepA*), iron storage (*ftnB*) and an activator of iron transport (*feaR*).

- **Genes involved in stress response:** Cranberry + propolis caused an up-regulation of genes involved in oxidative stress (*yggE*), and membrane stress (*rseB*, *spy* and *uspB*).
- **Genes involved in biofilm formation:** Cranberry + propolis resulted in a down115 expression of genes involved in the production of exopolysaccharide (*bscC*), the initiation of biofilm (*tqsA*), biofilm maintenance (*bdm*) and chemotaxis (*cheAR*, *malE*).

### Confirmation of gene expression changes by qRT-PCR

In order to confirm results obtained by microarray analysis, we determined the expression of randomly selected genes from each metabolic pathway (adhesion, motility, iron metabolism, stress response, and biofilm formation) and for each condition (cranberry, propolis and cranberry + propolis) using qRT-PCR.

For the cranberry and propolis alone, we confirmed the same gene expression changes as those observed with microarray regardless of the metabolic pathway (Figure 2).

For cranberry + propolis treatment, the majority of results were confirmed although some differences were noted, in particular for genes involved in iron metabolism. Indeed, whilst the majority of genes involved in this pathway were overexpressed (*ftnA*, *sodB*, *iscA*, *efeB*, *cpxR*), this up-regulation was non-significant for three genes (*iscA*, *efeB*, *cpxR*) (Figure 2). Moreover, two other genes (*iscS* and *feaR*) were down-expressed according to qRT-PCR, although these were also not significant. Also *lrhA* (motility) and *rseB* (stress response) gave opposite, although non-significant results on qRT-PCR compared to microarray. *csiD* is up-expressed according to microarray data upon exposure to propolis alone, but is significantly down expressed according to qRT-PCR data with propolis alone and cranberry + propolis.

### **Propolis potentiates the effect of cranberry on biofilm**

Biofilm formation results were confirmed with crystal violet experiments. Complete biofilm formation of untreated *E. coli* G50 was obtained after 48h (OD<sub>620</sub>= 1.02 ±0.35) (Figure 3A). Cranberry treatment had no significant effect on the formation of the biofilm (OD<sub>620</sub>= 0.81 ±0.19, p= NS), whereas propolis alone and combined with cranberry significantly inhibited biofilm formation (OD<sub>620</sub>= 0.28 ±0.12, p<0.001 and 0.59 ±0.16, p<0.01, respectively).

The Biofilm Ring test® was performed to evaluate the effect of cranberry + propolis on the capacity of *E. coli* to form biofilm. Untreated *E. coli* G50 constituted a largely complete biofilm by 2h and this biofilm was fully formed by 5h (BFI = 2.43 ±0.15 at 2h and 1.58 ±0.05 at 5h) (Figure 3B). Cranberry alone had no significant impact on biofilm formation kinetics, with cranberry-treated cells showing similar BFI values at both timepoints to that of untreated cells (3.68 ±0.6 at 2h and 2.05 ±0.2 at 5h, p= NS). In contrast, a slowdown of biofilm formation was detected for propolis-treated G50 after 2h and 5h (BFI= 5.10 ±1.7 and 4.22 ±2.9 respectively, p<0.05). This effect was significantly increased when G50 was incubated with cranberry + propolis (BFI= 7.08±0.15 at 2h and 3.28 ±0.79 at 5h, p<0.0001).

To further investigate the lack of impact of cranberry on biofilm formation, we observed the bacterial behavior with cranberry alone, propolis alone and both by an inverted microscope. As seen in Figure 4, we saw an immediate attraction of all the bacteria to each other. This attraction was not due to an active movement from the bacteria but more likely due to an electrostatic movement independent of bacteria. The bacteria formed a mass weaker than a biofilm, as brief agitation of the cells by vortex was sufficient to disperse the bacteria.

### **Propolis potentiates the effect of cranberry on motility**

To confirm the impact of cranberry and/or propolis on mobility, bacterial swimming and swarming motilities were quantified on soft agar at 48h.

As shown in Figure 5, cranberry and propolis alone did not affect the swimming behaviour although a non-significant decrease of swimming could be noted with propolis at 48h ( $43.1 \text{ cm}^2 \pm 1.9$  for untreated,  $42.1 \text{ cm}^2 \pm 0.5$  with cranberry,  $21.2 \text{ cm}^2 \pm 7.5$  with propolis;  $p = \text{NS}$ ). In contrast, the combined cranberry + propolis considerably affected swimming mobility ( $2.4 \text{ cm}^2 \pm 1.9$ ,  $p < 0.001$ ). Swarming behavior was not affected by propolis alone ( $2.9 \text{ cm}^2 \pm 0.5$  for the untreated vs  $1.9 \text{ cm}^2 \pm 1.1$  with propolis,  $p = \text{NS}$ ) at 48h, but a significant effect could be noted with cranberry alone and in combination with propolis ( $0.6 \text{ cm}^2 \pm 0.4$  with cranberry,  $p < 0.05$  and  $0.1 \text{ cm}^2 \pm 0.1$  with the combination,  $p < 0.001$ ).

## Discussion

This study confirms that propolis potentiates the effect of cranberry on transcriptional profiles of UPEC. Previously, we showed that administration of PACs plus propolis offers some protection against bacterial adhesion, bacterial multiplication and virulence in the urinary tract.<sup>15</sup> Here we observed for the first time that more bacterial pathways involved in UPEC pathogenicity (adhesion, motility, biofilm, stress) were affected by the combination of cranberry + propolis than those affected by the two components alone (Figure 6).

Some studies have already shown that PACs play a role in the modulation of adhesion in UPEC.<sup>9,19,22</sup> By taking a transcriptional approach, we confirmed that the inhibition of UPEC adhesion is not only due to an external modification of the bacterial membrane. This anti-adhesion is mainly the effect of PACs on “strategic” genes involved in *E. coli* adherence (Figure 6). Thus the two main adhesins (Type-1 and Pap fimbriae)-encoding genes (*fimH* and *pap*) were significantly down-regulated ( $p < 0.001$ ). Moreover, PACs also affected chaperone usher fimbriae proteins such as Yad. This protein is important in bacterial adhesion but also in biofilm formation and motility. By down-expressing *yad*, cranberry treatment affected all these downstream functions. Interestingly, in a murine model, *yad* gene knockouts reduced

bacteria fitness in bladder and kidney in comparison with wild-type.<sup>23</sup> The same results were observed for *hofBCQ*, Type-IV pili-encoding genes involved in mobility, adhesion, and biofilm formation, conferring an advantage to bacteria colonizing the kidney.<sup>24,25</sup> Our results also showed for the first time that propolis had UPEC anti-adhesion activity, notably on Type-I and Type-P fimbriae-encoding genes (*fimZ*, *papA*) and *ecpD* (*Escherichia coli* Common Pilus), an adhesion-encoding gene, which contributes to adhesion of human epithelial cells and biofilm development.<sup>26,27</sup> Interestingly, cranberry + propolis treatment increased this anti-adhesion effect, while some other fimbriae-encoding genes (*fimC*, *papF*) were also down-expressed. *csgB* gene was also down-regulated by the combined treatment. This gene encodes a subunit which composes the curli filament, an important factor involved in host cell adhesion, invasion and in biofilm formation.<sup>28</sup>

Other pathways were also affected by these treatments. We observed that cranberry alone caused down-regulation of genes involved in motility pathway such as *motAB*, two essential genes participating in flagellar rotation.<sup>29</sup> Moreover, cranberry exposure led to an up-regulation of *clpXP*, two protease-encoding genes controlling expression of flagellar genes by degradation of mRNA of genes related to flagellar biosynthesis (*flhCD*).<sup>30,31</sup> Cranberry treatment also resulted in up-regulation of *crl/rpoS* genes, which are mostly involved in stress response pathways during stationary and exponential phases. Both of these genes repress genes involved in motility (*fliA*, *flgM*).<sup>32</sup> Finally, cranberry treatment triggered up-regulation of *nsrR*, a nitric oxide-sensitive repressor of transcription-encoding gene. Previous experiments have shown that over-expression of *nsrR* led to a reduction of bacterial motility in low-agar plates experiments.<sup>33</sup> Interestingly, propolis also had an effect on genes involved in motility by causing a down-expression of *fliC*, encoding a protein of the flagella.<sup>34</sup>

Moreover, cranberry + propolis treatment had a significant impact on genes involved in motility, by down-regulating two other genes (*fliA* and *fliR*) participating in flagellar

biosynthesis.<sup>35</sup> A key repressor of motility, *lrhA* was also over-expressed under this condition. Knockout of this gene increases bacterial motility and chemotaxis.<sup>36</sup> To corroborate these results, we performed phenotypic experiments of bacterial swimming and swarming. The phenotypic assays confirmed that cranberry + propolis treatment significantly reduced bacteria swimming and swarming.

Secondly, we showed that many genes involved in iron metabolism were up-regulated by cranberry treatment, demonstrating that UPEC adapts to the presence of cranberry PACs by reducing iron storage and up-regulating iron acquisition systems. This confirms that cranberry limits iron availability.<sup>20,21</sup> For example, *fntA* which encodes a major iron storage protein,<sup>37</sup> is up-regulated. Moreover, *feoA* and *B* genes encoding proteins involved in transport of ferrous iron into bacteria,<sup>38</sup> were up-regulated, and their repressor (*feoC*) was down-regulated. Whilst propolis alone did not affect iron metabolism, the combination of cranberry + propolis significantly modified this pathway. Indeed more genes involved in iron transport (*efeB*, *fepA*),<sup>39,40</sup> and transcriptional activator for iron transporter (*feaR*),<sup>22</sup> iron storage (*fntB*)<sup>41</sup> are up-regulated than conditions with cranberry and propolis alone.

Thirdly, we observed that genes related to stress response were up-regulated in response to cranberry alone. For example, *oxyR*, a gene encoding a key regulator in the defense against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), was overexpressed.<sup>42</sup> The ARC two-components system (Anoxic Redox Control) allows facultative anaerobic respiration in response to a modification of nutrients condition. When this system is activated, respiratory metabolism is repressed and fermentative metabolism is promoted.<sup>43</sup> We observed that *arcA*, the gene encoding response regulator protein, was up-regulated in the cranberry-treated bacteria. As aerobic respiration is important for adhesion pathways and virulence of UPEC,<sup>44</sup> we assume that cranberry PACs could also decrease UPEC adhesion and virulence by promoting an anaerobic respiration.

Moreover, PACs have previously been shown to impact expression of *rpoS*, a gene involved

in stress response (pH, osmotic and oxidative stress) in the exponential phase,<sup>31</sup> and *cpxR*, a gene encoding a part of the CpxRA two-components system which is activated in response to membrane stress.<sup>45</sup> Microscopic observations confirmed that PACs induce an attraction between bacteria, possibly due to a modification of negative charged membranes of the bacteria and of surface hydrophobicity as previously observed.<sup>46</sup> Although propolis had a minor effect on UPEC stress response, cranberry + propolis impacted significantly more stress response genes than cranberry alone. For example, *rseB* gene, which is the sensor of environmental stress<sup>47</sup> and *spy*, a gene encoding an activator of *cpx*-controlled genes<sup>48</sup> were up-regulated in the combined treatment.

Finally, we observed that many genes involved in biofilm formation were modified. Most genes of this pathway were down-regulated, as previously noted for different pathogens (*E. coli*, *P. aeruginosa* or *Enterococcus* sp.),<sup>20,46,49-51</sup> although other studies contradict this.<sup>52,53</sup> Our results confirm that cranberry negatively impacts biofilm formation, by a reduction of exopolysaccharide, adhesin and chemoreceptors production. For example, poly-  $\beta$ -1,6-N-acetyl-D-glucosamine (PGA) is an adhesin which is necessary to maintain biofilm structure, and PgaC is required for the synthesis of this biofilm.<sup>54</sup> We observed that cranberry exposure caused down-regulation of the *pgaC* gene and the *yjbEFGH* operon. This operon is involved in the production of the exopolysaccharide, an important part of extracellular matrix involved in biofilm.<sup>55</sup> Finally *tsr* and *cheA* genes were down-expressed in cranberry alone treated cells. These genes encode two important chemoreceptors for bacterial communication and biofilm formation.<sup>56</sup> Propolis exposure also has an impact on biofilm formation; *emrE*, an efflux pump-encoding gene was down-regulated in response to propolis treatment. As previously shown, knockout of this gene strongly decreased biofilm formation.<sup>57</sup> However, as we noted for the other pathways, cranberry + propolis had an even greater impact on biofilm formation. In addition to the genes previously described, cranberry + propolis treatment triggered down-



regulation of: the *bscABZC* operon (involved in cellulose production),<sup>58</sup> *bdm* (biofilm-dependent modulation) gene (which is related to osmotic stress biofilm formation),<sup>59</sup> *tqsA* gene (involved in quorum-sensing) and other genes involved in adhesion and production of matrix (Table S1).<sup>60</sup> Biofilm formation is based on three major pathways: adhesion, motility and chemotaxis, all of which are negatively impacted by cranberry + propolis exposure. To corroborate these results, we performed two phenotypic experiments on biofilm formation. We confirmed that early biofilm formation and the final biofilm were strongly reduced by cranberry + propolis exposure.

In conclusion, this study highlighted that exposure to cranberry + propolis significantly increased the effect of cranberry on UPEC metabolism by down-regulation of genes involved in adhesion, motility and biofilm formation and by up-regulation of genes involved in stress responses and iron metabolism. In the context of increasing multidrug resistant bacteria and limited new antimicrobial solution, combined treatment could represent an interesting strategy to prevent UTI.

## Methods

**Bacterial strain, microbial culture and preparation of extracts.** All the assays were performed with an UPEC strain previously isolated from a patient with cystitis (G50).<sup>61</sup>

A mix of filtered urine (filtered by a vacuum-driven filtration system with a 0.22µm membrane, Millipore© (Billerica, Massachusetts, USA)) and Luria Broth (LB) growth medium (Invitrogen, Villebon sur Yvette) was used to culture bacteria for RNA extraction. Brain-Heart Infusion (BHI) growth medium (CondaLab, Madrid, Spain) was used to grow bacteria for Biofilm Ring-Test® and crystal violet experiments, and LB growth medium culture for microscopy and mobility assays.

Cranberry extract (*V. macrocarpon*) was obtained by dissolving dried cranberry (Exocyan

cran BL-DMAC 6% (Nexira, Rouen, France)) in phosphate buffered saline (PBS) and sterilized by filtration. The concentration of PAC-A was measured by BL-DMAC (colorimetric method<sup>62</sup>). Final concentration of PAC-A used was standardized to contain 190µg/L. The reconstituted PAC extract was stored at -20°C in the dark.

The propolis extract (Plantex, Sainte-Geneviève-des-Bois, France) used in this study is a hydroalcoholic extract of blended propolis from various origins mixed 60/40 w/w with carob. The propolis was diluted in 50 mL of PBS and incubated at 37°C with agitation at 100 rpm for 8 hours. Then, the solution was clarified by centrifugation (4000 rpm, 20°C, 10 min) and the supernatant sterilized by filtration.

**RNA extraction and cDNA synthesis.** G50 isolate was grown in urine and LB broth alone, with cranberry PAC extracts (190 µg/L), propolis extracts (102.4 µg/L) and both to an OD600 of  $\approx$  0.6. Total RNA from bacterial samples was extracted with Tryzol (Invitrogen) according to the manufacturer's instructions and samples were purified with the RNeasy mini kit (Qiagen, Courtaboeuf, France). All the RNA extraction experiments are performed in triplicate. RNA was treated with the RNase-Free DNase Set (Qiagen). Purity and concentration were determined using the Nanodrop<sup>TM</sup> 2000 spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA). cDNA was synthesized from 1µg of total RNA for each sample, using the iScript<sup>TM</sup> Select cDNA Synthesis kit (Bio-Rad, Hercules, CA) with random primers according to the manufacturer's instructions.

**Microarray analysis.** The one-color microarray-306 based prokaryote analysis protocol (FairplayIII Microarray labeling, Agilent) was used according to the manufacturer's instructions to synthesize and label cDNA. cDNA were hybridized onto slides (AMADID GE, *E. coli* 8X15K, Agilent®). The data were normalized using the robust multiarray average algorithm (RMA).<sup>63</sup> Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to obtain raw data and analyze the array images. GeneSpring software (version 12.5,

Agilent Technologies) was used to complete the analysis.

**Comparative real-time qRT-PCR.** To confirm the results found in microarray analysis, transcript levels analysis was performed by quantitative reverse transcription qRT-PCR using randomly selected genes involved in the main metabolic pathways of *E. coli* (Table S1). Real time PCR assays were performed in a LightCycler®480 device using the LightCycler FastStart DNA MasterPlus SYBRGreen I kit with 100 ng of cDNA and 10 pmol of target primers (Table S1). The specificity of the PCR products was tested by melting-point analysis. Amplifications were performed in duplicate from three different RNA preparations. The  $\Delta\Delta CT$  method was used to analyze transcriptional changes in target genes using *gapdh* as the housekeeping control gene. Data were log transformed to obtain a fold change difference between the different studied conditions.<sup>64,65</sup>

**Measure of constituted biofilm by crystal violet.** Biofilm development was also assessed by incubating bacterial cultures after an overnight incubation at 37°C and dilution to obtain a final optical density at OD600  $\approx$ 1, in 96-well microtiter plates in BHI. The plates were incubated at 37°C for 24h and 48h. After incubation, adherent cells were fixed with methanol (99%) washed three times with distilled water, then crystal violet (0.1%) added for 10 min at room temperature. The biofilm was then dissolved with acetic acid (33%) and crystal violet uptake was measured by taking the OD620.<sup>52</sup>

**Kinetics of biofilm formation.** Kinetics of early biofilm formation was explored using the Biofilm Ring Test® (BioFilm Control, Saint Beauzire, France) as previously described.<sup>66</sup> Briefly, standardized bacterial cultures were incubated at 37°C in 96-well microtiter plates in the presence of magnetic beads. At different time points (0, 2 and 5 h), plates were placed onto a magnetic block and placed in the reader (Epson scanner modified for microplate reading). The images of each well before and after magnetic attraction were analyzed with the BioFilm Control software generating a BioFilm Index (BFI). A high BFI value ( $> 7$ ) indicates

a high mobility of beads under magnet action, corresponding to an absence of biofilm formation, while a low value ( $< 2$ ) corresponds to a complete immobilization of beads due to sessile cells. Two independent experiments with at least two repeats were performed per condition (cranberry with/without propolis) and per incubation time.

**Motility assays.** The motility of the G50 strain in different conditions was evaluated using soft agar LB plates as described previously: swim plates containing 0.25% of agar and swarm plates containing 0.5% of agar supplemented with 0.5% of glucose.<sup>67</sup> Briefly, bacteria grown overnight in LB were diluted 1000-fold in LB and incubated at 37°C to an OD600 of  $\approx 0.7$ . Swarm plates were inoculated into the middle of soft agar by spotting with 5  $\mu$ l of standardized culture. Swimming plates were seeded with the same inoculum below the agar surface using a sterile inoculating needle. Plates were incubated for 48 h at 37°C. The diameter of the migration zones produced by the strain at different conditions was calculated using Image J software. Swimming and swarming experiments were performed independently three times.

**Optical microscopy.** A bacterial solution grown in LB alone, with cranberry PAC extracts (190  $\mu$ g/L), propolis extracts and both was prepared to an OD600 of  $\approx 0.1$  and observed on an inverted microscope (Leica). The different preparations were also observed after vortex of the solution.

**Statistical analysis.** Statistics and graphs were prepared using the software package GraphPad Prism 6.0. The effects of cranberry and/or propolis on the expression of selected genes and motility were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. Log-transformed data were used for real-time RT-PCR. Kinetics of biofilm formation were compared with a two-way ANOVA followed by Dunnett's multiple comparisons test. The crystal violet experiments were assessed using a Student's t-test.  $p < 0.05$  was considered to reflect a statistically significant difference.

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### **Author contribution statement**

JPL and AS designed the study protocols and secured funding; JR, LL and CDR undertook the experimental measurements; JR, CDR, JPL and AS collaborated on the manuscript preparation. All authors read and approved the final manuscript.

### **Competing financial interest**

Jeremy Ranfaing's work has been funded by Nutrivercell SA (France). He was recipient of a PhD grant (Bourse CIFRE) by this company. Other authors declare no potential conflict of interest.

## Figure legends

**Figure 1. Microarray results. Proportion of genes up-regulated and down-regulated by cranberry (190 µg/L) and propolis (102.4 µg/L) alone and combined.** Cranberry up-regulates 1,245 genes and down-regulates 2,190 genes. Propolis up-regulates 94 genes and down-regulates 1,384 genes. Cranberry + propolis up-regulate 2,950 genes and down-regulate 2,150 genes.

**Figure 2. Log relative fold-change in mRNA expression by qRT-PCR of genes involved in adhesion, motility, biofilm formation, iron metabolism and stress response for G50 strain after treatment with cranberry (190 µg/L) (A), propolis (102.4 µg/L) (B) and combined (C).** The average relative fold-change compared to the control urine/LB condition. The errors bars represent the standard deviation from three different experiments.

**Figure 3. Effect of cranberry, propolis and both on biofilm formation. (A).** Complete biofilm formation was determined by crystal violet experiment. The optical density (OD) is directly linked to the biofilm formation. **(B).** The kinetics of early stages of biofilm for G50 was determined by the Biofilm Ring Test at 2h and 5h. Biofilm Index (BFI) >7 indicates absence of biofilm and BFI <2 indicates a fully formed biofilm. Means and standard errors for three independent replicate are presented. Statistical differences between different growth conditions at each time were obtained by ANOVA.

**Figure 4. Image of strain G50 immediately obtained in a solution without (A), with cranberry (190 µg/L) (B), with propolis (102.4 µg/L) (C) and with cranberry + propolis (D) with an inverse microscope.**

**Figure 5. Comparative results of swimming and swarming assays for G50 in different growing conditions (cranberry, propolis, and combined) at 48h.** The errors bars represent the standard deviation from at least two independent assays.

**Figure 6. Simplified model for the metabolic pathways impacted by the association of cranberry (*Vaccinium macrocarpon*) and propolis.** Genes related to adhesion, motility, iron metabolism and biofilm formation are represented with their variation: + corresponds to an up-regulation; - to a down-regulation.

Figure 1

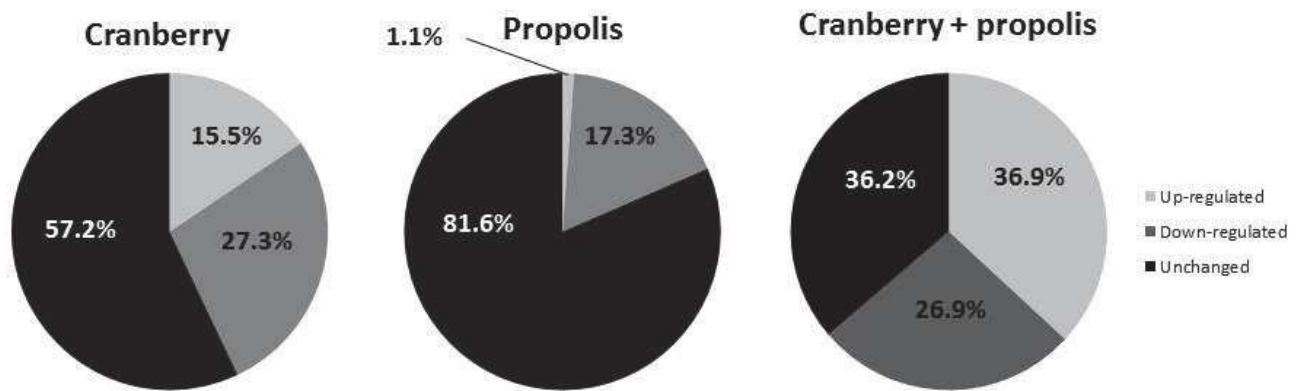




Figure 2

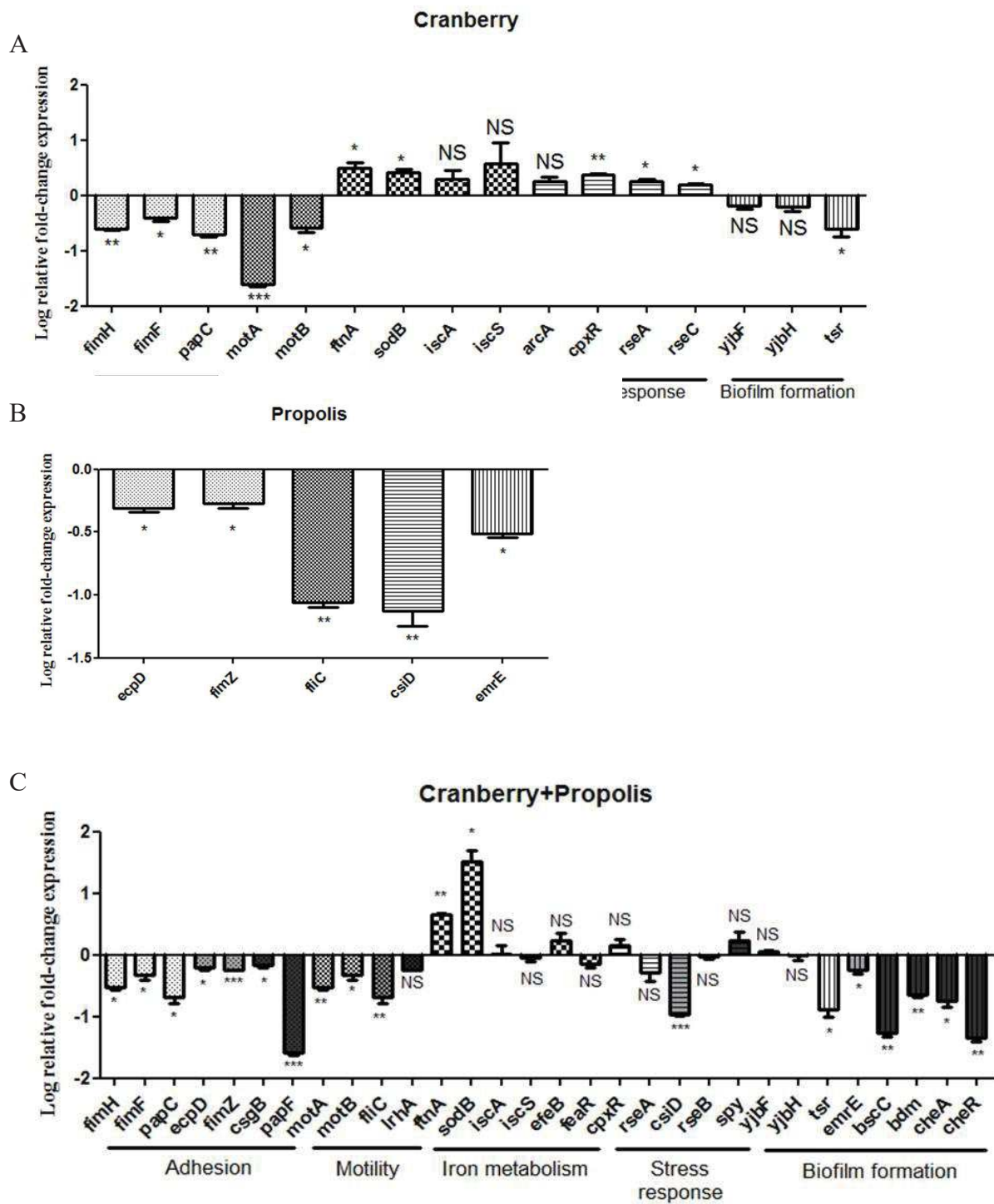
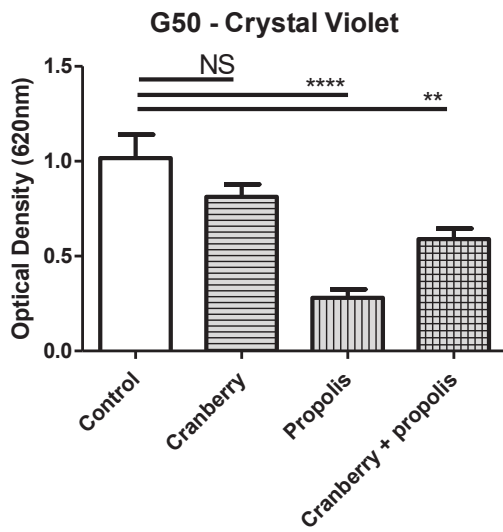


Figure 3

A



B

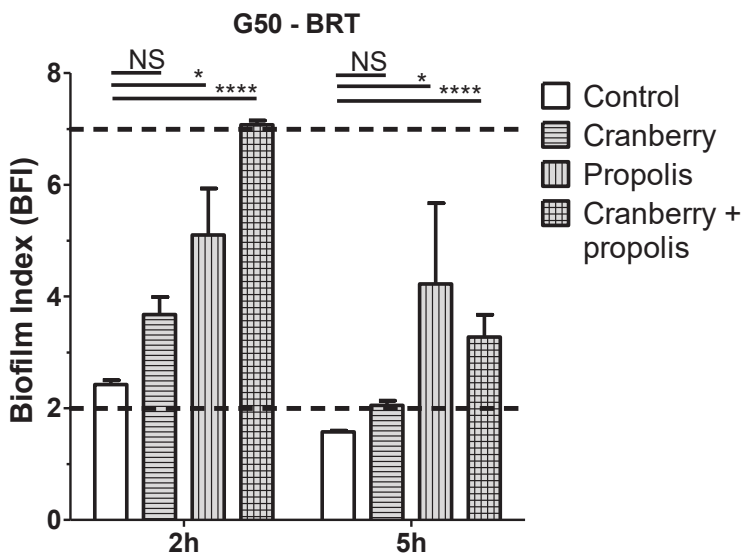


Figure 4

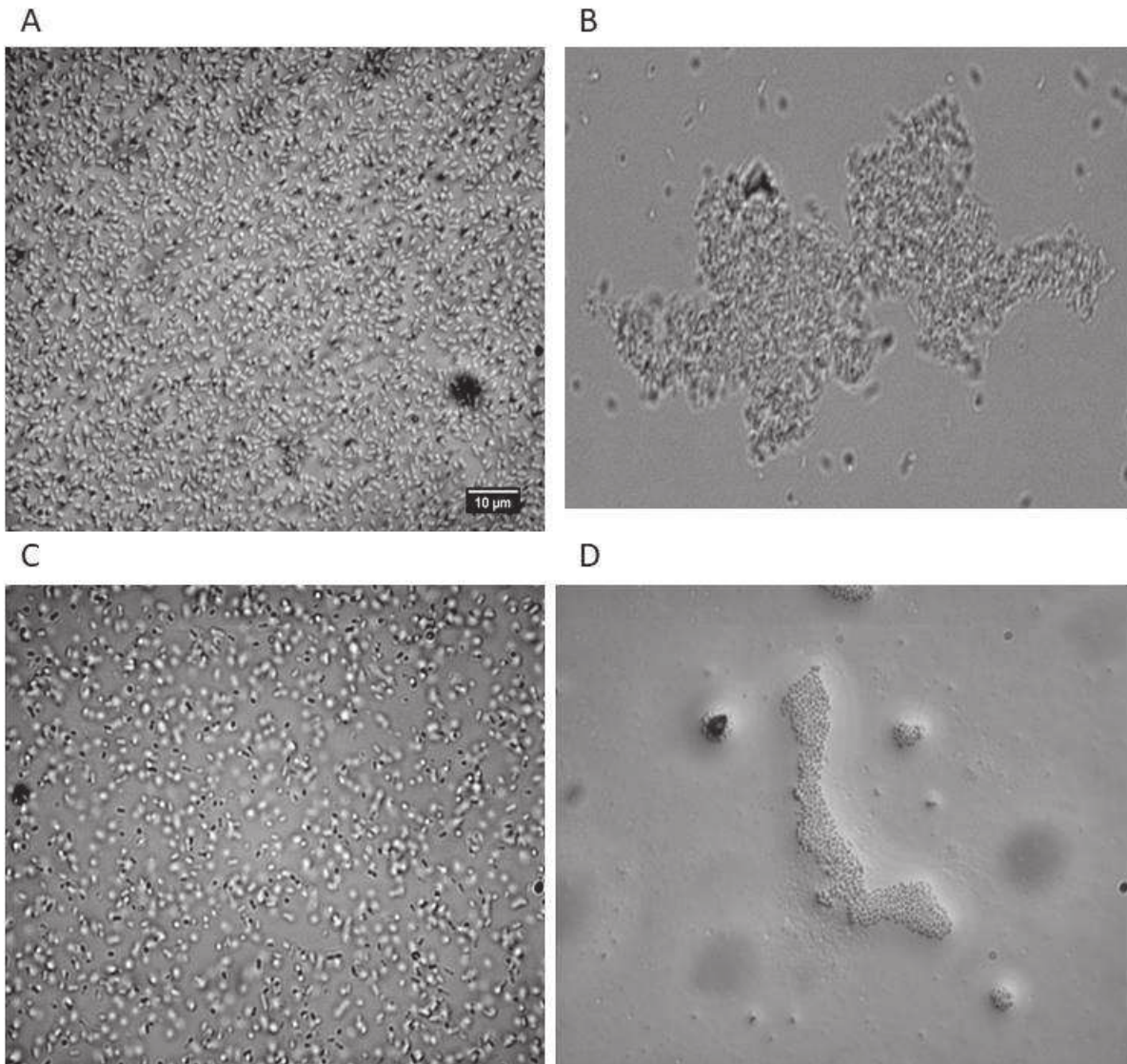


Figure 5

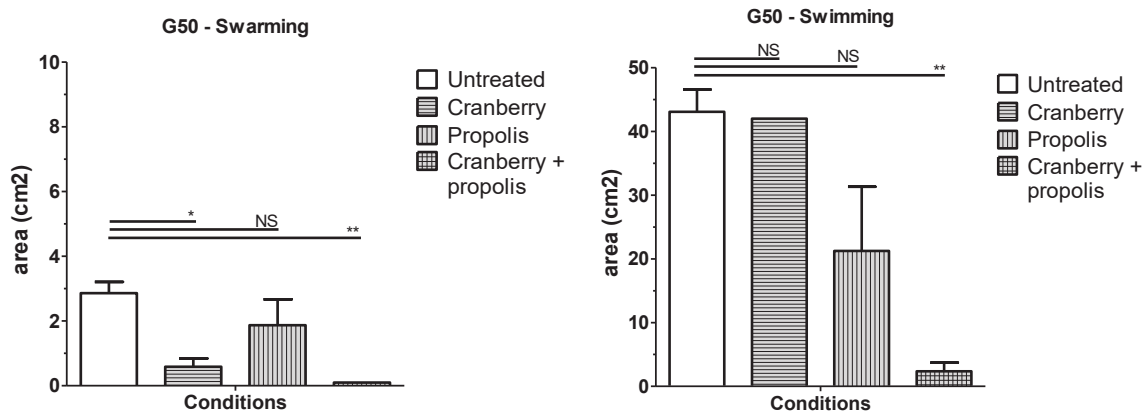
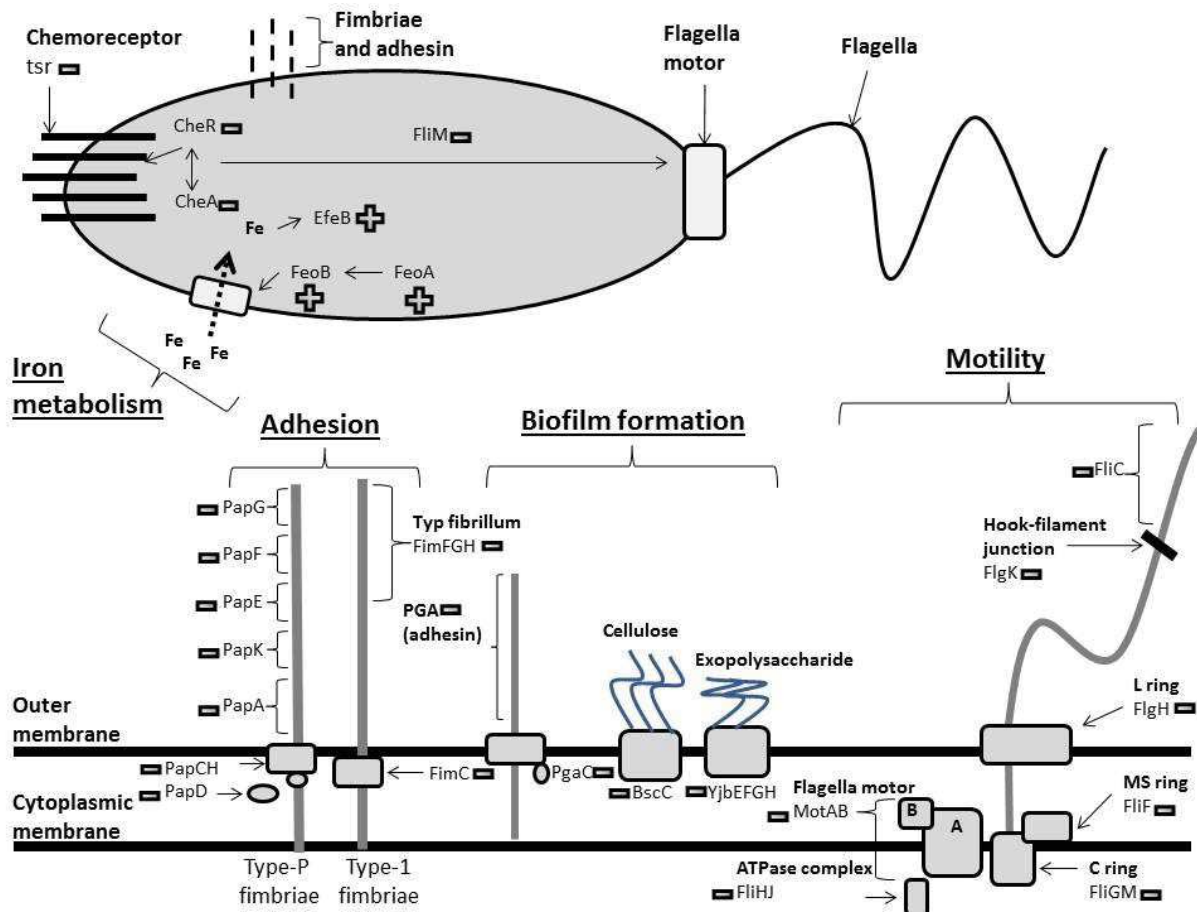


Figure 6



## **Travail n°3**

# **Action of propolis to increase the effect of cranberry in the reduction of the motility and the biofilm formation on a panel of Uropathogenic *Escherichia coli***

Auteurs : Jérémy Ranfaing, Catherine Dunyach-Remy, Jean-Philippe Lavigne, Albert Sotto

Article en cours de soumission

## Résumé :

La première partie de ce travail a permis de montrer que l'utilisation concomitante de canneberge et de propolis avait un effet significatif sur l'expression de gènes liés à des voies métaboliques impliquées dans la pathogénicité des infections urinaires (IU) chez une souche d' *E. coli* uropathogènes (UPEC). De plus, ces résultats de transcriptomique ont été confirmés par des tests phénotypiques axés sur la mobilité et la formation de biofilm. Ce travail avait donc montré l'intérêt potentiel de l'utilisation de l'association canneberge/propolis dans le traitement et la prévention des IU. Cependant, ce travail n'était basé que sur l'étude d'une seule souche d'UPEC, ne prenant donc pas en compte un comportement souche-dépendant.

L'objectif de ce deuxième travail était donc de tester différents paramètres impactés par l'association cranberry/propolis sur une collection d'UPEC isolées de patients ayant contracté une cystite, une pyélonéphrite ou présentant un portage asymptomatique. Des tests phénotypiques sur la mobilité et la formation de biofilm (précoce et complet) de 12 souches d'UPEC ont été réalisés. Les tests de mobilité ont été effectués dans des milieux Luria Broth (LB) contenant un pourcentage d'agar différent : 0,25% d'agar pour le milieu testant la mobilité de la bactérie dans un milieu liquide et 0,5% d'agar supplémenté de 0,5% de glucose pour le milieu correspondant à la mobilité par glissement de la bactérie. En parallèle, le Bofilm Ring® test ainsi qu'un test au cristal violet ont permis d'étudier respectivement la formation précoce et la formation tardive du biofilm.

Concernant la mobilité en milieu liquide, les résultats obtenus ont montré que l'impact de la canneberge et de la propolis était souche-dépendant. Cependant, l'association canneberge/propolis permettait de diminuer le « swimming » de toutes les souches testées. Le traitement par la canneberge a cependant permis de diminuer le « swarming » de l'ensemble des souches testées, l'ajout de propolis ayant alors un effet potentialisateur. La formation du biofilm était quant à lui diminuée après un traitement par l'association canneberge/propolis, la propolis ayant également un effet potentialisateur en comparaison à un traitement par de la canneberge seule.

Cette étude montre l'intérêt de l'utilisation concomitante de la propolis et de la canneberge dans le traitement et l'infection des IU récidivantes.

**Propolis potentiates the effect of cranberry (*Vaccinium macrocarpon*) in reducing the motility and the biofilm formation of uropathogenic *Escherichia coli***

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## Abstract

One strategy to prevent urinary tract infections is the use of natural products such as cranberry (*Vaccinium macrocarpon*) and propolis. The objective of this study was to evaluate the impact of these products alone and combined on the motility and biofilm formation of a collection of representative uropathogenic *Escherichia coli* (UPEC). Motility was evaluated by the swarming and swimming capacity of the isolates in presence/absence of cranberry ± propolis. Early and late biofilm formation was observed with the Biofilm Ring test (BioFilm Control®) and the crystal violet method. Cranberry alone was seen to have a variable effect on motility and biofilm formation unrelated to bacterial characteristics, but a reduced motility and biofilm formation was observed for all the isolates in the presence of cranberry + propolis. These results suggest that propolis potentiates the effect of cranberry on UPEC, representing a new strategy to prevent recurrent urinary tract infections.

## Keywords

Biofilm; Cranberry; *Escherichia coli*; Motility; Propolis; *Vaccinium macrocarpon*

## Highlights

- Cranberry (*Vaccinium macrocarpon*) had a variable effect on the motility of UPEC
- Cranberry (*Vaccinium macrocarpon*) had a variable effect on the biofilm formation of UPEC
- Cranberry and propolis impair the motility and biofilm formation of UPEC

## Abbreviations

BFI, BioFilm Index

BHI, Brain-Heart Infusion

CAUTI, catheter-associated UTI

LB, Luria Bertani

NS, Non significant

PAC-A, A-type proanthocyanidins

PBS, Phosphate Buffered Saline

SD, Standard Deviation

UTI, Urinary Tract Infections

UPEC, Uropathogenic *Escherichia coli*

## 1. Introduction

Urinary Tract Infections (UTIs) are the most common bacterial infections in both community and hospital settings (Silverman et al., 2013). It has been estimated that about 150 million people worldwide develop UTI each year, with high costs in terms of hospitalizations and medical expenses (Schwenger, Tedjani & Loewen, 2015; McLellan, & Hunstad, 2106; de Souza da-Silva et al., 2017). Among the common uropathogens associated with UTIs development, Uropathogenic *Escherichia coli* (UPEC) is the primary cause (Flores-Meireles et al., 2015). Effective methods to prevent UTI have been sought for many years, particularly since the beginning of the century due to the diffusion of multidrug resistance *E. coli* (Liu et al., 2006; Lavigne et al., 2011; Nicolas-Chanoine, Bertrand & Madec, 2014; O'Brien et al., 2015).

UPEC strains possess a plethora of both structural (e.g., fimbriae, pili, curli, flagella) and secreted (toxins, iron-acquisition systems) virulence factors that contribute to their pathogenicity. The ability to adhere to host epithelial cells in the urinary tract represents the most important determinant of pathogenicity (Terlizzi, Gribaudo & Maffei, 2017). Different strategies (e.g., vaccines, natural antimicrobial compounds, anti-adhesive peptides) have been proposed targeting these virulence factors (Terlizzi, Gribaudo & Maffei, 2017). Two natural substances were identified (cranberry (*Vaccinium macrocarpon* Ait.) and propolis) effective to prevent UTIs (Avorn et al., 1994; Foo et al., 2000a; Howell et al., 2010; Hidalgo, Chan, & Tufenkji, 2011; Lavigne et al., 2011; Boonsai, Phuwapraisirisan, & Chanchao, 2014). The antimicrobial effect of cranberry is largely due to the A-type proanthocyanidins (PAC-A), the dominating compound found in cranberries. PAC-As impair bacterial adherence by inhibiting Type-I fimbriae UPEC adhesion to uroepithelial cells (Howell et al., 2005). They also trigger cell rounding, thus reducing its surface of adherence (Ahuja et al., 1998). PAC-As reduce the

mobility of UPEC, a crucial step in the establishment of the ascending infection (passage between the ureter to the bladder and the bladder to the kidney), as seen in *E. coli* (Hidalgo, Chan, & Tufenkji, 2011) and in *Proteus mirabilis* (McCall et al., 2013). Finally, cranberry modifies biofilm formation, another important metabolic pathway involved in recurrent UTI and catheter-associated UTI (CAUTI) (Vollmerhausen et al., 2017), as has been observed in *Enterococcus faecalis* (Wojnicz et al., 2016), *E. coli* (Sun et al., 2015) and *Pseudomonas aeruginosa* (Ulrey et al., 2014). Propolis, a resinous material collected by bees from plants, has antimicrobial, anti-inflammatory, anti-tumour, immuno-modulatory and anti-oxidant activities (Viuda-Martos et al., 2008). Combined with cranberry, this substance can impact UPEC anti-adhesion activity, bacterial multiplication and virulence (Howell et al., 2005; Lavigne et al., 2008; Foo et al., 2010b; Gupta et al., 2012). However, the Cochrane Database concluded that no significant benefit was demonstrated for probiotics to prevent UTI (Schwenger, Tejani & Loewen, 2015).

Whilst the effect of cranberry on adhesion of UPEC has been extensively studied (Liu et al., 2006; Foo et al., 2000a; Gupta et al., 2004; Howell et al., 2005; Lavigne et al., 2011; Sun et al., 2015; de Llano et al., 2015), the data on motility and biofilm formation are scarce. The objective of this study was to evaluate the impact of cranberry, propolis and a combination of these components on motility and biofilm formation of a collection of UPEC strains.

## **2. Material and methods**

### **2.1. Bacterial strain, microbial culture and preparation of extracts**

All the assays were performed with a collection of 12 UPEC strains previously isolated from patients with cystitis, pyelonephritis or asymptomatic UTI (colonisation) (Lavigne et al., 2016) (Table 1).

A mix of filtered urine (filter by a vacuum-driven filtration system with a 0.22 $\mu$ m membrane, Millipore© (Billerica, Massachusetts, USA)) and Luria Bertani (LB) broth growth medium (Invitrogen, Villebon sur Yvette) have been used for the overnight culture of our strains. A Brain-Heart Infusion (BHI) growth medium (CondaLab, Madrid, Spain) has been used for Biofilm Ring-Test® and crystal violet experiments. A LB growth medium culture has been used for mobility assays.

Cranberry extract (*V. macrocarpon*) was obtained by using dried cranberry (Exocyan cran BL-DMAC 6% (Nexira, Rouen, France)) and phosphate buffered saline (PBS) and sterilized by filtration. The concentration of PAC-A was measured by BL-DMAC (colorimetric method (Prior et al., 2010)). Final concentration of PAC-A used was standardized to contain 190  $\mu$ g/L. Cranberry juice was stored at -20°C in the dark.

The propolis extract (Plantex, Sainte-Geneviève-des-Bois, France) used in this study is a hydroalcoholic extract of blended propolis from various origins mixed with carob in a proportion (60/40, w/w). The propolis was diluted in 50 mL of PBS and incubated at 37°C by shaking at 100 rpm for 8 hours. Then, the solution was clarified by centrifugation (4000 rpm, 20°C, 10 min). Supernatant was sterilized by filtration.

## **2.2.Motility assays**

Two forms of motility were studied: swimming and swarming. Swimming is used by the bacteria to move in a liquid medium and swarming in a semi-liquid medium. The motility of studied strains in different conditions was evaluated using soft agar LB plates as described previously (Dusane et al., 2014): swim plates containing 0.25% of agar and swarm plates containing 0.5% of agar supplemented with 0.5% of glucose. Briefly, bacteria grown overnight in LB were diluted 1000-fold in LB and incubated at 37°C to an OD600 of  $\approx$  0.7. Swarm plates were inoculated into the middle of soft agar by spotting with 5  $\mu$ l of

standardized culture. Swimming plates were seeded with the same inoculum below the agar surface using a sterile inoculating needle. Plates were incubated for 24 and 48 h at 37°C. The diameter of the migration zones produced by the strain at different conditions was calculated using Image J software. Swimming and swarming experiments were performed independently three times.

### **2.3. Kinetics of biofilm formation**

Kinetics of early biofilm was explored using the Biofilm Ring Test® (BioFilm Control, Saint Beauzire, France) as described (Chavant et al., 2007). Briefly, standardized bacterial culture were incubated at 37°C in 96-well microtiter plates in the presence of magnetic beads. After different time points (0, 2 and 5 h), plates were placed onto a magnetic block during 1 min then in the reader (Epson scanner modified for microplate reading). The images of each well before and after magnetic attraction were analysed with the BioFilm Control software giving a BioFilm Index (BFI). A high BFI value ( $> 7$ ) indicates a high mobility of beads under magnet action (corresponding to an absence of biofilm formation) while a low value ( $< 2$ ) corresponds to a complete immobilization of beads due to the sessile cells. Two independent experiments with at least two repeats were performed per conditions tested (cranberry with/without propolis) and per incubation time.

### **2.4. Measure of constituted biofilm by crystal violet**

Biofilm development was also assessed by incubating bacterial cultures, after an overnight incubation at 37°C and a dilution to obtain a final optical density at OD600 to  $\approx 1$ , in 96-well microtiter plates in BHI. The plates were incubated at 37°C for 24h and 48h. After incubation, adherent cells were fixed with methanol (99%) and after different washing steps with distilled water, the crystal violet (0.1%) was added for 10 min at room temperature. The biofilm was

then dissolved with acetic acid (33%). At least the liquid was read at OD620 (Ulrey et al., 2014).

## 2.5. Statistical analysis

Statistics and graphs were prepared using the software package GraphPad Prism 6.0. The effects of cranberry and/or propolis on motility were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. The comparisons were estimated between untreated and treated strains. Kinetics of biofilm formation were compared with a two-way ANOVA followed by Dunnett's multiple comparisons test. The crystal violet experiments were assessed using a Student's t-test.  $p < 0.05$  was considered to reflect a statistically significant difference.

## 3. Results

### 3.1. Panel of studied strains

The 12 strains were representative of the most commonly isolates found in UTI.

For resistance, the panel contains 5 strains susceptible to all the main antibiotic used in UTI (G10, G13, G24, G29, G46), 2 strains harbouring a penicillinase (G8 and G50), 2 strains harbouring a penicillinase and a chromosome-mediated resistance to fluoroquinolones (G03 and G19), 1 strain harbouring a penicillinase and an overexpression of efflux pump (G43) and finally 2 main Extended-spectrum  $\beta$ -lactamases (ESBL)-producing strains (G6 and G19), G6 belonging to the worldwide O25b-ST131-B2 clone (Nicolas-Chanoine et al., 2014)

For virulence traits, the half of strains belongs to B2 and D phylogroups related to the virulent UPEC and the other strains belong to the commensal *E. coli* (phylogroups A and B1). All the isolates harbour *fimH* gene which encodes the main pili of UPEC strains. Genes encoding other fimbriae (*papG2*, *papG3*) are differently distributed in the isolates. In the same way the genes involved in hemolysin (*hlyA*), capsule synthesis (*kpsM II*, *k2 kps*), iron acquisition

(*iroN*, *irp2*), and cytotoxicity (*cnf1*) have a variable distribution in correlation with the main UPEC strains found in UTI (Lavigne et al., 2016).

### **3.2. Treatment with cranberry + propolis inhibits UPEC motility**

To confirm the impact of cranberry ± propolis on bacterial mobility, swimming and swarming motilities were quantified on soft agar at 48h. Strains could be split into three different groups based on their motility without treatment and their response to the treatment, independent of virulence and resistance profiles (Figure 1). First, eight strains (G08, G10, G13, G19, G24, G29, G39 and G46) show a moderate swimming without treatment and impaired swimming upon exposure to cranberry, propolis and the combination of both (mean swimming diameter of untreated *E. coli*:  $3.34 \text{ cm}^2 \pm 0.29$ ; cranberry treatment:  $1.32 \text{ cm}^2 \pm 0.58$  ( $p < 0.0001$ ); propolis treatment:  $1.57 \text{ cm}^2 \pm 0.47$  ( $p < 0.0001$ ); cranberry + propolis treatment:  $0.15 \text{ cm}^2 \pm 0$  ( $p < 0.0001$ )). Two strains (G06 and G50) are a high swimming without treatment and are impacted only by the treatment with cranberry + propolis (mean swimming diameter of untreated:  $43.07 \text{ cm}^2 \pm 6.02$ ; vs. cranberry treatment:  $42.0 \text{ cm}^2 \pm 0$  ( $p = \text{Non significant (NS)}$ ); propolis treatment:  $21.23 \text{ cm}^2 \pm 17.45$  ( $p = \text{NS}$ ); cranberry + propolis treatment:  $2.36 \text{ cm}^2 \pm 1.93$  ( $p < 0.0001$ )). Finally, two strains (G03 and G43) are no motility and are not impacted by any of the treatments (mean swimming diameter of untreated:  $1.7 \text{ cm}^2 \pm 0.07$ ; cranberry treatment:  $0.41 \text{ cm}^2 \pm 0.15$  ( $p = \text{NS}$ ); propolis treatment:  $1.99 \text{ cm}^2 \pm 1.35$  ( $p = \text{NS}$ ); cranberry + propolis treatment:  $3.18 \text{ cm}^2 \pm 0.37$  ( $p = \text{NS}$ )).

A significant impact of cranberry alone and cranberry + propolis treatment on swarming was also observed for all strains (Figure 2). As a representative profile, strain G24 shows a mean swarming diameter untreated:  $5.19 \text{ cm}^2 \pm 1.77$ ; cranberry treatment:  $0.8 \text{ cm}^2 \pm 0.26$  ( $p < 0.0001$ ); propolis treatment:  $2.1 \text{ cm}^2 \pm 0.25$  ( $p < 0.05$ ); cranberry + propolis:  $0.1 \text{ cm}^2 \pm 0$  ( $p < 0.0001$ )). For five strains (G03, G10, G29, G39 and G50), the difference between the untreated condition and the propolis condition was not statistically significant.



In summary, although cranberry alone did not always display a significant effect on the motility of the studied UPEC, in combination with propolis, all strains showed impaired swarming.

### **3.3 Variability of the UPEC to form biofilm in presence of cranberry and/or propolis**

The effect of cranberry  $\pm$  propolis on early biofilm formation was assayed by the Biofilm Ring test® (Tables 2 and 3). The majority of the studied strains (7/12) showed early biofilm formation (G03, G08, G10, G19, G39, G43 and G50) (BFI < 2 after 5h of incubation) without treatment (Table 3). No significant effect could be noted after 2h (Table 2). Based on their response to cranberry  $\pm$  propolis after 5h, strains could again be classified into three groups, independent of the virulence and resistance profiles of the isolates (Table 3). Five strains (G03, G08, G10, G19, G39) are impacted by all the treatments (except G08 with propolis) with a strong reduction of early biofilm formation (Table 3, Figure 3). For three strains (G13, G29 and G50), an impact on the early biofilm formation has been only noted after treatment with propolis (Table 3). Finally, none of the treatments impacted early biofilm formation for four strains (G06, G24, G43 and G46). In summary, a great variability of action of cranberry and propolis was observed on early biofilm formation.

To evaluate whether these results extended to the formation of the complete biofilm, crystal violet assays were performed. Two profiles were observed (Figure 4). Half of the strains were significantly impacted by all the treatments compared to their standard behaviour (G03, G06, G13, G19, G29 and G43) ( $p < 0.001$ ) (Table 4). The other half of the studied strains was not impacted by the cranberry (G08, G10, G24, G39, G46 and G50) but was significantly affected by propolis alone and cranberry + propolis ( $p < 0.001$ ) (Table 4).

#### 4. Discussion

The aim of this study was to evaluate the impact of cranberry, propolis and the combination of both on two key metabolic pathways for a large panel of UPEC: motility and biofilm formation. Some studies have already shown the impact of the cranberry and the propolis on the motility of bacteria (Hidalgo, Chan & Tufenkji, 2011; Mirzoeva, Grishanin & Calder, 1997). Cranberry has been shown to reduce the expression of *fliC*, which encodes a major compound of the flagella, in *E. coli* CFT073 (Hidalgo, Chan & Tufenkji, 2011) and of *flaA*, *flhD* and *ureD* in *P. mirabilis* (McCall et al., 2013). However, this effect has not yet been confirmed in UPEC. Moreover, the impact of propolis on motility was noted on *Bacillus subtilis* (Pepeljnjak, Jalsenjak & Maysinger, 1982) and *Pseudomonas aeruginosa* (De Marco et al., 2017). Finally, no studies have investigated the effect of the combination of cranberry and propolis on the motility of a panel of UPEC.

Here, we observed the variability of the effect of cranberry  $\pm$  propolis on the motility of a panel of UPEC. Swimming and swarming are complementary in the pathogenesis of UTIs; swimming is related to the liquid displacement (spread in urine) and swarming to semi-solid displacement (spread at the surface of epithelial cells) (Dusane et al., 2014). We observed: 1) a variation of the effect of cranberry alone on the mobility of the panel of the UPEC strains; 2) a strong impact of cranberry + propolis on the studied strains. Importantly, despite swimming and swarming relying on different activation factors, cranberry + propolis treatment has a clear effect on both pathways in the majority of UPEC, regardless of their virulence and resistance profiles. This combination of compounds was not efficient against the swimming of only two strains (G03 and G43), but still inhibited swarming.

We also observed the same variability of the effect of cranberry  $\pm$  propolis on the biofilm formation of the studied strains. Biofilm formation has serious clinical implications. Biofilm in the uroepithelium of the bladder has been suggested as the mechanism responsible for

recurrent cystitis (Terlizzi, Gribaudo & Maffei, 2017; Azevedo et al., 2017). Moreover, CAUTIs are a serious problem during hospitalization, which is caused by a formation of a biofilm on the catheter (Azevedo et al., 2017). In this study, we investigated the early formation of biofilm (<5h) and the complete biofilm formation (48h). The cranberry alone had a slight impact on the biofilm formation; only half of the studied were impacted (early and complete biofilm formation). The propolis also had a moderate effect on early biofilm formation (seven strains affected). Moreover, propolis inhibits complete biofilm formation of all the studied strains. Interestingly, although we observed a variable effect of cranberry and propolis alone on biofilm formation, the combination clearly impairs formation, indicating that the propolis could potentiate the effect of cranberry on both the early and the complete biofilm formation. These results were expected because the biofilm formation is linked to other metabolic pathways impacted by the cranberry: motility and adhesion (Sobota, 1984; Foo et al., 2000a; Gupta et al., 2004; Howell et al., 2005; Liu et al., 2006; Howell et al., 2010; Lavigne et al., 2011; Sun et al., 2015; de Llano et al., 2015). The effects observed on motility and biofilm formation of our studied strains by cranberry + propolis suggest that the effects of these two compounds are not limited to adhesion, but have a global impact on the bacteria. A previous study has already recorded the negative impact of cranberry on complete biofilm formation on the reference *E. coli* strain (CFT073) (Mitra et al., 2013) and *E. faecalis* (Wojnicz et al., 2016).

Finally, one of the major findings in this study is the variation of effect of cranberry alone against a panel of 12 UPEC. This variation could be due to difference of baseline characteristics of these strains, the difference of clinical origins of the strains (colonisation, cystitis or pyelonephritis), or the difference of virulence and/or resistance profiles. However, no correlation was noted. The variability of the impact of the cranberry on the different UPEC may therefore be due to the presence/absence of a specific activator or regulator. This

important observation could explain the variability of results observed in clinical practice with cranberry (Schwenger, Tejani & Loewen, 2015). Nonetheless, the clear effect observed by the combination of propolis and cranberry suggests that this treatment represents an interesting solution to prevent UTIs.

## **5. Conclusion**

In conclusion, this study shows that the combination of cranberry and propolis has a strong impact on the motility and the biofilm formation of a collection of UPEC. This association could be a promising solution to prevent UTIs in the future, regardless of the virulence and resistance profiles of the UPEC.

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**Table 1.** Characteristics of the clinical Uropathogenic *Escherichia coli* strains used in this study (Lavigne et al., 2016).

	Clinical aspects	Resistance profile <sup>a</sup>	$\beta$ -lactam content	Phylogroup	Main virulence factors
G03	Pyelonephritis	AMX, TIC, NAL, OFX, SXT, FUR	TEM-1	D	<i>papG2, papA, papE, fimH, iroN, KpsM II, K2 Kps, iutA, traT, malX, irp2, cnf1</i>
G06	Pyelonephritis	AMX, AMC, TIC, TCC, TZP, CAZ, CTX, CXM, NAL, OFX, SXT	TEM-24	A	<i>papG2, fimH, iroN, KpsM II, iutA, traT, malX</i>
G08	Cystitis	AMX, TIC	TEM-1	A	<i>fimH, iroN, malX, irp2</i>
G10	Colonisation	-	-	A	<i>fimH, iroN, traT, malX</i>
G13	Cystitis	-	-	A	<i>papA, papC, fimH, iroN, kpsM II, k2 kps, iutA, traT, malX, irp2</i>
G19	Pyelonephritis	AMX, TIC, NAL, OFX	TEM-1	B1	<i>fimH, iroN, iutA, traT, malX, irp2</i>
G24	Colonisation	-	-	B1	<i>fimH, traT, malX</i>
G29	Colonisation	-	-	D	<i>fimH, iroN, KpsM II, K2 Kps, iutA, traT,</i>
G39	Colonisation	AMX, AMC, TIC, TCC, TZP, CAZ, CTX, CXM, FEP, NAL, OFX, CIP, FUR	CTX-M-15, TEM-1	B2	<i>fimH, KpsM II, iutA, traT, malX, irp2</i>
G43	Colonisation	AMX, AMC, TIC, TCC CXM, SXT	TEM-1	B2	<i>papG2, papA, papC, papE, fimH, hlyB, iroN, kpsM II, k2 kps, iutA, traT, Irp2, cnf1</i>
G46	Cystitis	-	-	B2	<i>papG2, papA, papC, papE, fimH, iroN, kpsM II, k2 kps, iutA, traT, malX, irp2,</i>
G50	Cystitis	AMX, TIC, SXT	TEM-1	B2	<i>papG3, papA, papC, papE, fimH, hlyB, iroN, kpsM II, k2 kps, iutA, traT irp2, cnf1, sfa</i>

<sup>a</sup>AMX, amoxicillin; AMC, amoxicillin+clavulanic acid; TIC, ticarcillin; TCC, ticarcillin+clavulanic acid; TZP, tazocillin; CAZ, ceftazidime; CTX, cefotaxime; CXM, cefixime; NAL, nalidixic acid; OFX, ofloxacin; CIP, ciprofloxacin; SXT, cotrimoxazole; FUR, furadantin

**Table 2.** Early biofilm formation (at 2h) of a panel of uropathogenic *Escherichia coli* studied without treatment, with cranberry, propolis and a combination of both. The early biofilm was explored using the Biofilm Ring Test®. The results are presented by means and standard deviation of the values of Biofilm Formation Index (BFI).

Strains	Biofilm ring test at 2h								<i>P</i>		
	Untreated (U)		Cranberry (C)		Propolis (P)		Cranberry + Propolis (C+P)		U vs C	U vs P	U vs C+P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
G03	12.30	0.86	8.38	0.19	11.85	1.37	8.58	0.83	NS	NS	NS
G08	8.31	1.00	7.33	0.73	12.90	1.73	9.43	1.23	NS	NS	NS
G10	8.58	1.00	7.85	1.03	11.83	1.88	9.48	0.88	NS	NS	NS
G19	9.71	0.92	7.43	1.09	11.65	0.57	8.18	0.59	NS	NS	NS
G39	10.01	0.21	9.45	0.21	11.00	1.46	10.13	1.04	NS	NS	NS
G50	2.43	0.15	3.68	0.63	5.1	1.67	7.08	0.15	NS	NS	NS
G13	7.62	1.08	7.77	1.14	11.70	0.45	8.38	0.86	NS	NS	NS
G29	11.28	0.26	9.34	0.73	12.40	1.07	7.60	1.20	NS	NS	NS
G06	9.32	0.68	10.62	1.08	7.53	1.17	5.45	1.31	NS	NS	NS
G24	11.27	0.54	8.58	0.40	11.70	0.69	8.90	0.63	NS	NS	NS
G43	2.26	0.29	5.58	1.04	2.08	0.56	5.23	0.55	NS	NS	NS
G46	12.07	0.92	9.17	0.34	12.75	1.14	8.81	0.37	NS	NS	NS

**Table 3.** Early biofilm formation (at 5h) of a panel of uropathogenic *Escherichia coli* studied without treatment, with cranberry, propolis and a combination of both. The early biofilm was explored using the Biofilm Ring Test®. The results are presented by means and standard deviation of the values of Biofilm Formation Index (BFI).

Strains	Biofilm ring test at 5h								P		
	Untreated (U)		Cranberry (C)		Propolis (P)		Cranberry + Propolis (C+P)		U vs C	U vs P	U vs C+P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
G03	1.68	1.10	8.85	0.70	11.98	1.85	8.33	0.32	<0.0001	<0.0001	<0.0001
G08	1.57	0.10	8.60	0.49	3.85	1.91	8.28	0.29	<0.0001	NS	<0.0001
G10	1.90	0.25	8.18	1.36	9.03	1.29	10.48	0.62	<0.0001	<0.0001	<0.0001
G19	1.65	0.10	9.30	1.16	7.95	2.91	9.48	0.83	<0.0001	<0.0001	<0.0001
G39	1.48	0.09	9.78	0.92	10.10	2.12	7.83	0.45	<0.0001	<0.0001	<0.0001
G50	1.58	0.05	2.05	0.17	4.23	2.89	3.28	0.79	NS	0.004	NS
G13	3.98	1.11	2.62	0.41	9.34	1.25	6.54	0.33	NS	<0.001	NS
G29	4.88	1.14	4.50	1.37	9.60	1.20	5.42	1.40	NS	<0.0001	NS
G06	9.62	0.15	10.90	0.56	11.91	0.48	5.73	1.60	NS	NS	NS
G24	7.81	3.89	8.61	1.75	12.82	0.45	5.61	1.44	NS	NS	NS
G43	1.50	0.01	1.78	0.08	2.15	0.52	1.63	0.13	NS	NS	NS
G46	8.83	1.12	7.25	0.51	10.88	0.95	8.43	0.94	NS	NS	NS

**Table 4. Determination of complete biofilm formation of a panel of uropathogenic *Escherichia coli* studied without treatment, with cranberry, propolis and a combination of both.** The complete biofilm was explored using the crystal violet method. The results are presented by means and standard deviation of the values of OD620. SD, Standard Deviation; NS, not significant

Strains	Crystal Violet								<i>P</i>		
	Untreated (U)		Cranberry (C)		Propolis (P)		Cranberry + Propolis (C+P)		U vs C	U vs P	U vs C+P
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
G03	0.819	0.232	0.396	0.129	0.306	0.138	0.451	0.131	<0.0001	<0.0001	0.0016
G06	0.709	0.310	0.372	0.038	0.379	0.147	0.401	0.101	0.0052	0.0055	0.0084
G13	0.719	0.147	0.555	0.108	0.387	0.161	0.332	0.104	0.0052	0.0012	<0.0001
G19	0.712	0.278	0.375	0.036	0.425	0.231	0.306	0.131	0.0045	0.0087	0.0022
G29	1.194	0.727	0.579	0.143	0.451	0.131	0.488	0.204	0.0143	0.0072	0.0092
G43	1.096	0.443	0.737	0.124	0.450	0.072	0.530	0.220	0.0127	0.0028	0.006
G08	0.852	0.201	0.728	0.055	0.349	0.130	0.259	0.067	NS	0.004	<0.0001
G10	0.621	0.318	0.517	0.190	0.360	0.125	0.329	0.095	NS	0.0053	0.0026
G24	0.855	0.571	0.602	0.208	0.347	0.172	0.479	0.157	NS	<0.0001	0.0044
G39	1.282	0.457	0.981	0.191	0.313	0.057	0.434	0.147	NS	<0.0001	0.0053
G46	1.014	0.440	0.762	0.203	0.386	0.131	0.529	0.182	NS	<0.0001	0.0012
G50	1.017	0.349	0.812	0.185	0.281	0.124	0.590	0.158	NS	<0.0001	0.0071

**Figure Legends**

**Figure 1. Representation of 3 different profiles observed on swimming capacity of a panel of uropathogenic *Escherichia coli* in presence of cranberry, propolis and both.**

Comparative results of swimming assays for representative strains in different growing conditions (cranberry, propolis, and both) at 48h.

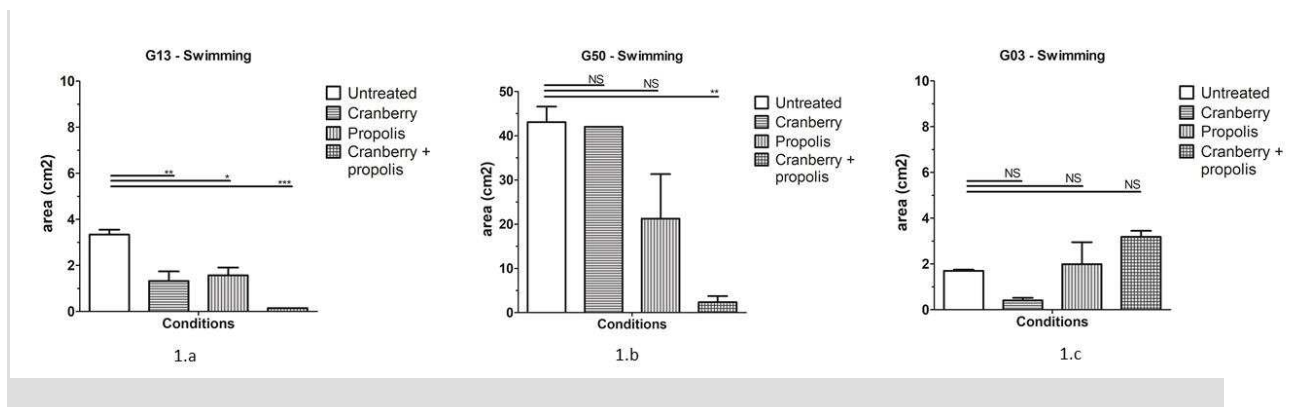
1a) Profile of the swimming of the G13 strain

1b) Profile of the swimming of the G50 strain

1c) Profile of the swimming of the G03 strain

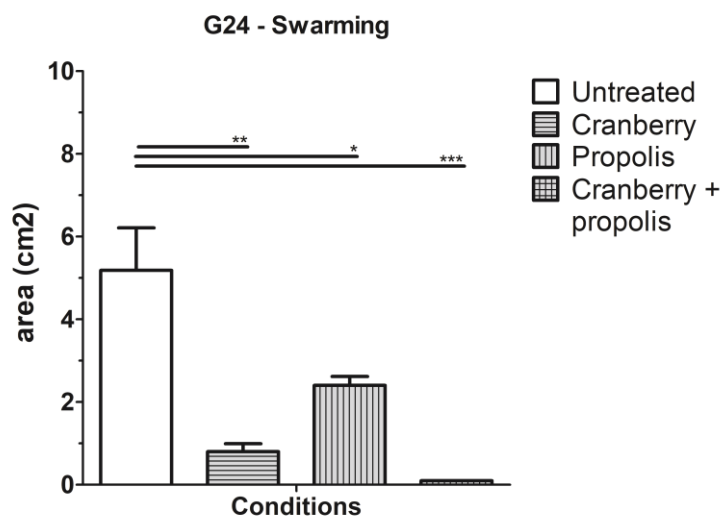
The errors bars represent the standard deviation from at least two independent assays

p<0.01; \*\*, p<0.001; \*\*\*, p<0.0001; NS, not significant



**Figure 2. Effect of cranberry, propolis and both on swarming capacity of uropathogenic *Escherichia coli*.**

Comparative results of swarming assays for a representative strain (G24) in different growing conditions (cranberry, propolis, and combination of both) at 48h. The errors bars represent the standard deviation from at least two independent assays.



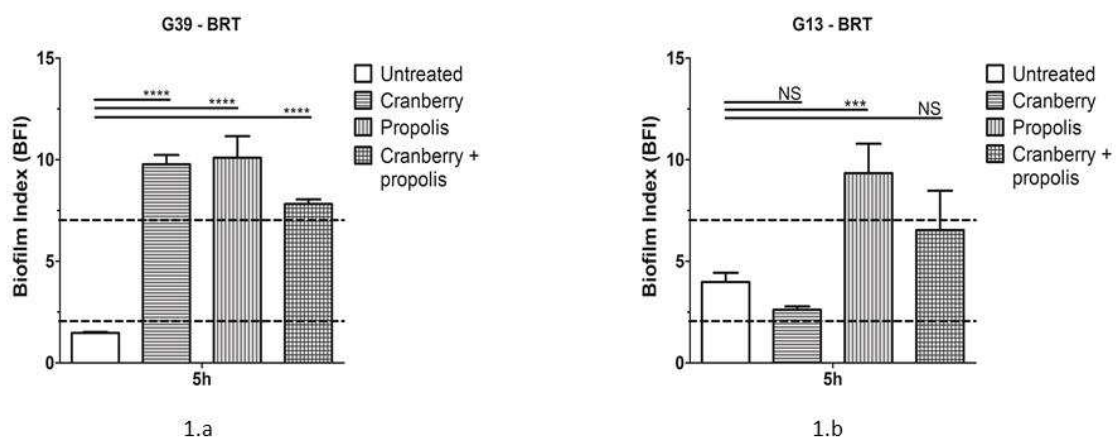
**Figure 3. Effect of cranberry, propolis and both on early biofilm formation of a panel of uropathogenic *Escherichia coli*.**

The kinetics of early stages of biofilm was determined by the Biofilm Ring Test. Biofilm Index (BFI) >7 indicates an absence of biofilm and BFI <2 indicates a fixed biofilm.

1a) Profile of the G39 strain impacted by all of the treatments.

1b) Profile of the G13 strain impacted only by the treatment with propolis.

Means and standard errors for three independent replicate are presented. Statistical differences between different conditions at each time were obtained by ANOVA.





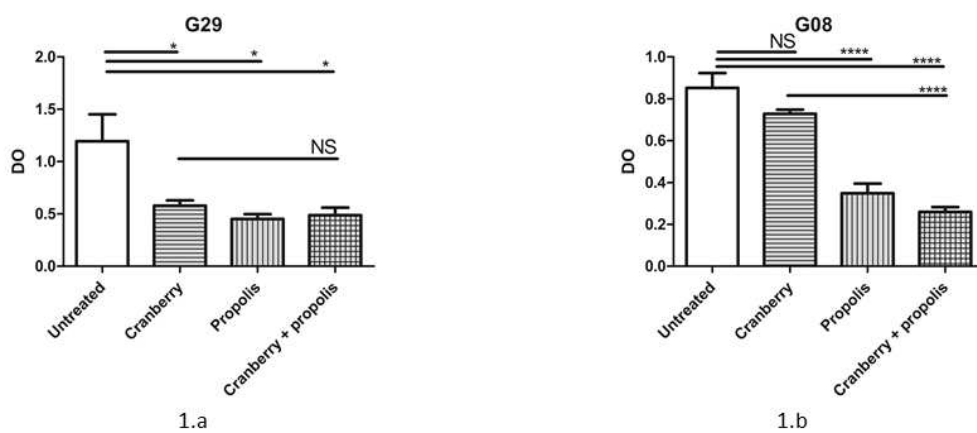
**Figure 4. Effect of cranberry, propolis and both on complete biofilm formation of a panel of uropathogenic *Escherichia coli*.**

The kinetics of the complete biofilm formation were determined by crystal violet experiment. The optical density (OD) is directly linked to the biofilm formation.

1a) Profile of the G29 strain impacted by all the treatments.

1b) Profile of the G08 strain impacted only by the treatment with propolis alone and the combination of cranberry and propolis.

Means and standard errors for three independent replicates are presented. Statistical differences between different conditions at each time were obtained by ANOVA.



## **Travail n°4**

**A quantitative Real-Time PCR assay to measure the anti-bacterial activity of a powder based on dried cranberry.**

Auteurs : Jérémy Ranfaing, Catherine Dunyach-Remy, Jean-Philippe Lavigne, Albert Sotto

Ce travail fait l'objet d'un dépôt de brevet.

## Résumé :

Les propriétés anti-bactériennes de la canneberge (*Vaccinium macrocarpon*) sont liées à une de ses molécules qui la compose : la proanthocyanidine de type A (PAC-A). Cependant, après ingestion, il est impossible de détecter cette molécule dans les urines. La quantification des PAC est difficile à cause de la forte teneur en pigments. Cela rend les méthodes de dosage par chromatographie et spectrophotométrie moins efficaces. Il existe deux méthodes actuellement validées: la chromatographie liquide avec spectrométrie de masse (LC-MS) ou la méthode colorimétrique modifiée 4-diméthylaminocinnamaldehyde (DMAC). Les difficultés de quantification amènent des disparités entre différents produits commerciaux et peuvent expliquer la différence d'efficacité observées entre différentes études [1].

L'objectif de ce travail a été de développer un outil permettant d'évaluer l'efficacité de différentes préparations de canneberge en se basant sur son impact sur le transcriptome d'une souche d'UPEC plutôt que sur sa concentration en PAC-A. Les résultats de transcriptomique que nous avons obtenus précédemment nous ont permis de sélectionner des gènes cibles dont l'expression pourrait potentiellement varier en fonction de la teneur en PAC dans le milieu de culture.

Quatre gènes impliqués dans la physiopathologie de l'IU ont été sélectionnés (liés au métabolisme du fer et à la motilité). La variation de leur expression a été analysée en PCR en temps réel après exposition d'une souche d'UPEC à des concentrations croissantes d'une préparation de canneberge diluée dans un milieu de culture LB (Luria Broth) et dans un milieu composé d'urine préalablement filtrée additionné de 5% de LB. Ce test a été réalisé également à partir d'échantillons d'urine filtrée de patients n'ayant pas d'IU recueillis avant et 4h après la prise d'un complément alimentaire à base de canneberge, le DUAB®, afin de vérifier s'il était possible de doser directement chez le patient l'efficacité de cette préparation. En parallèle, des échantillons « contrôles » d'urine de patients recueillis avant et 4h après la prise d'un placebo ont également été testés. Ces échantillons ont été obtenus au cours de la visite d'inclusion de l'étude clinique CYSDUA qui avait pour but de tester l'efficacité d'une prise continue de DUAB© (composé de canneberge, de propolis et de zinc) sur une période de 6 mois sur la prévention des infections urinaires récidivantes.

Les résultats obtenus ont montré qu'après exposition d'une souche d'UPEC à des concentrations croissantes de canneberge diluées dans du milieu LB et du milieu urine filtrée, la variation

d'expression des gènes analysés étaient bien corrélés à la concentration en canneberge des préparations testées, permettant d'en déduire également une concentration efficace en PAC-A. D'autre part, les résultats obtenus à partir des échantillons de patients ont montré qu'il existait une grande variabilité dans l'expression des gènes ciblés suivant les prélèvements testés. Parmi les 4 gènes ciblés, seule l'expression du gène *ftnA* varie de façon identique aux tests préalables après culture dans des urines de patients ayant pris du DUAB©. De plus, la moyenne de la variation de l'expression de chaque gène au sein des deux groupes a permis de constater des différences notables entre les groupes DUAB© et placebo mais ces variations étaient opposées à celles trouvées lors de nos expériences *in vitro*. Cette différence peut s'expliquer par la composition du complément alimentaire DUAB©. Ce dernier contient de la canneberge et de la propolis alors que notre outil a été mis au point avec uniquement de la poudre de canneberge non complémenté en propolis. Cependant, des tests préalables avaient démontré que la propolis potentialisait l'effet de la canneberge sur l'expression des gènes testés. La composition même des urines des patients inclus pourrait expliquer également la variabilité des résultats obtenus.

En conclusion, l'outil développé permet de doser *in vitro* dans un milieu LB standardisé et dans un milieu non standardisé telles que les urines, l'efficacité de différentes préparations de canneberge en s'affranchissant du dosage analytique de la PAC-A. Cet outil n'est cependant pas adapté au suivi de l'efficacité du traitement directement à partir des urines de patients ayant pris par voie orale de la canneberge. Seule l'évaluation de l'expression du gène *ftnA* permettrait d'évaluer cette efficacité.

**CE DOCUMENT EST CONFIDENTIEL**  
**ET NE DOIT PAS ETRE DIFFUSE**

**A quantitative Real-Time PCR assay to measure the anti-bacterial activity of a powder based on dried cranberry**

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**Keywords:** Cranberry, *Escherichia coli*, Real-time PCR,

### Abstract of the invention

The anti-adherential power of the cranberry on Uropathogenic *Escherichia coli* (UPEC) has been known for many years and we also know that type-A proanthocyanidin (PAC-A) is the active component which carries the antibacterial effect of cranberry. So, the anti-bacterial activity of a powder based on dried cranberry is characterized by its PAC-A concentration. The major problem is the difficulty to measure the concentration of PAC-A and the lack of normalization. So to pass over this problem, a technique to measure the anti-bacterial activity of a powder based on dried cranberry has been developed by measuring the modification of the expression of genes involved in the pathogenesis of bacteria by qRT-PCR.

Based on our previous work about the transcriptomic impact of the cranberry, 4 genes involved in the iron metabolism and in the motility of UPEC have been chosen. These results have been confirmed in two medium: a LB (Luria Broth) medium and in a medium composed by filtered urine and 5% of LB. Then, this test has also been realized with the urines of patients, before and 4h after ingestion of a dose of the food supplement based on cranberry: DUAB©. Groups of patients who have taken a placebo have also been included.

The results shown that, after the exposition of an UPEC with different concentrations of cranberry diluted in a LB medium and an urine medium, a correlation between the variation of the expression of the targeted genes and the concentration of cranberry was highlighted. On the other hand, the results obtained from the patient samples showed that there was great variability in the expression of the targeted genes according to the samples tested. Among the 4 targeted genes, only the expression of the *ftnA* gene was varied in the same way as the preliminary tests after culture in urine of patients taking DUAB®. The composition of the urine of the included patients could also explain the variability of the results obtained.

In conclusion, the developed tool makes it possible to determine the efficacy of different cranberry preparations *in vitro* in a standardized LB medium and in a non-standardized medium such as urine, by avoiding the analytical assay of PAC-A. However, this tool is not suitable for monitoring the efficacy of treatment directly from the urines of patients taking

oral cranberry. Only evaluation of the expression of the *ftnA* gene would make it possible to evaluate this efficiency.

## 1. Introduction

Urinary tract infections (UTI) are considered to be the most common human infectious diseases [2]. Approximately 150 million cases per year have been counted [3]. It is estimated that 50% of women and 5% of men will develop an UTI during their life [4].

Most UTI are due to colonization of the urethra by faecal bacteria, and spreading up in the urinary tract to the bladder as well as to the kidneys. The difference in the length of urethra explains why women are more sensitive to UTIs than men [5]. Depending on the site of the infection, UTIs are classified in cystitis (bladder) or pyelonephritis (kidneys).

Uropathogenic *Escherichia coli* (UPEC) are the major pathogens involved in UTIs [6]. These strains are able to invade bladder epithelial cells, providing the bacteria a protected niche where they can persist for long times, unperturbed by host defences and protected from antibiotic treatments [7]. Thus UPEC can persist within the urinary tract and may serve as a reservoir for recurrent infections and serious complications.

For many years, these bacteria have developed mechanisms of resistance against  $\beta$ -lactams and fluoroquinolones, the antimicrobial agents usually used in these infections [4][8]. The incidence of multidrug resistant *E. coli* has dramatically increased since the beginning of the century. In this context, it seems essential to test new strategies to prevent or treat UTIs.

Adhesion of UPEC to host cells is a crucial step for their pathogenicity, many scientists and industrials are already searching for molecules inhibiting bacterial adhesion. Among others, cranberry extracts showed efficiency in preventing UTI in susceptible population [9].

For some years, evidence suggests that cranberry is effective to prevent UTIs [10][11]. The main efficacy is related to the antiadherence properties of cranberry (*Vaccinium macrocarpon*). The A-type proanthocyanidin (PAC-A) in cranberry have been implicated as important inhibitor of Type-I fimbriae *E. coli* adhesion to uroepithelial cells. This effect is due

partly to a modification of bacteria form, the cell becomes round and reduces its surface of adherence [12].

To have an anti-bacterial effect on the bacteria, a cranberry solution needs to have a final concentration of PAC-A of 190 µg/L (measured by BL-DMAC) [13][14]. Nevertheless, the method to measure the concentration of PAC-A in a cranberry extract is not standardized. In addition, cranberry efficacy was shown to be optimal 4 hours after of oral ingestion [13].

The aim of this work is to develop a genomic approach for measuring, by qRT-PCR, the anti-bacterial activity of dried cranberry powder based on its impact on the expression of genes involved in the pathogenesis of UPEC.

## **2 Material and methods**

### **2.1 Bacterial strain, microbial culture and preparation of extracts**

All the assays were performed with an UPEC strain previously isolated from a patient with cystitis (G50) [15]. Luria Broth (LB) growth medium (Invitrogen, Villebon sur Yvette) and a mix of filtered urine (filter by a vacuum-driven filtration system with a 0.22µm membrane, Millipore© (Billerica, Massachusetts, USA)) complemented by LB growth medium (Invitrogen, Villebon sur Yvette) have been used for RNA extraction.

In parallel, this test was also performed from filtered urine samples from patients without UTI collected before (T0) and 4 h (T4h) after taking a cranberry-based dietary supplement (DUAB®) (n=9) to see if it was possible to measure the efficacy of this preparation directly in urine of patient. In parallel, urines "control" samples from patients (n=6) collected before (T0) and 4h (T4h) after taking placebo were also tested. These samples were collected the day of inclusion in CYSDUA study. This study was designed to test the efficacy of continuous use of DUAB® over a 6-month period on the prevention of recurrent urinary tract infections. All the patients had contracted at least four cystitis in the year before their inclusion in CYSDUA study. Median age of the placebo and DUAB groups was 60 and 54 years respectively.

Cranberry extract (*V. macrocarpon*) was obtained by using dried cranberry (Exocyan cran BL-DMAC 6% (Nexira, Rouen, France)) and phosphate buffered saline (PBS) and sterilized by filtration. The concentration of PAC-A was measured by BL-DMAC (colorimetric method



[1]). Final concentration of PAC-A used was standardized to contain 190 µg/L. Cranberry juice was stored at -20°C in the dark.

A cranberry solution at 100% is equivalent to a final concentration of PAC-A at 190 µg/L. A cranberry solution at 50% is equivalent to a final concentration of PAC-A at 95 µg/L. A cranberry solution at 10% is equivalent to a final concentration of PAC-A at 19 µg/L.

## 2.2 RNA extraction and cDNA synthesis

G50 isolate was grown in LB broth medium, in urine medium complemented with LB broth alone and with cranberry proanthocyanidins extracts to an OD600 of  $\approx$  0.6. For clinical samples, G50 isolate was grown in urine samples of patients obtained before and 4 h after taking DUAB® or placebo. Total RNA from bacterial samples was extracted with Tryzol (Invitrogen) according to the manufacturer's instructions and samples were purified with the RNeasy mini kit (Qiagen, Courtaboeuf, France). All the RNA extraction experiments are performed on triplicate. RNA was treated with the RNase-Free DNase Set (Qiagen). Purity and concentration were determined using the Nanodrop™ 2000 spectrophotometer (Fisher Scientific, Pittsburgh, PA, USA). cDNA was synthesized from 1µg of total RNA for each sample, using the iScript™ Select cDNA Synthesis kit (Bio-Rad, Hercules, CA) with random primers according to the manufacturer's instructions.

## 2.3 Selection of target genes

Genes have been selected based on transcriptional analysis of an UPEC strain after a treatment with a cranberry solution. These genes have been chosen for their relation to the pathogenicity of UPEC. The genes selected are: *tsr* (chemoreceptor) Forward primer: CAACGAGTTCTTTGATCAGC, Reverse primer: TACCAGCGAGGCTTTAATAC, *ftnA* (iron storage), Forward primer: AATACCGTTGAATCTCCGTT, Reverse primer: GCTCAGAAACATACCATTGC, *fecB* (siderophore), Forward primer: GCACGTTTACTACTCGAAAAA, Reverse primer: GATCAGGTCTGGTTTCAGAG and *feoB* (Iron transport), Forward primer: GAAAGACACCTTCAGCCTTA, Reverse primer: TGAGTAAGCGATATTCAGCC.

## 2.4 Comparative real-time qRT-PCR

Transcript levels analysis was performed by quantitative reverse transcription qRT-PCR using selected genes (as described previously). Real-time PCR assays were performed in a LightCycler®480 device using the LightCycler FastStart DNA Master<sup>Plus</sup> SYBRGreen I kit with 100 ng of cDNA and 10 pmol of target primers (as described previously). The specificity of the PCR products was tested by melting-point analysis. Amplifications were performed in duplicate from three different RNA preparations. The  $2^{-\Delta\Delta CT}$  method was used to analyze transcriptional changes in target genes using *gapdh* as the housekeeping control gene. For clinical samples, calculation of fold change are based on the comparison of data obtained before (T0) and 4h (T4h) after taking DUAB® or placebo. Data were log transformed to obtain a fold change difference between the different studied conditions [20,21].

## 2.5 Statistical analysis

Statistics and graphs were prepared using the software package GraphPad Prism 6.0. The effects of cranberry on the expression of selected genes were assessed using one-way ANOVA followed by Dunnett's multiple comparisons test. Log-transformed data were used for real-time RT-PCR.  $P < 0.05$  was considered to reflect a statistically significant difference.

## 3. Results

### 3.1 Evaluation in LB medium

In a first time, the impact of a cranberry powder on the expression of target genes after incubation in a LB medium has been tested (Figure 1).

These results show an over-expression of the *ftnA* gene and a down-expression of the *tsr*, *fecB* and *feoB* genes after over-night incubation in a solution of LB supplemented with cranberry solution at 100% of effective dose. In addition, results obtained with different concentrations of cranberry show that these variations of gene expression are dose-dependant. Indeed, with a cranberry solution at 10%, results are inverted: *ftnA* gene is down-regulated and *tsr*, *fecB*, *feoB* genes are up-regulated. However, only expression of *tsr* gene is significantly correlated at cranberry concentrations whatever tested concentrations.

### 3.2 Evaluation in urine medium

In a second time, the same experiment has been made after an incubation of G50 strain in a medium compounded mainly of filtered urine and increasing concentrations of cranberry (Figure 2).

Same results than in the previous experiment with the LB medium have been obtained: an over-expression of the *ftnA* gene and a down-expression of the *tsr*, *fecB* and *feoB* genes after over-night incubation in a solution of sterile urine supplemented with a cranberry solution at 100%. Results obtained with different concentrations of cranberry show a dose-dependent effect. However, we do not notice a significant difference between the expression of the gene *feoB* after incubation with a cranberry solution at 100% and a cranberry solution at 50% or 10%. In this case also, expression of *tsr* gene is significantly correlated at cranberry concentrations whatever tested concentrations.

### 3.3 Evaluation with clinical samples

This test was also performed using filtered urine samples from patients without UTI collected before (T0) and 4 h (T4h) after taking a cranberry-based dietary supplement, DUAB®, to determine if it was possible to dose directly the efficacy of this preparation to the urine of patient. In parallel, "control" samples of patients collected before and 4h after taking placebo were also tested.

The urines of 15 patients (two samples by patient T0 and T4h) have been sterilized by filtration and the same protocol previously described without supplementation of cranberry was used. Nine patients took DUAB® (501, 5014, 506, 507, 510, 511, 515, 516, 517) and 6 patients took placebo (502, 503, 508, 509, 512, 513).

The results obtained from the patient samples showed that there was great variability in the expression of the targeted genes according to the samples tested. Among the 4 targeted genes, only the expression of the *ftnA* gene was varied in the same way as the preliminary tests after culture in urine of patients taking DUAB®. A notable difference between the placebo group and the DUAB group has been observed also for the genes *tsr* and *feoB* but an up-regulation

of these genes has been observed instead of a down-regulation, which has been observed in the *in vitro* tests.

## Discussion

The aim of the first part of this work has been to develop a genomic method to measure the quality and efficiency of a powder based on dried cranberry using qRT-PCR. This method is not based on measuring of the concentration of A-type proanthocyanidin (PAC-A) in the sample but on measuring the impact of the cranberry solution on the expression of target genes involved in the pathogenicity of UPEC (iron metabolism and motility).

After an incubation in a standard medium (LB broth medium) qRT-PCR assays show a significant difference between a treatment with a cranberry solution with a PAC-A concentration of 190 µg/L and a PAC-A concentration of 19 µg/L. We find similar results after incubation in a sterile urine medium. In a standardized medium our technique is able to evaluate the anti-bacterial activity of a cranberry powder regardless of the concentration of PAC-A in the sample.

The PAC-A is the active component of the cranberry, so the efficiency of a powder based on this product is directly linked to this molecule. A study has shown that a minimum concentration of PAC-A is required to observe an impact on the adhesion of bacteria (60 µg/L) [10]. These experiments *in vitro* confirm that at concentration  $\leq 50$  µg/L of PAC-A, few variation of the expression of target genes is observed. Moreover, at low concentration (10 µg/L of PAC-A), the impact of cranberry on variation of the expression of all genes tested is inverted showing the importance of cranberry concentration, so A-PAC concentration, on efficacy of these cranberry preparations on pathogenicity of UPEC. *tsr* gene appears to be the best candidate. Other cranberry preparations will have to be tested in order to assess the relevance of simultaneously following the expression of the 4 targeted genes or to follow only the expression of the *tsr* gene.

On the other hand, the PAC-A cannot be found in human urine [16] so it is very difficult to determine an optimal concentration of this component to design an efficient food supplement. A previous work of our team has been done to determine this optimal concentration. The anti-

adherential effect of urines from healthy patients collected after the consumption of solutions with different concentration of cranberry has been tested. They demonstrate that the optimal daily quantity of cranberry necessary to have an impact on the adhesion of bacteria is 190 µg/L [13]. Our technique confirms these results by a method by on the transcriptomic instead of a phenotypical effect.

In a second part of this work, this tool has also been realized with the urines of patients, before and 4h after ingestion of a dose of the food supplement based on cranberry: DUAB©. Groups of patients who have taken a placebo have also been included. Results obtained from the patient samples showed that there was great variability in the expression of the targeted genes according to the samples tested. The variability of the urine and the composition of the food supplement can explain these differences.

Nevertheless another explanation can be express. Studies of our team demonstrate the anti-adhesion activity of an urine medium after the consumption of cranberry [12][13]. The anti-adhesion effect can be carried by an undetected metabolite of PAC-A which do not carries all the anti-bacterial activities of PAC-A. It can explain the results of the studies the leak of specificity of our targeted genes (excepted *ftnA*) because they do not linked to the adhesion. Other genes, linked to the adhesion, can be selected to detect the activity of a cranberry powder in urine.

To conclude, this technique based on the variation of the expression of specific UPEC genes involved in their pathogenicity can measure the anti-bacterial activity of a powder of cranberry regardless the measure of its concentration in PAC-A. Nevertheless, this technique cannot determine the efficacy of a food supplement from an urine sample of a patient. Only evaluation of the expression of the *ftnA* gene would make it possible to evaluate this efficiency.

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### Figure legends

**Figure 1:** Log relative fold-change mRNA expression of target genes (*ftnA*, *tsr*, *fecB*, *feoB*) for G50 strain after a treatment with a cranberry solution at different concentrations (100%, 50% and 10%) in a LB medium. Bars represent the average of relative fold-change (expression of genes in LB/cranberry medium in comparison of expression of genes in LB medium). The error bars represent the standard deviation from three different experiments.

**Figure 2:** Log relative fold-change mRNA expression of target genes (*ftnA*, *tsr*, *fecB*, *feoB*) for G50 strain after a treatment with a cranberry solution at different concentration (100%, 50% and 10%) in urine/LB (5%) medium. Bars represent the average of relative fold-change (expression of genes in urine/cranberry medium in comparison of expression of genes in urine medium). The error bars represent the standard deviation from three different experiments.

**Figure 3:** Mean of the log relative fold-change mRNA expression of target genes (*tsr*, *ftnA*, *fecB*, *feoB*) for G50 strain after incubation in the urines of patients 4 hours (T4h) after taking DUAB® (n=9) or placebo (n=6). Bars represent the average of relative fold-change (expression of genes in T0 urines

in comparison of expression of genes in T4h urines). The error bars represent the standard deviation from the variation between the patients of each group.

**Figure 1**



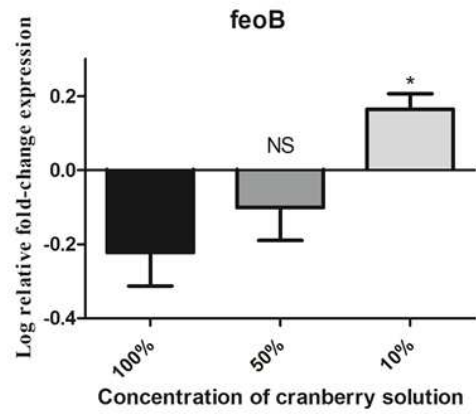
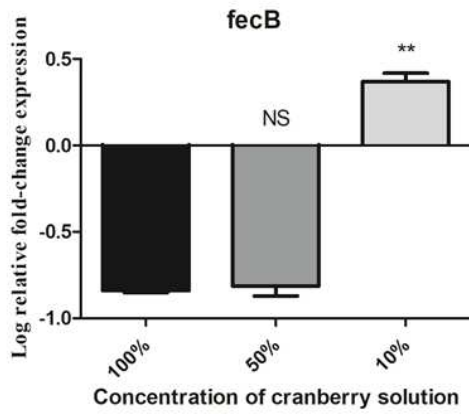
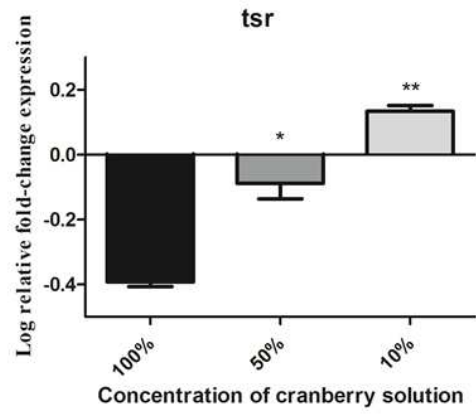
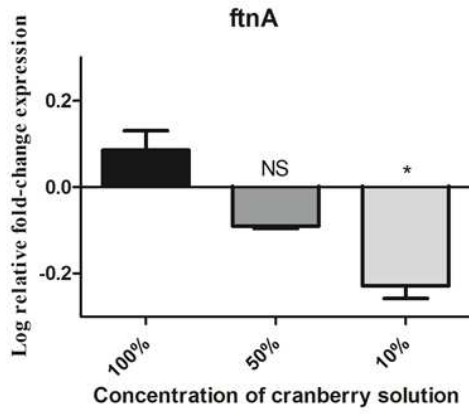


Figure 2

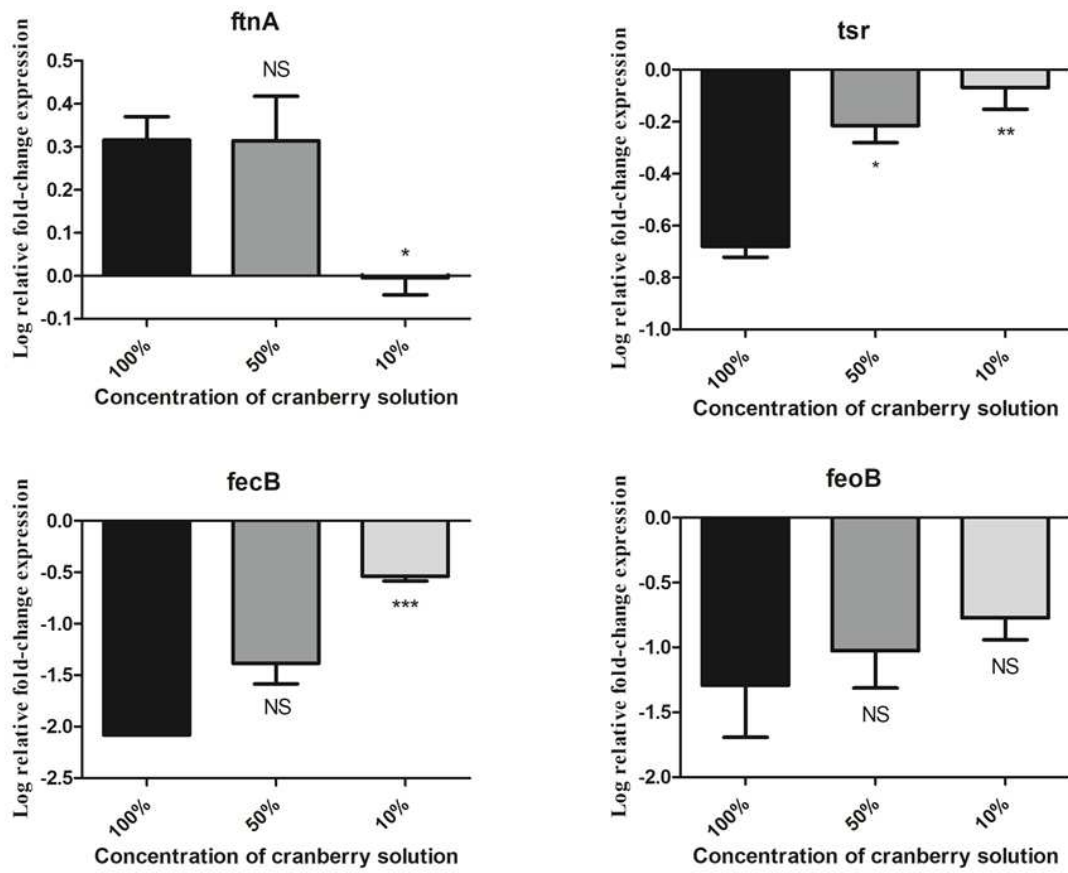
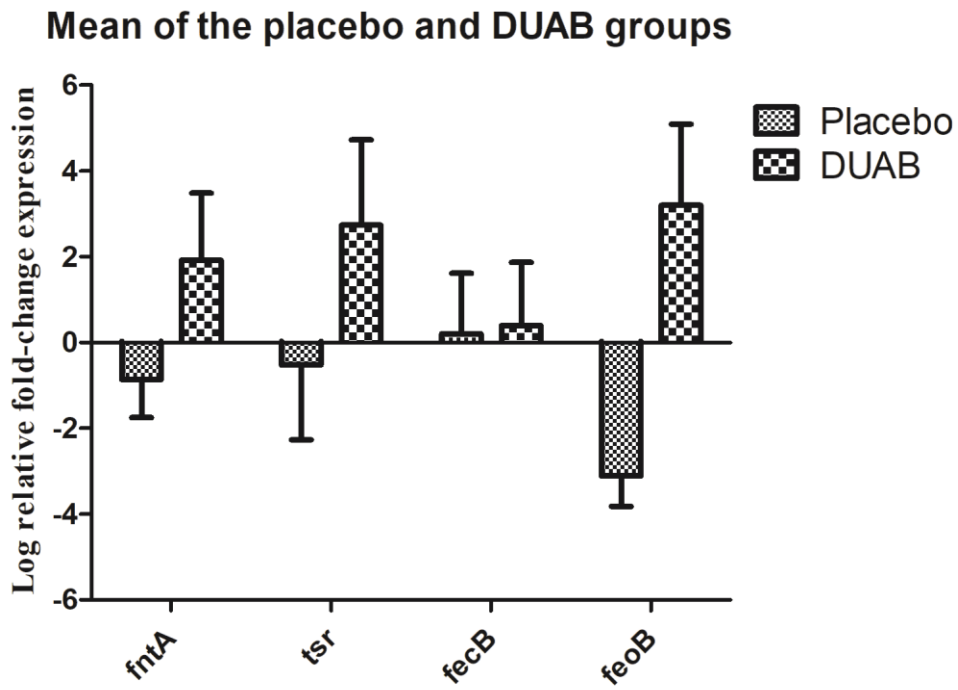


Figure 3



## **Travail n°5**

### **Efficacy of antibiotics used for Urinary Tract Infection is improved by the propolis**

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Article en cours d'écriture

**RESUME :**

Les deux premières parties de ce travail ont été axées sur les effets de l'association canneberge – propolis, d'un point de vue transcriptomique et phénotypique, sur des souches d'*E. coli* uropathogènes (UPEC). Un effet potentialisateur de la propolis a pu être observé. A partir de ces résultats nous avons voulu explorer davantage cet effet potentialisateur et expérimenter cet effet sur des antibiotiques utilisés en pathologie infectieuse urinaire. L'objectif ici a été de démontrer que, en plus d'avoir un rôle dans la prévention, l'association canneberge – propolis pouvait améliorer le traitement des IU en améliorant l'efficacité du traitement antibiotique.

Quatre antibiotiques utilisés en pathologie infectieuse urinaire ont été testés : ceftriaxone, fosfomycine, ertapénème et ofloxacine. Pour chacun de ces antibiotiques, l'impact de la propolis sur deux propriétés de ces antibiotiques a été évalué : la concentration minimale inhibitrice (CMI) et l'effet bactéricide. Des souches d'UPEC sensibles et possédant des mécanismes de résistance aux antibiotiques ont été testées pour nos expériences.

Un effet potentialisateur de la propolis sur l'action de la majorité des antibiotiques testés a été montré. Après addition de propolis, la ceftriaxone, l'ertapénème et l'ofloxacine ont eu un impact significatif sur des souches disposant de mécanismes de résistances. Cependant, cet effet n'a pas été constaté avec la fosfomycine.

Cette étude montre, qu'en plus de l'effet direct de la propolis sur la bactérie, la propolis peut améliorer l'effet des antibiotiques et ainsi rendre le traitement plus efficace. Si on ajoute cela aux précédents résultats, l'association canneberge et propolis pourrait présenter un intérêt significatif dans le traitement des infections urinaires ainsi que dans la lutte contre l'émergence des souches résistantes aux antibiotiques.

## **Efficacy of antibiotics used for Urinary Tract Infection is improved by the propolis**

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**Abstract**

**Scope:** Urinary Tract Infections (UTIs) are the most common bacterial infections in the world. Uropathogenic *Escherichia coli* (UPEC) is the pathogen which is mostly isolated and this bacteria has become increasingly resistant to antibiotics to antibiotics prescribed to treat UTIs. Because of the rarefaction of new antibiotics molecules, new strategies have to be found to improve the efficacy of the actual treatment such as the utilization of propolis.

**Methods and results:** Minimal Inhibitory Concentration (MIC) and bactericide curve of fosfomycin, ceftriaxone, ertapenem and ofloxacin, with and without propolis, have been measured. A significant diminution of the MIC has only been observed when we have added propolis with ceftriaxone. Nevertheless, a slight improvement of all the other antibiotics has been observed after the addition of propolis. The bactericide effect of all the antibiotics (except the fosfomycin) has been significantly improved after addition of propolis.

**Conclusion:** This study demonstrates the impact of propolis on the efficacy of antibiotics. The use of this natural product in addition to the usual treatment could compensate for the increase in resistance to antibiotics.

## 1. Introduction:

Urinary Tract Infections (UTIs) are the most common bacterial infections [1]. These infections affect 9 million women in America and 150 million people in the world each year [2][3]. They mainly concern women [4]. Around 50% of women will present an UTI during their life and 25% of these women will will develop recurrent UTIs [2][5]. Uropathogenic *Escherichia coli* (UPEC) are the major pathogen involved in UTIs [6]. For many years, these bacteria have developed mechanisms of resistance against  $\beta$ -lactams and fluoroquinolones, the antimicrobial agents usually used in these infections [5][7]. The incidence of multidrug resistant *E. coli* has dramatically increased since the beginning of the century. In addition, the discovery of new antibiotics has become rare in the last decade. In this context, it seems essential to test new strategies to prevent or treat UTIs.

One of these strategies is the utilization of nutraceuticals. Nutraceuticals gather all the food or food products which can provide medical benefits and be delivering under medical form. The most studied is the cranberry (*Vaccinium macrocarpon*). Numerous evidences suggested that cranberry has an in vitro action on UPEC adhesion. In vitro and in vivo studies of our team have demonstrated the impact of cranberry [8][9]. Nevertheless, the clinical results are currently contradictory. It is therefore difficult to conclude that an in vivo effect occurs during urinary tract infections [10][11]. The propolis is also a natural compound which has an anti-bacterial effect. It is a resinous material collected by bees from plants then mixed with wax and bee enzymes[12]. Propolis has been used for several years for its antiseptic and local anesthetic properties. It has also antimicrobial, anti-inflammatory, anti-tumour, immunomodulatory and anti-oxidant activities, among others [13]. Interestingly, we led a study that showed that propolis could potentiate the anti-adhesion activity of proanthocyanidins on UPEC [14]. Indeed, the cranberry-propolis combination led to a decrease in the cell adhesion capacity of the UPEC strains and a decrease in their virulence compared to cranberry alone.

Based on these results, the aim of the present study was to investigate the impact of the association of propolis and antibiotics used to treat UTIs (ofloxacin, ceftriaxone, ertapenem and fosfomycin). To evaluate this impact, two experiments have been conducted: (i) the



measurement of minimal inhibitory concentration (MIC) and the monitoring of bactericide curve on UPEC with different resistance profiles.

## **2. Material and methods**

### **2.1 Bacterial strains, microbial culture and preparation of extracts**

Six strains of UPEC have been used in this study with different resistance profiles: 2 strains sensitive to antibiotics (NECS892841 and NECS30090), 2 strains quinolone-resistant and 2 strains producing extended spectrum beta-lactamase (ESBL). CFT073 has been used here as a reference strain for the UPEC.

Mueller-Hinton (MH) growth medium (Invitrogen, Villebon sur Yvette) has been used for MIC experiment and for bactericide curve.

The propolis extract (Plantex, Sainte-Geneviève-des-Bois, France) used in this study is an hydroalcoholic extract of blended propolis from various origins mixed with carob in a proportion (60/40, w/w). The propolis was diluted in 50 mL of PBS and incubated at 37°C by shaking at 100 rpm during 8 hours. Then, the solution was clarified by centrifugation (4000 rpm, 20°C, 10 min). Supernatant was sterilized by filtration.

### **2.2 MIC experiments**

All the strains have been cultivated in a MH growth medium overnight at 37°C. Then a bacterial solution with a final concentration of bacteria of  $10^8$  bacteria/ml (OD600 of 0.35) has been prepared.

Then, in 96-well microtiter plates, a serial dilution of each antibiotic has been done. The concentration started at 5196 mg/L and was going to 0.5 mg/L. Depending on the strains, the concentration of propolis was 120 or 250 mg/L. Three conditions have been studied: antibiotics, propolis and association of both.

The bacterial solution of each strain has been grown in the three conditions and they have been incubated for 24h. Then the well without bacterial growth with the lower concentration of antibiotic determines the MIC. Each experiment has been assayed in triplicate.

### **2.3 Bactericide curve**

All the strains have been cultivated in a MH growth medium under agitation at 37°C. The MIC of the propolis has been determined at 256 mg/L (1X concentration). First the bactericide impact of the propolis has been tested with different concentration: 0.5X, 1X, 2X, 4X, 16X. All the strains have been cultivated in 4 conditions: in a MH medium (untreated condition), with propolis (MIC 1X), with each antibiotics tested (0.5X MIC) and with the combination propolis (MIC 1X) and antibiotics (MIC 0.5X)

Samples of bacterial solutions with the different conditions have been taken at different time points: 0h, 1h, 2h, 3h, 5h, 6h and 24h. These samples have been plated and, after an incubation of 24h at 37°C, colonies have been counted.

### **2.4 Statistical analysis**

For the MIC experiments, we assumed that a difference of 3 dilutions or more between 2 conditions is considered like a significant result.

## **3. Results**

### **3.1 Determination of the MIC of each strains**

First, the determination of the MIC of the propolis has been determined. A high MIC of propolis has been observed.

Then, we evaluate the impact of the addition of a solution of propolis on the MIC of each antibiotic tested. A decrease of all the MICs has been observed (Table 1). However, the difference was not significant in all the conditions.

The combination with propolis did not have a significant impact on the MIC of ofloxacin, ertapenem or fosfomycin (diminution of the MIC less than 3 dilutions). Nevertheless the addition of propolis has a significant impact of the ceftriaxone MIC. Interestingly, propolis was also effective on strains producing ESBL.

### **3.2 Monitoring of bactericide curve with a treatment propolis and antibiotic**

A study of the bacteria survival with a propolis solution has been conducted prior to study the impact of the association of propolis and antibiotics on the survival of bacteria.

This first experiment (Figure 1) revealed two information: i) the propolis has a bactericide effect on UPEC; ii) the properties of this effect was not linked to the concentration but it was linked to the time of incubation.

Then, the study of the impact of the combination of propolis and the antibiotics tested on our UPEC strains has been conducted (Figure 2, 3, 4, 5).

An improvement of the efficacy of antibiotics has been observed after the addition of propolis to the solution. The synergetic impact of propolis was important with the ceftriaxone and the ertapenem whatever the strain tested. This synergetic impact with the ofloxacin depended to the sensibility of the strain tested. This synergetic impact has not been observed with the fosfomycin.

## **4. Discussion**

The aim of this study was to determine if the propolis could improve the efficacy of antibiotics used to treat UTIs. Four antibiotics have been studied: ofloxacin, ceftriaxone, ertapenem and fosfomycin.

Fosfomycin is the antibiotic which is used in a first intention to treat acute uncomplicated cystitis [15]. Observations which come from North America during the years 2010 to 2013 showed a high susceptibility to this antibiotic (about 99.4%)[16]. Moreover, European studies also confirmed that fosfomycin was also effective against strains producing ESBL and cephalosporinases [17][18]. This antibiotic has been included in this study because of its prevalent utilization to treat UTI, however the propolis does not improve its activity.

Ofloxacin is frequently used in urinary tract infections. This last decade, a rise of the utilization of fluoroquinolone to treat UTIs has been noticed in North America, which leads to an increase of the resistance rate. Moreover, this rise did not only affect the strains isolated from healthcare associated infections but also, community strains [20]. In association with propolis, the MIC of ofloxacin decreased, even with ESBL producing strains. Nevertheless, the bactericide effect is slightly improved after the addition of propolis against resistant strains of UPEC. However, the association was very effective against sensitive strain. It could be interesting to use this association to avoid the emergence of resistance. Indeed, if the MIC of the antibiotic is decreased after the addition of propolis, the circumstances in which the concentration of the antibiotic is subinhibitory will be limited. Which lead to a diminution of the conditions favorable for the emergence of antibiotic resistance.

Ertapenem belongs to the carbapenem family which has the greatest level of activity against the strains producing ESBL[21]. The use of carbapenems should be limited in order to limit the emergence of resistance strains for this family of antibiotics. The association with propolis was more effective than the antibiotic alone. First, a reduction of the MIC for the profile of strains has been demonstrated. Then, the bactericide effect of ertapenem was significantly improved by the addition of propolis.

Like fluoroquinolones, ceftriaxone is frequently prescribed during UTIs [22]. However, the rate of resistance become higher for the strains ESBLs producer [23]. So, this antibiotic has to be used wisely. The addition of propolis with a treatment of ceftriaxone could be a good option. It decreases significantly the MIC against strains which produced ESBLs and the propolis improved the bactericide effect of this antibiotic.

With the rise of antibiotic resistance we have to use antibiotics wisely. There are still antibiotics effective against UPEC producing ESB, however, the evolution of resistance is of concern. In this context, in order to improve the action of the antibiotics used, for some, frequently during UTIs, we studied their association with a natural compound: propolis. Two characteristics of the antibiotics have been studied: the MIC and the bactericide effect. Except for the fosfomycin, a significant improves of the antibiotics properties have been demonstrated after an addition of propolis. So a combination of propolis and the antibiotics used to treat UTIs could be a good solution to improve the efficacy of actual antibiotics and counter the rise of antibiotics resistance.

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## **Legends**

**Figure 1:** Impact of the propolis on the survival of UPEC.

Different concentrations of propolis have been used here: 128mg/L (0.5X), 256mg/L (1X), 512mg/L (2X), 1024 mg/L (4X) and 4096 mg/L (16 X). Each hour the concentration of bacteria has been determined by counting the Colonies Forming Unit (CFU).

**Figure 2:** Impact of the association of propolis and ofloxacin on the survival of UPEC

For these experiments a concentration of 256mg/L for the propolis and a concentration equal to half of the MIC have been used for the ofloxacin. In the figure 2a the reference strain CFT073 has been used. In the figure 2b a resistance strain has been used. In the figure 2c a strain which produced ESBL has been used. Each hour the concentration of bacteria has been determined by counting the Colonies Forming Unit (CFU).

**Figure 3:** Impact of the association of propolis and ceftriaxon on the survival of UPEC

For these experiments a concentration of 256mg/L for the propolis and a concentration equal to half of the MIC have been used for the ceftriaxon. In the figure 3a the reference strain CFT073 has been used. In the figure 3b a resistance strain has been used. In the figure 3c a strain which produced ESBL has been used. Each hour the concentration of bacteria has been determined by counting the Colonies Forming Unit (CFU).

**Figure 4:** Impact of the association of propolis and ertapenem on the survival of UPEC

For these experiments a concentration of 256mg/L for the propolis and a concentration equal to half of the MIC have been used for the ertapenem. In the figure 4a the reference strain CFT073 has been used. In the figure 4b a resistance strain has been used. In the figure 4c a strain which produced ESBL has been used. Each hour the concentration of bacteria has been determined by counting the Colonies Forming Unit (CFU).

**Figure 5:** Impact of the association of propolis and fosfomycin on the survival of UPEC

For these experiments a concentration of 256mg/L for the propolis and a concentration equal to half of



the MIC have been used for the fosfomycin. The reference strain CFT073 has been used. Each hour the concentration of bacteria has been determined by counting the Colonies Forming Unit (CFU).

**Table 1:** Summary of the MIC of antibiotic, propolis and combination of both for each strain tested. All the data are in mg/L. The annotation of (S) means that the strain is sensitive. The annotation of (R) means that the strain is resistant.

Strains	Propolis	Oxfloxacin	Oxfloxacin + Propolis	Ceftriaxon	Ceftriaxon + Propolis	Ertapenem	Ertapenem + Propolis	Fosfomycin	Fosfomycin + Propolis
CFT073	256	0.25 (S)	0.06 (S)	0.5 (S)	0.125 (S)	0.06 (S)	<0.03 (S)	8 (S)	4 (S)
NECS892841	256	0.5 (S)	0.123 (S)	1 (S)	0.125 (S)	0.06 (S)	0.03 (S)	512 (R)	128 (R)
NECS30990	128	0.25 (S)	0.06 (S)	0.5 (S)	0.06 (S)	0.03 (S)	<0.03 (S)	8 (S)	8 (S)
NECS858785	256	2 (R)	0.5 (S)	0.25 (S)	<0.06 (S)	0.06 (S)	<0.03 (S)	128 (R)	64 (S)
NECS864598	256	32 (R)	8 (R)	0.5 (S)	0.06 (S)	0.125 (S)	0.06 (S)	512 (R)	256 (R)
NECS892420	256	>32 (R)	8 (R)	>32 (R)	2 (R)	0.25 (S)	0.06 (S)	8 (S)	8 (S)
NECS118564	256	>32 (R)	8 (R)	>32 (R)	2 (R)	0.5 (S)	0.125 (S)	512 (R)	128 (R)

Figure 1

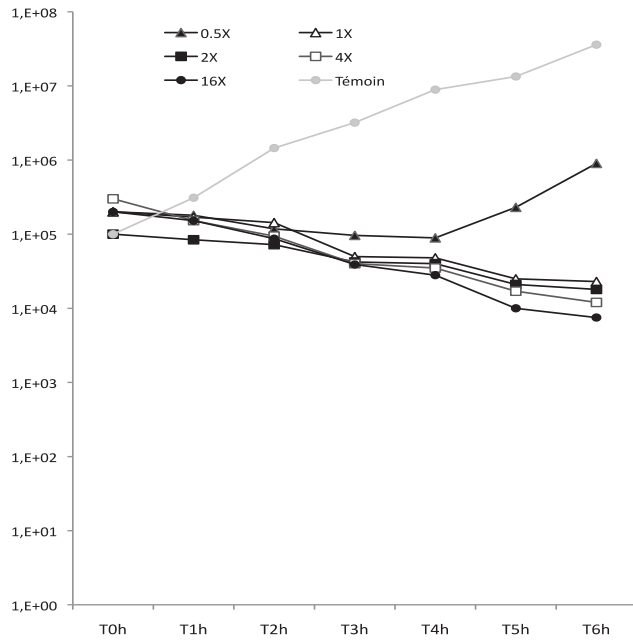


Figure 2

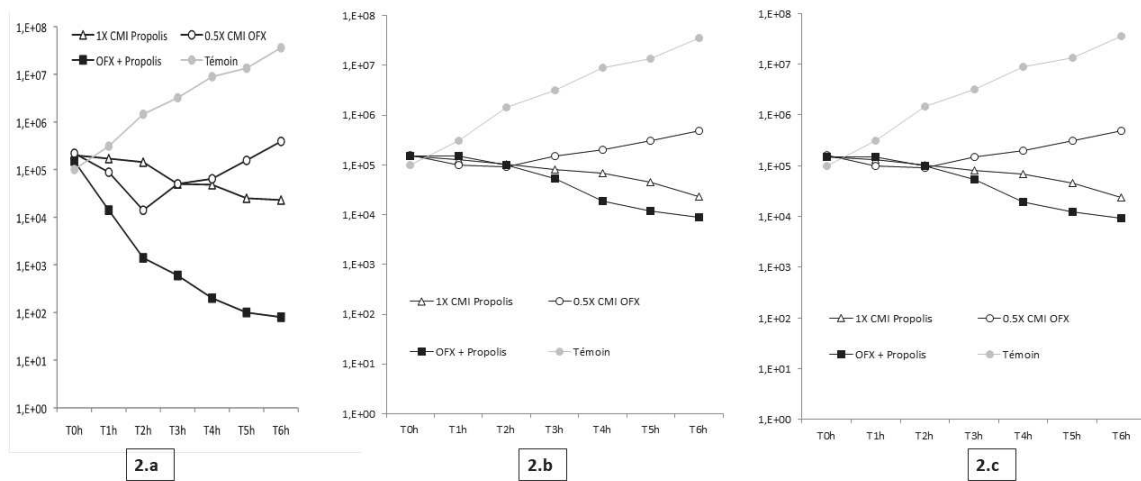


Figure 3

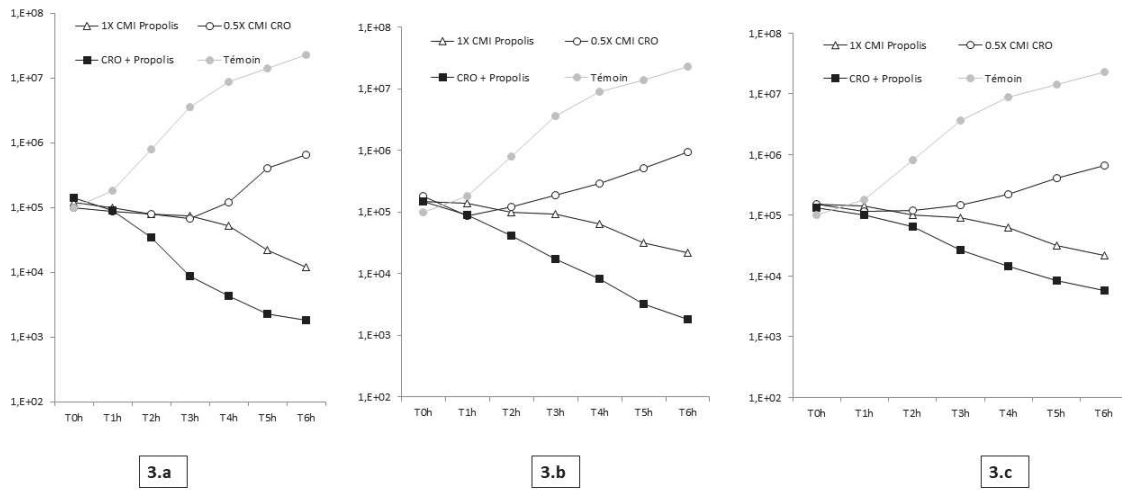
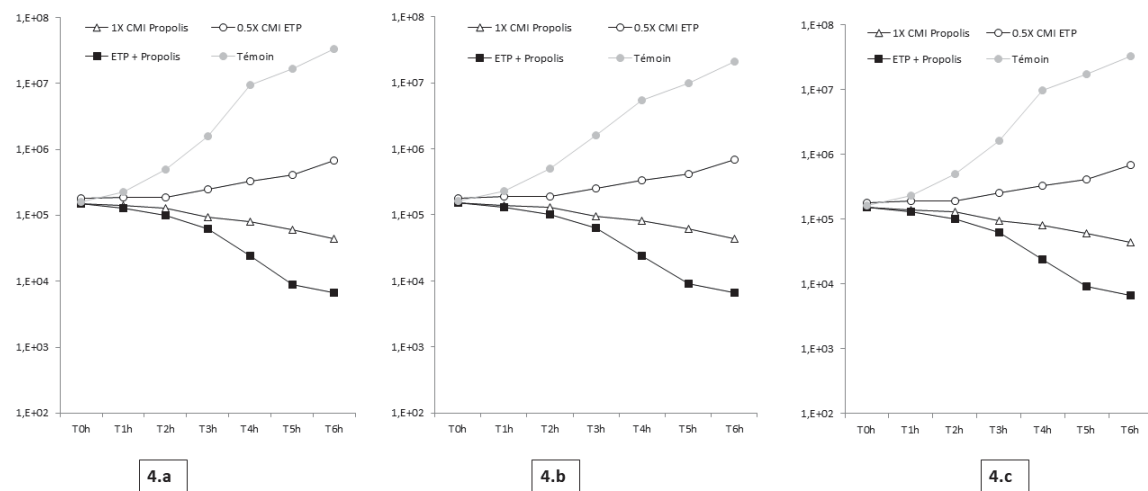
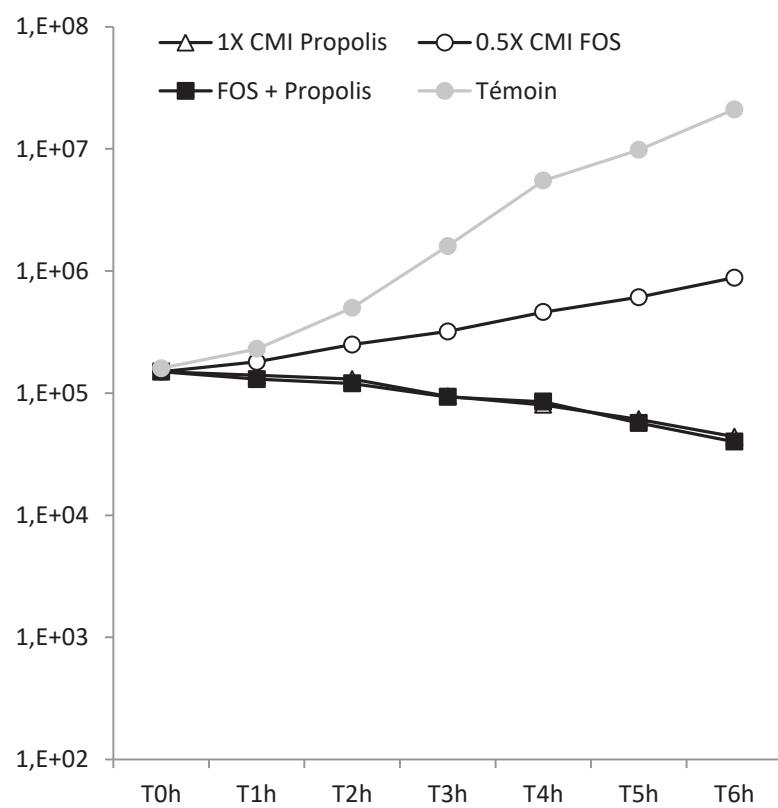


Figure 4



**Figure 5**



## **DISCUSSION - PERSPECTIVES**

Les IU sont un problème majeur de Santé publique, sa forme la plus courante étant la cystite aiguë. Une des particularités de la cystite est sa propension à récidiver. Le traitement préconisé, en dehors de mesures générales de prévention est une prise d'antibiotiques à chaque épisode. Lorsque les rechutes deviennent trop fréquentes, une antibioprofylaxie au long cours est habituellement mise en place. Elle peut être prescrite sur une période de plusieurs mois induisant le risque d'émergence de souches bactériennes résistantes aux antibiotiques. Les bactéries les plus fréquemment isolées sont des *E. coli* UPEC. Ces dernières années ces bactéries ont développé de nombreux mécanismes de résistance, dont le plus répandu est la sécrétion de BLSE.

De nouvelles stratégies doivent être développées afin de prévenir et traiter les infections urinaires. Parmi ces différentes stratégies, l'utilisation de produits naturels tels que la canneberge (*Vaccinium macrocarpon*) apparaît comme prometteuse. En effet, l'action de la canneberge sur l'adhésion est connue depuis de nombreuses années. Les premières études datent de 1984 avec les travaux de Sobota *et al.* [80]. A partir de là, des expériences visant à démontrer l'impact de la canneberge sur l'adhésion des UPEC à des cellules uroépithéliales ont été menées et ont pu confirmer son effet anti-adhérentiel [7][104]. Cet effet anti-adhérentiel est porté par la proanthocyanidine de type A (PAC-A). Une étude récente a montré récemment que l'effet de la canneberge sur l'adhésion de la bactérie était dû à une modification des propriétés électrostatiques de la membrane, une modification de la forme de la bactérie et surtout une inhibition de la synthèse des fimbriae [83]. Cependant plusieurs points restent à éclaircir quant aux propriétés anti-bactériennes de la canneberge. En effet, même si son effet sur l'expression de gènes liés à l'adhésion des bactéries a été partiellement décrit par notre équipe, peu d'études se sont intéressées aux impacts que ce produit peut avoir sur d'autres voies métaboliques. En parallèle, une étude menée par notre équipe a montré que l'effet de la canneberge sur l'adhésion et la virulence de souches d'*E. coli* uropathogènes pouvait être potentialisé par l'ajout d'un autre composé naturel : la propolis. Cela désigne un ensemble de produits résineux récoltés par les abeilles sur certains arbres. Depuis l'Antiquité ses propriétés anti-bactériennes sont reconnues et des études plus récentes ont démontré son impact sur des bactéries à Gram positif mais également sur deux bactéries à Gram négatif : *E. coli* et *Pseudomonas aeruginosa*.

Ce travail de thèse s'est donc inscrit dans l'étude de la mécanistique de la canneberge seule ou en association à la propolis sur la pathogénicité d'UPEC. La première question que nous nous sommes posés était de savoir quel était l'impact de la canneberge, de la propolis et de leur association sur le transcriptome d'une souche d'*Escherichia coli* uropathogène? L'étude transcriptomique que nous avons menée a non seulement confirmé l'effet anti-adhérentiel de la canneberge mais a également mis en évidence que d'autres gènes liés à l'adhésion étaient également impactés. D'autre part, d'autres gènes codant pour des protéines impliquées dans la mobilité, fonction clé dans la physiopathologie des IU, étaient impactés par un traitement à base de canneberge. Des études antérieures avaient souligné que la canneberge avait un effet sur la mobilité des bactéries. Cependant ces études étaient peu nombreuses et se limitaient à des tests phénotypiques [105][106]. Une seule étude avait exploré l'impact de la canneberge sur l'expression de gènes liés à la mobilité chez *E. coli*, mais cette étude ne s'était intéressée qu'à un seul gène et aucune confirmation phénotypique n'avait été menée [107]. Cette étude transcriptomique a permis de confirmer et d'approfondir ces résultats en montrant que les gènes liés à la structure du flagelle n'étaient pas les seuls touchés par l'action de la canneberge. Toute la biosynthèse des composants du flagelle ainsi que des protéines impliquées dans la rotation (*motAB*) était négativement impactées par la canneberge. Ces résultats ont été partiellement confirmés par des tests phénotypiques sur une collection de 12 souches d'UPEC, confirmant une réduction de la mobilité des bactéries. Nous avons également mis en évidence l'impact de la canneberge sur des gènes codant pour des protéines impliquées dans la formation de biofilm. Cette voie métabolique est très importante dans le cadre d'IU récidivantes [54][108]. Des travaux avaient montré l'impact de la canneberge sur la formation de biofilm de pathogènes urinaires [109][110][105] mais restaient limitées à des tests phénotypiques sur des souches de référence. Notre étude du transcriptome d'une souche d'UPEC après un traitement par la canneberge a montré que celle-ci réduisait l'expression de gènes codant pour des protéines liés à la chimotaxie et à la production d'exopolysaccharides, deux éléments importants (en plus de l'adhésion et de la mobilité) dans la formation de biofilm. Ces résultats ont ensuite été confirmés par des tests phénotypiques sur la formation de biofilm précoce et complète sur une collection de 12 souches d'UPEC. Enfin, nous avons constaté que la canneberge augmentait l'expression de certains gènes codant pour des protéines liées au métabolisme du fer et à la réponse aux stress. L'effet de la canneberge sur le métabolisme du fer était déjà connu [111] et nous avons pu confirmer ces résultats et apporter quelques précisions. L'augmentation de l'expression de gènes liés à ces deux voies métaboliques montre qu'en présence de canneberge, les UPEC sont dans un état de stress important.

De plus, ce travail a démontré que la propolis potentialisait l'effet de la canneberge, tant sur le plan transcriptomique que phénotypique. Ces derniers tests ont été confirmés partiellement par des expériences menées sur 12 souches d'UPEC. Les résultats obtenus ont montré qu'il existe un effet souche-dépendant. En effet, les tests phénotypiques réalisés sur une collection de 12 souches d'UPEC ont pointé que certaines souches ne modifiaient pas leur métabolisme en présence de canneberge. En revanche, dans l'ensemble de ces cas, l'ajout de la propolis à la canneberge permettait d'obtenir l'effet attendu. La cause de cette variabilité reste inconnue. L'ensemble des 12 souches a été isolé à partir de patients ayant développé une cystite, une pyélonéphrite ou une bactériurie asymptomatique. Les gènes de virulence et de résistance de ces souches sont connus mais aucune corrélation n'a pu être faite entre la présence ou l'absence d'un de ses gènes et la variabilité observée lors des tests phénotypiques. Il serait intéressant de faire une analyse du transcriptome complet de chacune de ces souches afin de déterminer précisément quelles sont les différences transcriptomiques qu'il peut y avoir et expliquer ainsi la variabilité de réponse après un traitement avec l'association canneberge - propolis.

D'autre part, des études ont précédemment montré une différence d'efficacité en termes d'impact sur l'adhésion des bactéries entre différents compléments alimentaires contenant de la canneberge. Cette différence pourrait être expliquée par la concentration en PAC-A des différents compléments alimentaires testés [84][85]. Cependant son dosage dans un échantillon est difficile [89] et il n'est pas mis en évidence de traces de cette molécule dans les urines [112]. Nous nous sommes donc demandé s'il était possible de déterminer la qualité d'un complément alimentaire à base de canneberge en ciblant l'expression de gènes d'*E. coli* impliqués dans l'activité anti-bactérienne de celle-ci ? Cela a donc fait l'objet de la deuxième partie de cette thèse qui a été consacrée au développement d'un outil permettant de mesurer l'activité anti-bactérienne d'une poudre de canneberge en s'affranchissant de la mesure de la concentration en PAC-A. Sur la base de l'étude transcriptomique que nous avons menée, 4 gènes codant pour des protéines impliquées dans la pathogénicité des UPEC et dont l'expression était significativement impactée par un traitement par de la canneberge ont été sélectionnés. L'expression de ces gènes a été évaluée chez une souche d'UPEC cultivée dans deux milieux différents (LB et urine filtrée) avec différentes concentrations de PAC-A. Les résultats que nous avons obtenus ont permis de mettre en évidence un effet dose-dépendant de la canneberge sur l'expression des gènes sélectionnés. De plus, en présence d'une concentration faible de canneberge, un effet antagoniste sur l'expression des gènes sélectionnés a été mis en évidence, montrant l'importance de l'utilisation d'une concentration optimale en



canneberge. L'outil ainsi optimisé a été testé directement sur des échantillons urinaires de patients ayant pris 4h auparavant soit une dose optimale de DUAB<sup>®</sup>, une formulation commerciale contenant de la canneberge et de la propolis, soit un placebo. Pour cela, la souche d'UPEC précédemment testée a été cultivée dans un milieu composé d'urines stériles de patients. Notre technique a ensuite été appliquée suivant le même protocole que précédemment mais sans ajout de solution de canneberge eu milieu de culture. L'objectif était ici de vérifier si notre technique pouvait également détecter l'activité de la canneberge dans des urines après ingestion.

Les résultats obtenus ont montré qu'il existait une grande variabilité dans l'expression des gènes ciblés suivant les prélèvements testés. Parmi les 4 gènes ciblés, seule l'expression du gène *ftnA* variée de façon identique aux tests préalables après culture dans des urines de patients ayant pris du DUAB<sup>®</sup>. De plus, la moyenne de la variation de l'expression de chaque gène au sein des deux groupes a permis de constater des différences notables entre les groupes DUAB<sup>®</sup> et placebo mais ces variations étaient opposées à celles trouvées lors de nos expériences *in vitro*. Cette différence peut s'expliquer par la composition du complément alimentaire DUAB<sup>®</sup>. Ce dernier contient de la canneberge et de la propolis alors que notre outil a été mis au point avec uniquement de la poudre de canneberge non complétement en propolis. Cependant, des tests préalables avaient démontré que la propolis potentialisait l'effet de la canneberge sur l'expression des gènes testés. La composition même des urines des patients inclus pourrait expliquer également la variabilité des résultats obtenus. L'outil qui a été développé permet donc d'évaluer l'efficacité d'une poudre de canneberge en s'affranchissant du dosage des PAC-A mais seule l'évaluation de l'expression du gène *ftnA* permettrait d'évaluer l'efficacité d'un complément alimentaire à base de canneberge après une prise orale de ce complément.

La troisième partie de ce travail s'est plus particulièrement intéressée à l'effet potentialisateur de la propolis. En effet, après avoir observé cet effet sur l'action antibactérienne de la canneberge, des expériences ont été réalisées afin de démontrer si cet effet pouvait être observé avec des antibiotiques, le but étant d'améliorer l'efficacité de ces molécules. Les résultats obtenus ont montré que pour la majorité des antibiotiques testés (excepté la fosfomycine), leur efficacité était améliorée par addition de propolis (diminution de la CMI ou amélioration de l'effet bactéricide). Ces résultats prometteurs pourraient permettre de positionner la propolis comme un complément alimentaire de choix pouvant être proposé au cours d'une antibiothérapie, en particulier dans le cadre du traitement de cystites récurrentes. Cet effet potentialisateur de la propolis pourrait permettre de prévenir l'apparition de résistances et également la formation de biofilm ou de micro-

colonies intra-cellulaires. D'autres tests devront cependant être réalisés afin de comprendre le mode d'action de la propolis sur la bactérie en présence d'antibiotiques : existe-t-il un réel effet potentialisateur de ce produit naturel dû à une interaction entre l'antibiotique et la propolis ou cet effet reflète-t-il uniquement l'effet antibactérien de la propolis?

Les résultats de ce travail montrent que l'association canneberge/propolis a un potentiel très intéressant pour la prévention des infections urinaires et, plus particulièrement, des cystites récidivantes. Nos études se sont focalisées sur *E. coli* car c'est le pathogène le plus fréquemment isolé dans cette pathologie. Cependant *E. coli* n'est pas le seul pathogène impliqué, et il serait intéressant d'étudier l'impact de cette association sur les autres espèces bactériennes responsables d'infections urinaires.

Nous avons mené des études préliminaires sur quelques souches de *K. pneumoniae*, *K. oxytoca*, *S. aureus*, *S. saprophyticus*, *P. mirabilis*, *P. aeruginosa* et *E. faecalis*. Les mêmes tests phénotypiques sur la mobilité et la formation de biofilm ont été réalisés sur ces souches. Les résultats obtenus montrent que la formation de biofilm des souches de *S. aureus* et *S. saprophyticus* sont impactés par l'association canneberge - propolis. L'impact sur *S. aureus* nous a particulièrement intéressés. Des souches de *S. aureus* résistant à la méthiciline et des souches sécrétrices de leucocidine de Panton-Valentine (PVL) ont été testées. Elles étaient également impactées par cette association. Des applications à des pathologies autres que l'infection urinaire pourraient donc être envisagées. En effet, les biofilms bactériens sont impliqués dans de nombreuses pathologies bactériennes. La plaque et les caries dentaires sont en partie provoquées par une bactérie qui développe un biofilm afin de se préserver des agressions du milieu extérieur : *Streptococcus mutans* [113]. L'utilisation de la canneberge a déjà été envisagée et testée dans le cadre de la santé bucco-dentaire. Des études ont montré que la prise quotidienne de bain de bouche à base de canneberge diminuait la concentration de *S. mutans* dans la salive. Des résultats *in vitro* ont confirmé que l'adhésion de ces souches était diminuée [114]. Une étude plus récente a permis de montrer que la masse de biofilm présente à la surface de caries dentaires (expériences menées sur des rats) était réduite significativement après deux prises quotidiennes de canneberge [115]. Nos résultats ainsi que les expériences sur la plaque dentaire ont montré que la canneberge a un impact important sur les cocci à Gram positif et que son domaine d'action n'est pas limité aux pathologies urinaires. L'association canneberge - propolis pourrait potentiellement être utilisée dans d'autres infections dues à *S. aureus* telles les infections ostéo-articulaires [116]. Ces bactéries sont de grandes productrices de biofilm, ce qui explique la difficulté qu'il existe à traiter des infections liées à des prothèses contaminées [117]. Le traitement

classique de ces infections comprend une intervention chirurgicale afin de retirer la prothèse ainsi qu'une antibiothérapie prolongée [118]. L'antibiothérapie a pour but de prévenir la formation d'un nouveau biofilm, et ainsi limiter le risque d'une réinfection ultérieure. Cependant deux problèmes se posent : la formation précoce d'un biofilm par *S. aureus* diminue l'efficacité de l'antibiotique [119] (ii) la présence de mécanismes de résistance chez les souches de staphylocoques à coagulase négative ainsi que chez les souches de *Staphylococcus aureus* [120]. Dans ce contexte il serait possible d'imaginer un traitement des prothèses avec une solution à base de canneberge ou même d'incorporer directement des produits dérivés de la canneberge (telle que la PAC) à la couche superficielle de la prothèse. Cela pourrait permettre de limiter fortement le développement du biofilm et ainsi améliorer l'efficacité de l'antibiothérapie. A terme cela permettrait de diminuer le nombre d'interventions chirurgicales causées par la récurrence de ces infections. Mais cela reste à démontrer.

L'idée d'associer la PAC avec du matériel médical a déjà été envisagée dans le cadre des infections urinaires liées à la pose d'un cathéter. Des études ont montré qu'après traitement de deux matériaux synthétiques, le polyvinyl chloride (PVC) et le polytetrafluoroethylene (PTFE) avec de la PAC extraite de canneberge, une nette diminution de l'adhésion des souches testées (*E. coli* et *E. faecalis*) avait été observée [121]. Une autre étude a montré que l'ajout de PAC à du silicone diminuait la mobilité des souches testées (*E. coli* et *P. mirabilis*) par une diminution de l'expression des flagellines majeures [122]. La fabrication de cathéters (pas seulement urinaires) imprégnés de PACs n'a pas encore été développée mais ces résultats montrent la potentielle efficacité de cette méthode.

Jusqu'ici nous avons pu constater que la canneberge avait un spectre d'action très étendu. Des expériences ont également été menées sur le potentiel antiviral de la canneberge. Une étude réalisée sur l'effet du jus de canneberge sur la pathogénicité des rotavirus sur des cellules de foie de singe (MA-104) a montré des résultats intéressants [123]. Une inactivation très importante de ces virus a été observée, ainsi qu'un effet sur leur réplication, après une exposition d'une heure à une solution de canneberge. Cet effet était rapide et dépendant de la concentration. Une autre étude a montré des résultats similaires sur le virus de la grippe. Une réduction de l'adhésion et de la réplication du virus a été observée sur des cellules de foie de chien (MDCK) [124].

Pour conclure, ce travail de thèse a permis de décrire de façon plus précise l'impact de l'association canneberge et propolis sur le transcriptome des UPEC. Les résultats obtenus ont montré l'intérêt d'utiliser la canneberge en association avec la propolis afin i) de potentialiser l'effet de canneberge

tant sur le point transcriptomique que sur le point phénotypique non seulement sur l'adhésion des UPEC mais également sur d'autres voies métaboliques moins étudiées jusqu'alors telles que la mobilité et la formation de biofilm ; ii) d'atténuer l'effet souche-dépendant de la canneberge qui a été décrit d'un point de vue phénotypique sur la mobilité et la formation de biofilm. D'autre part, ce travail montre également l'intérêt de l'utilisation de la propolis en complément d'une antibiothérapie afin de potentialiser l'effet des antibiotiques prescrits. L'association de ces deux composés naturels a donc toute sa place dans la stratégie thérapeutique de prise en charge des IU seule ou en association avec une antibiothérapie.

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## Résumé :

L'infection urinaire (IU) est un problème majeur de Santé publique. La cystite aiguë touchant principalement les femmes est la plus fréquente des IU. La bactérie la plus fréquemment isolée au cours de ces IU est *Escherichia coli*. Une des particularités de la cystite est sa propension à récidiver. Le traitement préconisé pour ces infections est la prise d'antibiotiques, qui peut être fréquente en cas de cystites récidivantes. C'est dans ce contexte que de nouvelles stratégies doivent être développées afin de prévenir et traiter les IU récidivantes. Parmi ces différentes stratégies, l'utilisation de produits naturels tels que la canneberge (*Vaccinium macrocarpon*) apparaît comme prometteuse. En effet, des études antérieures ont montré que la canneberge a un effet négatif sur l'adhésion des bactéries aux cellules superficielles de l'épithélium vésical facilitant l'élimination des bactéries par le flux urinaire. Cette activité est portée par la proanthocyanidine de type A (PAC-A). D'autre part, une étude menée par notre équipe a montré que l'effet de la canneberge sur l'adhésion et la virulence de souches d'*E. coli* uropathogènes pouvait être potentialisé par l'ajout d'un autre composé naturel : la propolis. Depuis l'Antiquité ses propriétés anti-bactériennes sont reconnues et des études plus récentes ont démontré son impact sur des bactéries à Gram positif mais également sur deux bactéries à Gram négatif : *E. coli* et *Pseudomonas aeruginosa*. Ce travail de thèse a permis : i) de décrire l'impact de la canneberge, de la propolis et de leur association sur le transcriptome d'une souche clinique d'*E. coli* uropathogène. Cette analyse transcriptomique a montré que la canneberge entraînait une sous-expression de gènes codant pour des protéines liées à l'adhésion, mais également de gènes codant pour des protéines liées à la mobilité et à la formation de biofilm. En revanche, la canneberge augmentait l'expression des gènes liés au métabolisme du fer ainsi qu'à la réponse au stress. Ces effets étaient potentialisés par l'ajout de la propolis. En parallèle, des tests phénotypiques menés sur une collection de souches d'*E. coli* uropathogènes sur la mobilité et la formation de biofilm ont confirmé les résultats précédents ; ii) de développer un test, basé sur les précédents travaux de transcriptomique, permettant une évaluation standardisée de l'effet de la PAC-A sur *E. coli*, indépendamment de sa concentration car il n'existe pas de techniques standardisées pour doser cette molécule. C'est ainsi que 4 gènes (*tsr*, *ftnA*, *fecB*, *feoB*) ont été sélectionnés, le suivi de leur expression permettant une mesure de l'activité anti-bactérienne de la canneberge; iii) de mesurer l'effet potentialisateur de la propolis sur l'activité des antibiotiques utilisés dans le traitement des IU. C'est ainsi qu'il a été montré que l'ajout de la propolis permettait d'augmenter l'activité bactéricide des antibiotiques testés et de diminuer les concentrations minimales inhibitrices de ces antibiotiques.

## Abstract:

Urinary Tract Infection (UTI) is a major problem of public health. Acute cystitis which touches mostly women is the most common form of UTI. The bacteria which is mostly isolated in an UTI is *Escherichia coli*. A particularity of cystitis is to come back. In this context news strategies have to be developed to prevent and cure recurrent UTI. One of these strategies is the utilization of natural products like the cranberry (*Vaccinium macrocarpon*) which is promising. Indeed, previous studies showed the negative impact of cranberry on the adhesion of bacteria on the superficial cells of bladder which help the elimination of bacteria by the urinary flux. This activity is carried by the type A proanthocyanidin (PAC-A). Moreover, a study lead by our team has demonstrated an improvement of the activity of cranberry on the adhesion and the virulence of uropathogenic *E. coli* (UPEC) by another natural product: the propolis. Since Antiquity its antibacterial activities have been recognize and more recent studies have demonstrated its impact of Gram positive bacteria and also on two Gram negative bacteria: *E. coli* and *Pseudomonas aeruginosa*. This thesis has allowed for: i) the description of the impact of cranberry, propolis and its association on the transcriptome of a clinical strain of UPEC (G50). This transcriptomic analyze have shown that the cranberry down regulated genes linked to the adhesion and also genes linked to the motility and biofilm formation. However the cranberry up regulated genes linked to the iron metabolism and the stress response. These effects are improve by the addition of propolis. Concurrently phenotypics tests have been conducted on a collection of UPEC on the

motility and the biofilm formation and they confirmed the previous results; ii) the development of a test, based on our transcriptomic results, enable to performed a standardized evaluation of the impact of PAC-A on *E. coli*, independently of its concentration. Indeed, this molecule cannot be measure in a standard way. Four genes have been selected (*tsr*, *ftnA*, *fecB*, *feoB*), the monitoring of their expression allow us to measure the anti-bacterial efficiency of the cranberry; iii) the measurement of the potential effect of the propolis of the antibiotic's activities used to treat UTIs. Thus it have been observed that the addition of propolis improve the bactericide activity of the antibiotics tested and reduces the minimal inhibitory concentration of these antibiotics.