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Susana M. MARTIN-ORUE, profesora asociada, del departamento de Ciencia Animal y de los Alimentos de la Facultad de Veterinaria de la Universitat Autonoma de Barcelona,

Certifica:

Que la memoria titulada "Screening funcional ingredientes for the prevention of diarrhoea caused by ETEC K88 in miniaturized models", presentada por Yanan Zhu con la finalidad de optar al grado de Doctor en Veterinaria, ha estado realizada bajo mi dirección y, considerándola acabada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que así conste para los efectos oportunos, firmo la presente en Bellaterra a 18 de Septiembre de 2018.

Dra. Susana M. Martin Orue

SUMMARY

The aim of this Thesis was to explore new feed strategies to fight digestive pathogens in piglets and contribute to reduce the use of antibiotics at weaning. Based on previous research findings of our research group, that evidenced the ability of wheat bran to interfere with the attachment of ETEC K88 to intestinal epithelial cells (IPEC-J2 cells), in this Thesis we also explore the potential of other potential macro-ingredients to prevent the intestinal adhesion of this pathogen. In previous works it was also evidenced the ability of microbial exopolysaccharides (EPS) isolated from the industrial fermenters of table green olive, to attach specifically ETEC K88 and also to block its adhesion to IPEC-J2 cells. In this Thesis we go one step further and we study the chemical composition and structure of this by-product of the olive industry and its variability between different industrial fermenters. Moreover, new strategies to improve the functionality of these microbial EPSs are explored as the use of different mates of *Lactobacillus pentosus* and *Candida boidinii* strains to be used as starters in the fermentation of table green olives.

In order to achieve these main objectives a set of three trails (chapters 4 to 6) were designed.

Trial I was design to evaluate *in vitro,* with different miniaturized models, the ability of different common ingredients used in the formulation of piglet diets, and particularly different kinds of cereal brans, to attach ETEC K88 (adhesion test, AT) and to block its attachment to intestinal epithelial cells (IPEC-J2) (blocking test, BT). For the common feed ingredients, wheat, corn, oats, barley, rye, soybean meal 44%, extruded full-fat soybean meal, as well as sweet whey powder, all demonstrated a clear affinity to attach ETEC K88. Regarding rye, oats and also wheat, all of them could also reduce ETEC K88 adhesion to IPEC-J2 cells. Among differently tested cereal brans, wheat bran, spelt bran, kamut bran, rye bran, oat bran and rice bran all demonstrated an ability to attach ETEC K88. Also, all of them were able to reduce ETEC K88 attachment to IPEC-J2 cells, except rice bran.

Trial II aimed to explore biological functions against enterotoxigenic *E. coli* (ETEC) K88 of the microbial exopolysaccharides (EPSs) produced during the industrial fermentation of table green olives. Exopolysaccharides were isolated from the brines obtained from five industrial fermenters. Analysis of their monosaccharide composition by GLC revealed that the main components were glucose and galactose followed by rhamnose and arabinose. The

^{1}H NMR spectrum showed a very similar profile between samples, and a more in-depth analysis revealed the presence of an α-pyranose in the form of α-D-Glc*p*-(1→) and two different α-furanoses, with chemicals shifts values, suggesting the presence of α-D-Glc*f* and α-D-Gal*f*. Miniaturized *in vitro* tests included adhesion test (AT) to evaluate the ability of EPS to attach specifically ETEC K88 and three different approaches to evaluate its interference on adhesion of the pathogen to IPEC-J2 cells: competition test (CT), exclusion test (ET) and displacement test (DT). Results from AT demonstrated the ability of EPS samples to attach specifically to ETEC K88 with variable intensity. The CT, was not able to show ability to block the ETEC K88 adhesion to IPEC-J2 cells, however in the DT, all EPS samples were shown to effectively remove the pathogen once attached to the cells.

Trial III was designed to evaluate the potential of different starters to modulate the fermentation of table green olives and to improve the anti-adhesive characters of the EPSs isolated from the fermentation brines. A strain of *Candida boidinii* TOMC-Y13 and four *Lactobacillus pentosus* strains were used as starters in different combinations giving a total of 10 treatments in a 2 x 5 design. Different physicochemical parameters were registered along the fermentation process including pH and plate-counts of LAB and yeast. The pH values, yeast and LAB numbers on olive surface at the 60th day were modified by these starters. Some particular strains were able to improve the anti-adhesive characters of EPSs: *L. pentosus* 119WT and specially *L. pentosus* 119-14MT were the most effective starters improving antiadhesive properties of EPSs. Combination of 119-14MT strain with *C. boidinii* TOMC-Y13 reversed this improvement.

Results in this Thesis showed that wheat, rye, oat and their brans could be regarded as potential ingredients to prevent of diarrhoea in weaning piglets if used preferably in the formulation of diets. Moreover microbial EPS, obtained as by-products from the industrial production of table green olives, is suggested as a potential in-feed additive to be used in the prevention of post-weaning diarrhoea. These EPSs are demonstrated to be quite similar between different industrial olive fermenters regarding their chemical composition and structure determined by ^{1}H NMR spectrum. Different EPS tested also showed consistent anti-adhesive abilities although of variable intensity depending on the industrial fermenter. Using *L. pentosus* strains and *C. boidinii* TOMC-Y13 as starters, are demonstrated to

influence the fermentation process modifying pH and the attached numbers of yeast and LAB on olive surfaces. Anti-adhesive abilities could also be improved by the use of some particular *L. pentosus* strains. The above-mentioned results will be helpful to explore new feed strategies and the development of new feed additives in the prevention of post-weaning colibacillosis induced by ETEC K88.

RESUMEN

El objetivo de esta tesis ha sido explorar nuevas estrategias nutricionales frente a patógenos de relevancia en lechones durante el periodo post-destete y así poder contribuir a la reducción del uso de antibióticos durante esta etapa. Partiendo de los resultados obtenidos previamente en nuestro grupo, con los que se evidenció la capacidad del salvado de trigo de interferir en la adhesión de *Escherichia coli* enterotoxigénica (ETEC) K88 a las células epiteliales intestinales (IPEC-J2), en esta tesis se evaluó el potencial de otros macro-ingredientes en la prevención de la adhesión intestinal de este patógeno. Asimismo, en trabajos anteriores, también se evidenció la capacidad de los exopolisacáridos (EPS) de origen microbiano, aislados de fermentadores industriales destinados a la producción de las aceitunas verdes, de adherirse específicamente a ETEC K88 y así bloquear su adhesión a las células IPEC-J2. En esta Tesis se propuso explorar estos compuestos en mayor profundidad y se estudió su composición química y su estructura analizando la variabilidad existente entre diferentes fermentadores industriales. Además, se implementaron nuevas estrategias a fin de mejorar la funcionalidad de estos EPS microbianos mediante el uso de diferentes cepas de *Lactobacillus pentosus* y *Candida boidinii* como starters en la fermentación de las aceitunas verdes de mesa.

Con el fin de cumplir con los principales objetivos mencionados, se diseñaron tres pruebas distintas (Capítulos 4 a 6).

El **Experimento I** se concibió con la finalidad de evaluar *in vitro*, mediante diferentes modelos miniaturizados, la capacidad de diferentes ingredientes, comúnmente usados en la formulación de las dietas para lechones, y particularmente diferentes tipos de salvados de cereales, de adherirse a ETEC K88 (test de adhesión, AT) y bloquear su adhesión a las células epiteliales intestinales (IPEC-J2) (test de bloqueo, BT). En cuanto a los ingredientes habituales, el trigo, maíz, avena, cebada, centeno, harina de soja 44%, haba de soja extrusionada, así como el lactosuero dulce, todos demostraron una clara afinidad de adhesión a ETEC K88. Con respecto al centeno, la avena y también el trigo, se observó como los tres ingredientes redujeron la adhesión de ETEC K88 a las células IPEC-J2. Entre los distintos salvados de cereal testados, los salvados de trigo, espelta, kamut, centeno, avena y arroz

demostraron ser capaces de adherirse a ETEC K88, además de reducir la adhesión de ETEC K88 a las células IPEC-J2, a excepción del salvado de arroz.

El **Experimento II** fue diseñado para explorar las funciones biológicas de los exopolisacáridos (EPS) microbianos, producidos durante la fermentación industrial de las aceitunas verdes de mesa, frente a *E. coli* enterotoxigénica (ETEC) K88. Los exopolisacáridos fueron asilados de la salmuera procedente de cinco fermentadores industriales. El análisis de la composición de monosacáridos mediante GLC reveló que los componentes principales correspondían a la glucosa y galactosa, seguidos de la ramnosa y arabinosa. El espectro ^{1}H NMR mostró un perfil muy similar entre las diferentes muestras, y el análisis posterior más detallado reveló la presencia de α-piranosa según la forma α-D-Glcp-(1→) y dos tipos distintos de α-furanosas, lo que sugiriere la presencia de α-D-Glcf y α-D-Galf. Los tests *in vitro* incluyeron el test de adhesión (AT) para evaluar la capacidad del EPS de adherirse específicamente a ECET K88, y tres estrategias diferentes para evaluar la interferencia en la adhesión del patógeno a las células IPEC-J2: test de competición (CT), test de exclusión (ET), y test de desplazamiento (DT). Los resultados del AT demostraron la capacidad de las muestras de EPS de adherirse específicamente a ETEC K88 con intensidad variable. En el caso del CT, no se pudo mostrar la capacidad de bloquear la adhesión de ETEC K88 a las células IPEC-J2, sin embargo, en el test DT, todas las muestras de EPS demostraron ser eficaces eliminando el patógeno ya adherido a las células.

El **experimento III** fue diseñado para evaluar el potencial de diferentes cultivos starter para modular la fermentación de las aceitunas verdes de mesa, así como mejorar las características anti-adhesivas de los EPS aislados de las salmueras de fermentación. Para tal fin, se usaron distintas combinaciones de una cepa de *Candida boidinii* TOMC-Y13 y cuatro cepas de *Lactobacillus pentosus* como starters dando lugar a un total de 10 tratamientos bajo un diseño de 2 x 5. Durante el proceso de fermentación, se registraron diferentes parámetros físico-químicos que incluyeron el pH y los recuentos en placa de la LAB y de levaduras. Los starters modificaron el pH, los recuentos de levaduras y de LAB en la superficie de las aceitunas a día 60. Concretamente, algunas cepas fueron capaces de mejorar las características físico-químicas de los EPS: *L. pentosus* 119TW y especialmente *L. pentosus* 119-14-MT fueron los starters más eficaces en la mejora de las propiedades anti-adhesivas de los EPS. Por otro lado,

la combinación de la cepa *L. pentosus* 119-14-MT con *C. boidinii* TOMC-Y13 revirtió esta mejora.

Los resultados de esta Tesis mostraron que el trigo, centeno, avena, y sus salvados podrían tenerse en cuenta como posibles ingredientes a considerar y a incluir en la formulación de las dietas para la prevención de diarreas en los lechones destetados. Por su parte, el EPS microbiano, co-producto obtenido de la producción industrial de aceitunas verdes de mesa, también demostró tener potencial para su uso como aditivo en las dietas post-destete. Los EPS presentaron una composición similar entre los diferentes fermentadores industriales en cuanto a su análisis químico y su estructura determinada mediante el espectro de ^{1}H NMR. Los distintos EPS testados también demostraron poseer capacidades anti-adhesivas, aunque con intensidad variable entre los diferentes fermentadores. El uso de las cepas starters de *L. pentosus* y *C. boidinii* TOMC-Y13 demostró su influencia en la fermentación mediante la modificación del pH y de los recuentos de levaduras y LAB adheridos a las superficies de las aceitunas. Fue posible además mejorar la capacidad anti-adhesiva de los EPS mediante el uso de cepas específicas de *L. pentosus*. Los resultados expuestos pueden ser de utilidad para el desarrollo de nuevas estrategias nutricionales y aditivos dirigidos a la prevención de la colibacilosis post-destete causada por ETEC K88.

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GENERAL INDEX

ABBREVIATIONS

AMR: antimicrobial resistance

AT: adhesion test

BT: blocking test

CA: combined acidity

CFU: colony-forming units

CGMP: Casein Glycomacropeptide

DMBQ: 2, 6-dimethoxy-1, 4-benzoquinone

DSS: dextran sodium sulfate

DMEM: Dulbecco´s Modified Eagle Medium

DT: displacement test

ECM: extracellular cell matrix

EPEC: Enteropathogenic *E. coli*

EPS: exopolysaccharides

ET: exclusion test

GP: Glycoprotein

GSL: Glycolipids

HePs: heteropolysaccharide

HMOs: human milk oligosaccharides

HoPS: homopolysaccharide

IPEC-J2: porcine intestinal epithelial cell line J2

LAB: Lactic acid bacteria

LB: Luria broth

MLNs: mesenteric lymph nodes

NF: non-fimbriated

NMR: nuclear magnetic resonance

OD: optical density

PRRs: patter recognition receptors

ROS: reactive oxygen species

RSE: Residual standard error

SCFA: short-chain fatty acids

SED: subepithelial dome

TA: titratable acidity

TDA: thymus-dependent area

WB: wheat bran

WHO: World Health Organization

YM: yeast-malt-peptone-glucose medium

TABLES AND FIGURES INDEX

1. General introduction

In pig nutrition, feed macro-ingredients have been considered traditionally just as vehicles of energy and nutrients, being their possible functional properties generally ignored or neglected. However it is known that food can provide a repertoire of trace compounds some of them with meaningfully bioactive characters (Jansman, 2016). Some particular carbohydrates, glycoproteins, peptides and polyphenols, among others, have been shown to have anti-inflammatory, bactericidal or prebiotic roles contributing to maintain intestinal health (Puupponen-Pimiä et al., 2002). Especially, the ability of some compounds to interfere with the adhesion of pathogens to the intestine has recently attracted attention (Lane et al., 2010).

Antibiotics were discovered about 90 years ago and since then they have been extensively used in human and veterinary medicine for the prevention and treatment of diverse bacterial infections. In livestock, antibiotics have been also used as growth promoters included as in-feed additives in animal diets, although since the year 2006 their use was forbidden in the EU (EPC, 2005). Parallel to the use of antibiotics, microorganisms have evolved developing different molecular ways of resistance. Every year, in the EU more than 25 000 people die due to multidrug-resistant bacteria with €1.5 billion health care costs (Alvana et al., 2011). The antimicrobial resistance (AMR) of pathogenic bacteria is nowadays a global threat that has lead the World Health Organization to establish a Global Action Plan in 2015 (WHO, 2015). Within their strategic objectives it is stated the need to increase investment in new medicines, diagnostic tools, vaccines and other interventions able to reduce the need of use antibiotics.

Weaning is one of the most critical periods in the pig life. A number of stressors, such as social, environmental and dietary stress, could cause intestinal and immune system dysfunctions facilitating pathogenic infections (Campbell et al., 2013). Weaning-associated infectious pathologies are one of the main responsible for the use of antibiotics in livestock (Mathew et al., 2007). Improving management and nutrition, during this period of pig live, seems essential in any reduction plan. From this point of view, a rational use of feed ingredients in the piglet diet, aimed to improve natural resistance of the animal to disease, appears as an attractive strategy.

In intestinal bacterial infections, attachments or adhesion of pathogens to the intestinal epithelial cells is one of the first and required steps to colonize the gut and cause disease (Ofek et al., 2003). Attachment is mediated by bacteria fimbriae/adhesins and their specific receptors on cell surfaces. Literature have pinpointed carbohydrates, proteins or glycoconjugates as the main biological structures responsible of the interactions between adhesins and intestinal cells (Sharon and Lis, 2004; Day et al., 2015). Taking this into account, it has been proposed that providing exogenous molecules with similar structure to the microbial adhesins or epithelial receptors, could correspondingly bind with any of them interfering in the bacteria-intestine attachment. Providing these exogenous analogues through the diet could therefore inhibit the first step of pathogenic colonization preventing disease (Cozens and Read, 2012). In addition, these agents would not kill bacteria like antibiotics, not being expected to cause selective pressure for the appearance of resistant bacteria (Ofek et al., 2003).

Varity of macro-ingredients of vegetable, animal origin have been described as possible anti-adhesive providers (Sun and Wu, 2017). Particularly lot of research can be found regarding the potential of human milk oligosaccharides to assist neonates in the prevention of diarrhoeal pathogens (Coppa et al., 2006). Also, specific carbohydrates, exopolysaccharides (EPS), and proteins from microbial origin can be add to the list of potential competitive analogues (Wang et al., 2010; Sun et al., 2017).

In the latest years, the Animal Nutrition and Welfare research group of the UAB have tried to explore natural ingredients with anti-adhesive properties. In the frame of previous projects (AGL 2007-60851/GAN: Evaluation of different functional ingredients to improve the health status of pigs; AGL2009-07328: Evaluation of Zn and blocking substrates of microbial adhesion in the feed of the post-weaning piglet) Molist et al. (2011) found that wheat bran (WB) included in the piglet diets reduced fecal *E. coli* counts in weaning piglets and also in ileal digesta after an oral challenge with ETEC K88 (Molist et al., 2010). Hermes et al. (2011) also showed *in vitro* how WB, Casein Glycomacropeptide (CGMP) or locust bean were able to inhibit the ETEC K88 attachments to IPEC-J2 cells, also modulating the innate inflammatory response. Results from Gonzalez-Ortiz et al. (2013) also verified the anti-adhesive abilities of these ingredients, and moreover Hermes et al. (2013) and Gonzalez-

Ortiz et al. (2014a, b) showed that CGMP and WB were able to reduce the adhesions of this bacteria to piglet mucosa *in vitro* and *in vivo*. More recently in the frame of a later project (AGL2012-3192: Two new strategies to improve the adaptation of piglets to weaning: the optimizations of Zn status and the EPS from fermentation of green olives) it was evidenced the potential of microbial EPS isolated from olive fermentation brines to interfered with ETEC K88 attachment to IPEC-J2 cells (Gonzalez-Ortiz et al., 2014a).

Based on results from previous studies, and framed in this last project (AGL2012-3192), it is developed the research of this PhD. With this work it is aimed to explore the potential of common macro-ingredients in the piglet diets to act as anti-adhesive agents, and more deeply evaluate the potential of microbial EPSs obtained from olive fermentation brines.

2. LITERATURE REVIEW

2.1. BACTERIAL ADHESION

2.1.1. INTRODUCTION

The intestine is inhabited by a large population of bacteria that coexist in symbiosis with its host. Actually bacterial cells in the gut are higher in numbers than eukaryotic cells in the body (Rosner, 2014). Bacteria in the gut can play numerous beneficial functions to the host, including the fermentation of some undigested food material and the synthesis of some vitamins, like vitamin k or folic acid (Cummings and MacFarlane, 1997). Together to these digestive functions, commensal bacteria are able to cross talk with the host through different molecular ways sharping and influencing the immune system (Canny and McCormick, 2008; Nicholson et al., 2012). Some particular microbial groups had been demonstrated to have anti-inflammatory effects and also to be able to exclude pathogens (Canny and McCormick, 2008; Hill and Artis, 2010). However, under particular conditions, some opportunistic pathogens can colonize the gut and cause disease. The competitive balance maintained by commensal and opportunistic pathogens is vital for gut health (Guarino et al., 2012).

It is a well-accepted theory that for many pathogens the interaction with epithelial cells is a critical and first step for performing their actions. This adhesion would be mediated by different bacterial appendages able to recognize host receptors on cellular surfaces (Soto and Hultgren, 1999; Sharon. 2006). In the following chapters we will review how bacteria can interact with the gut and particularly how some intestinal pathogens rely on this ability to attach to intestinal epithelial cells and cause disease.

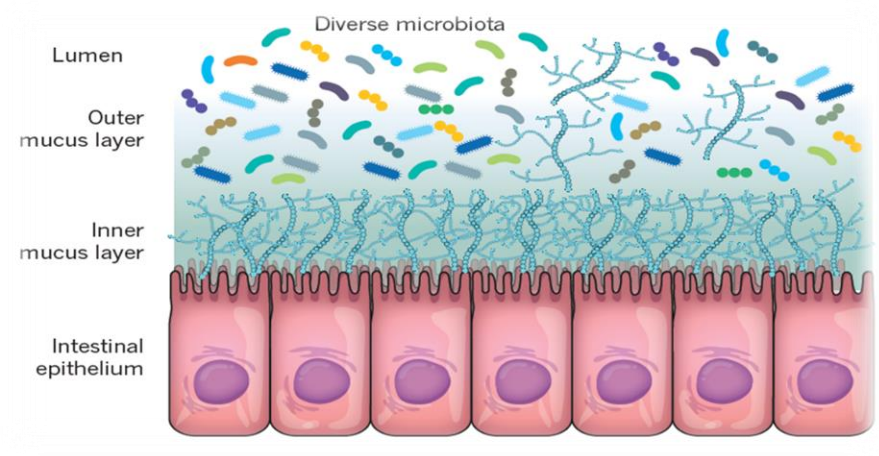


Figure 2.1. A diverse and non-disturbed microbiota in the intestinal epithelium (cited from Baümler and Sperandio, 2016).

2.1.2. BACTERIA-HOST INTERACTIONS

One of the main mechanisms by which bacteria interact with its host is by the expression of different molecules and structures on their surface that make them able to specifically recognize receptors on the host epithelial cells. These structures are generally known as adhesins (Nougayrede et al., 2003; Kline et al., 2009; Solanki et al., 2018). Interaction of bacteria with the epithelium in this way can exert different responses in the immune system of the host training it to distinguish between commensal and potential pathogenic bacteria (Kline et al., 2009). However adhesion of bacteria to the intestinal surface can also turn on a way for pathogens to resist physical removal (Klemm and Schembri, 2000) avoiding bacterial to be swept out by the digesta flow. In this way adhesins could assist bacteria to resist defence from host by bonding receptors on the host surface and increasing the opportunities of gut colonization.

2.1.2.1. CATEGORIZATION OF ADHESINS

Depending on the structures exhibited on the bacterial surfaces, adhesins could be divided into pili/fimbriae and nonpilus/afimbrial adhesins. Pili/fimbriae are extracellular hairy appendages. Afimbrial adhesins, contrary to pilus adhesins, without appendages outside the bacterial membrane, are like rivets into bacterial surfaces (Finlay and Cossart, 1997; Soto and Hultgren, 1999). Adhesins of Gram-negative bacteria are pili/fimbriae (Patel et al.,

2017), but pili also have been discovered in some Gram-positive bacteria, and they also play vital roles in microbial adherence (Pizarro-Cerdá and Cossart, 2006; Mandlik et al., 2008).

Depending on the chemical nature of adhesins and receptors we can distinguish multiple kinds of interactions.

2.1.2.1.1. LECTIN-CARBOHYDRATE INTERACTION

Lectins were first described more than 100 years ago originating from a series of research regarding hemagglutinins (Sharon and Lis, 2004). They were defined as carbohydrate-binding proteins existing in plants, animals and bacteria. Lectin-carbohydrate interactions between microorganisms and cell membrane could be meditated by two ways: bacterial lectins bonding with cell surface carbohydrates or cell lectins bonding with bacterial carbohydrates (Figure 2.2) (Sharon and Lis, 2004; Ohlsen et al., 2009).

2.1.2.1.2. PROTEIN-PROTEIN INTERACTION

Bacteria surface proteins can also help bacteria to adhere the host by connecting with the extracellular cell matrix (ECM), which mainly consist of some fibrous proteins (Chagnot et al., 2012). Moreover, some transmembrane proteins can also act as receptors of bacterial surface proteins, such as $\alpha_5\beta_1$ integrin bounding with Ipa proteins of *Shigella* (Watarai et al., 1996), E-cadherin bounding with internalin of *L.monocytogenes* (Mengaud et al., 1996).

2.1.2.1.3. CARBOHYDRATE-CARBOHYDRATE INTERACTION

Carbohydrate-carbohydrate interactions are also important mechanisms in biological processes including cell adhesion and communications/recognitions (Rojo et al., 2002). Considered as a week interaction, carbohydrate-carbohydrate interactions between bacteria and host cells have not been attracted more attentions. However, a recent study conducted by Day et al. (2015) firstly illustrated interactions between some bacterial glycan and host glycan could be as effective as protein-carbohydrate interactions. These glycan-glycan interactions also interfered with bacterial attachments to Caco-2 cells. Belotserkovsky et al. (2018) verified glycan-glycan interactions existed in the binding of *Shigella* to CD^+ T lymphocytes.

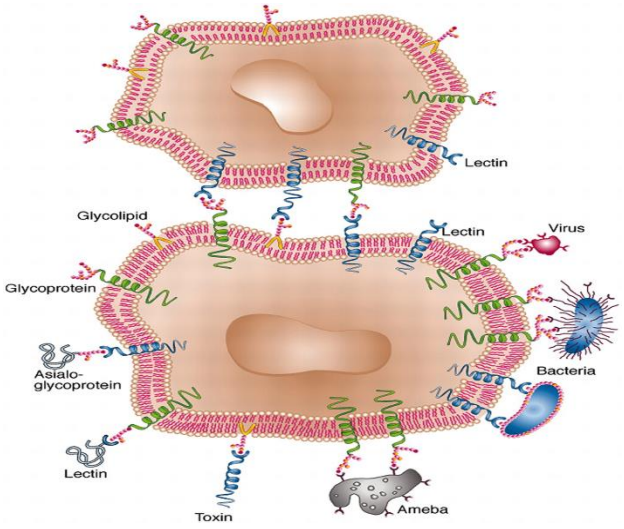


Figure 2.2. Cell surface lectin-carbohydrate interactions. Bacterial lectins connect with carbohydrate sites of cellular surface carbohydrates of glycoproteins or glycolipids. Lectins on cell surface also could attach with outside carbohydrates of bacteria. (cited from Sharon and Lis (2004); Based on an original diagram from BioCarbAB (Lund, Sweden)).

2.1.2.1.4. OTHER MECHANISMS

Bacteria could also 'generate' and 'send' their own receptors to host. Regarding this aspect, Kenny et al. (1997) revealed intimin of Enteropathogenic *E. coli* (EPEC) interact with Hp90 on mammalian cells, and actually this Hp-90 was a 90 k Da protein secreted by EPEC. Bacterial outer membrane vesicles also seemed as similar actors. Vesicles released by growing bacteria could interact with host cells and enhance their bacterial attachments to host cells (Kuehn and Kesty, 2005).

2.1.3. RECEPTORS IN BACTERIA-HOST INTERACTIONS

Corresponding to adhesins, surface receptors are the other fundamental structures responsible for bacterial surface adherence (Jones and Isaacson, 1982). Bacteria attachments could not occur if cells would not offer specific receptors (Nizet et al., 2017). As known, bioactive components of receptors are highly related with carbohydrates (Kato and Ishiwa, 2015). As the most well studied receptors, glycoproteins and glycolipids (proteins and lipids linked with

carbohydrates) mainly constitute the glycocalyx on cellular surfaces (Ohlsen et al., 2009; Kato and Ishiwa, 2015).

Carbohydrate linkages in glycoproteins and glycolipids could be of different nature (Wyss and Wagner, 1996; Sharon, 2006; Gabius et al., 2016), several examples are given in Table 2.1. *N*- and *O*-linked glycans are common found forms (Yang et al., 2017). In term of *N*-linkage, Asn-Xaa-Ser/Thr is a necessary 'unit', and glycans bond with side-chain nitrogen of asparagine in this 'unit'. *O*-linked glycans consisted of 1-20 residues, and their linkages do not need specific 'unit' of membrane proteins (Wyss and Wagner, 1996; Juge, 2012).

2.1.4. ANTI-ADHESIVE STRATEGIES IN THE PREVENTION OF DIGESTIVE PATHOGENS

Based on the mechanism of adhesin-host interactions, the oral supplementation with adhesin/receptor analogues has been proposed as a competitive strategy to reduce pathogen virulence by blocking bacterial receptors or bacterial adhesins (Kelly and Younson, 2000). The principal advantage of this alternative therapy, compared to antibiotics is that it would not be expected to cause the selection of bacterial resistance as it not compromise the growth or survive of bacteria (Ofek et al., 2003; Sharon, 2006).

Literature have revealed many compounds that could be used as anti-adhesive agents, especially natural compounds obtained from plant, dairy products or microorganism. Molecule structures of these analogues usually resemble structures of adhesins or bacterial receptors (Kelly and Younson, 2000; Krachler and Orth, 2013). Among these agents, carbohydrates and their related materials have been widely investigated. Between them methyl α -D-mannopyranoside (αMM) was the first recorded anti-adhesive material *in vivo* preventing pathogen infection (Aronson et al., 1979). In the following chapters there will be reviewed the potential anti-adhesive properties reported for different natural ingredients. It will be also explored the properties of specific carbohydrates-exopolysaccharides (EPSs), produced by certain microorganisms, as receptor/adhesins analogues.

Table 2.1. Carbohydrates as attachment sites for bacterial pathogens on animal tissues.

Organism	Target tissue	Carbohydrate	Form[a]
C. jejuni	Intestinal	Fucα2GalβGlcNAc	GP
E. coli Type 1	Urinary	Manα3Manα6Man	GP
P	Urinary	Galα4Gal	GSL
S	Neural	NeuAc (α2–3)Galβ3GalNAc	GSL
CFA/1	Intestinal	NeuAc (α2–8)–	GP
F1C	Urinary	GalNAcβ4Galβ	GSL
F17	Urinary	GlcNAc	GP
K1	Endothelial	GlcNAcβ4GlcNAc	GP
K99	Intestinal	NeuAc(α2–3)Galβ4Glc	GSL
H. influenzae	Respiratory	$[NeuAc(\alpha2–3)]_{0,1}$ Galβ4GlcNAcβ3Galβ4GlcNAc	GSL
H. pylori	Stomach	NeuAc(α2–3)Galβ4GlcNAc	GP
		Fucα2Galβ3(Fucα4)Gal	GP
K. pneumoniae	Respiratory	Man	GP
N. gonorrhoea	Genital	Galβ4Glc(NAc)	GSL
N. meningitidis	Respiratory	$[NeuAc(\alpha2–3)]_{0,1}$ Galβ4GlcNAcβ3Galβ4GlcNAc	GSL
P. aeruginosa	Respiratory	L-Fuc	GP
	Respiratory	Galβ3Glc(NAc)β3Galβ4Glc	GSL
S. typhimurium	Intestinal	Man	GP
S. pneumoniae	Respiratory	$[NeuAc(\alpha2–3)]_{0,1}$ Galβ4GlcNAcβ3Galβ4GlcNAc	GSL
S. suis	Respiratory	Galα4Galβ4Glc	GSL

[a]Predominant form in tissue: GP: Glycoprotein; GSL: Glycolipids. Cited from Sharon (2006).

2.2. POTENTIAL ROLE OF DIETARY MACRO-INGREDIENTS IN INTESTINAL HEALTH

Cereals and vegetable and animal sources of protein have been widely used in the formula of pig diets. These ingredients are important origins of carbohydrates, proteins, fat, mineral, and even vitamin for pigs. However no only that, different studies have described how some feed ingredients could also promote animal health together providing nutrients (Jansman, 2016). In this part, some simple descriptions would focus on reported functions for some different ingredients used in the piglet diets, such as cereals, vegetable and animal sources of protein or dairy products. Main functions to be reviewed will include effects on mucosa architecture, immune response, antiadhesive properties against pathogens and intestinal microbiota.

2.2.1. INGREDIENTS AND MUCOSA ARCHITECTURE

The integrity of mucosa of the small intestine is vital for the digestive function (Bach Knudsen et al., 2012). During the piglet weaning period, mucosal architecture is greatly affected by the food change from the milk to a vegetable based dry diets (Bach Knudsen et al., 2012). Research have exhibited how some ingredients, especially cereals, can have an influence on the mucosa architecture during this period.

It has been proved in piglets that intake of cereals increase crypt depth without creep feed previously (Torrallardona et al., 2015). However other researchers did not find differences between cereals (Boudry et al., 2002) with no change in jejunal structure among wheat-, barley- or milk- diets. Brunsgaard. (1998) comparing barley and wheat diets did not found differences associated to the cereal type on the mucosal architecture, epithelial cell proliferation, or production and composition of the mucins in the large intestine of weaning pigs, but they did related to the particle size of each cereal. Regarding the use of cereal by-products, the study conducted by Hedemann et al. (2006) also verified that feeding newly weaned pigs with barley hull could result in higher villous height, which determines post weaning weight gain and improves gut morphology, increase the activity of the peptidase, aminopeptidase N and dipeptidyl peptidase TV.

As the above descriptions, changes of mucosa architecture could be however consequence of many factors. The kind of carbohydrates in the cereals could be determinant (Bach Knudsen et al., 2012). In large intestine, indigestible carbohydrates are the main fermentation substrates for bacteria, and SCFA the final metabolites. Although they were produced in the large intestine, SCFA could also promote cell proliferation and growth of the small intestine (Montagne et al., 2003). For example, arabinoxylan and β-glucans are some of these non-digestible carbohydrates from cereals that could promote, in appropriate doses, these effects. Its presence in the different kind of cereals is variable being present for example in oats in a high percentage (Bach Knudsen et al., 1993 a; Wilczak et al., 2015). Moreover, soluble polysaccharides could also increase the viscosity in the intestinal digesta (Mudgil and Barak, 2003) and promote changes. Higher viscosity in weaning piglet intestine induced by the addition of carboxymethylcellulose has been demonstrated to increase the total number of ileal goblet cells (Piel et al., 2005).

It is worthy also to mention that indigestible lectins from legumes, cereals and some other plants could also cause intestinal damages (Zárate et al., 2017). In the study conducted by de Oliveira et al. (1988), purified lectins from kidney bean leaded to intestinal hypertrophy and hyperplasia in rats. Results from Sun et al. (2008) confirmed that soybean β-conglycinin in both 1% and 3% levels caused intestinal morphology damage. However most of the antinutritional factors from legumes can be inactivate by thermal processing. In this regard more than 80% of the antinutritional protein in soya bean meal can be abolished by dry heating (Yin et al., 2011). Supplementing piglets with heated pigeon pea seed meal did not cause significant difference in term of villus height, cell area and cell mitosis compared to the control group, but piglets in raw pigeon pea seed meal group without removing the anti-nutritional factors showed lower villus height, cell area and cell mitosis (Mekbungwan and Yamauchi, 2004).

2.2.2. IMPROVEMENTS IN THE IMMUNE LOCAL RESPONSE

Local immune system is an important part of the immune response when fighting intestinal pathogen infections (Mowat, 2003). The mucosa-associated lymphoid tissues lining the gut are known as gut-associated lymphoid tissue or GALT (Garside et al., 2004). An extremely important site for the induction of immune responses in the small intestine are lymphoid

follicles-Peyer's patch and leukocytes in epithelium and lamina propria (Jung et al., 2010; Mowat, 2003). It has been described in the literature the potential of different ingredients to modulate this mucosal immune organ by different ways.

Regarding cereal ingredients, β-glucan is one of the most studies polysaccharides with bioactive properties. Barley β-glucan upregulate the expression of surface molecule CD86 in dendritic cells (Wismar et al., 2011). Combinations of barley β-glucans and probiotics have been described to modulate the expression of immune genes of human THP-1 cells macrophages, and differences of these immune-regulating functions were found between oat (1-3, 1-4)-β-glucan and barley (1-3)-β-glucan (Arena et al., 2016). In rat enteritis model, oat β-glucans with high and low molecular weights significantly reduced the increased number of total lymphocytes T, B and granulocytes induced by LPS (Suchecka et al., 2015).

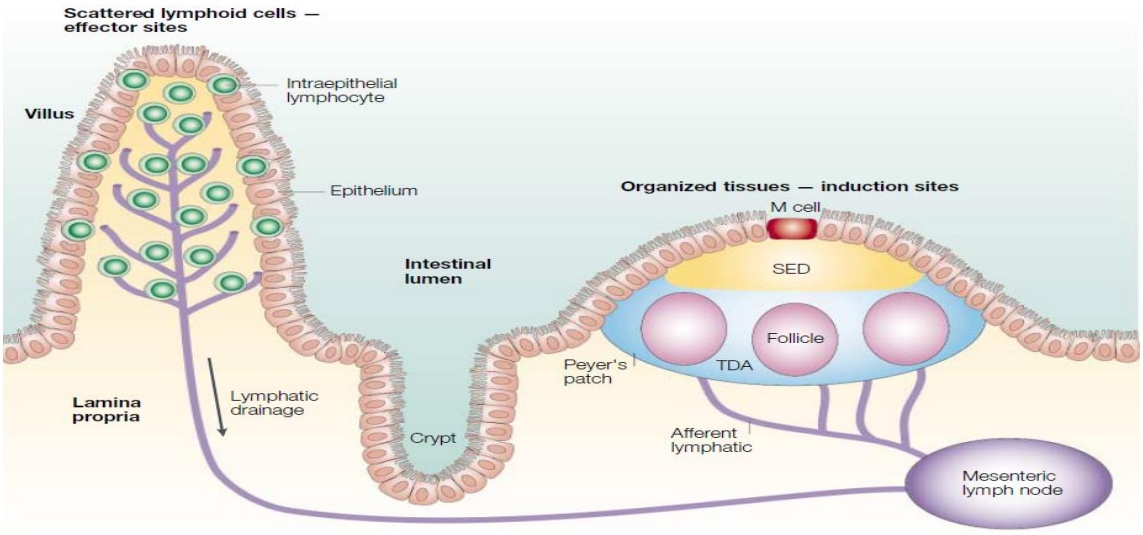


Figure 2.3. Schematic representation of the lymphoid elements of the intestinal immune system (Mowat, 2003).

The organized tissues of the Peyer's patches and mesenteric lymph nodes (MLNs) are involved in the induction of immunity and tolerance, whereas the effector sites are scattered throughout the lamina propria and epithelium of the mucosa. Both the Peyer's patches and villus lamina propria are drained by afferent lymphatics that go to the MLNs. SED, subepithelial dome; TDA, thymus-dependent area (cited from Mowat, 2003).

Some β-glucans–connecting receptors have been found on the membrane of immune system cells (Suchecka et al., 2017). Dectin-1 is one of these receptors, which could recognize β-glucans of many fungal species (Shinkai et al., 2016). Sonck et al. (2009) found dectin-1 expressed in the porcine small intestine, colon, rectum and the mesenterial lymph nodes. Moreover Tada et al. (2009) demonstrated that barley β-glucans could activate NF-κB in 293T cells when dectin-1 was transfected into the cells together with Syk, CARD9 and Bcl 10. Oat β-glucan after endo-glucanase treatment showed more activation of the Dectin-1 receptors in human dendritic cells. The endo-glucanase treatment may change the size and molecular weight of β-glucan, and help to expose the β - (1-3) linkages. Authors regarded these changes as one of the main reasons for the enhancement of immune activity (Sahasrabudhe et al., 2016a).

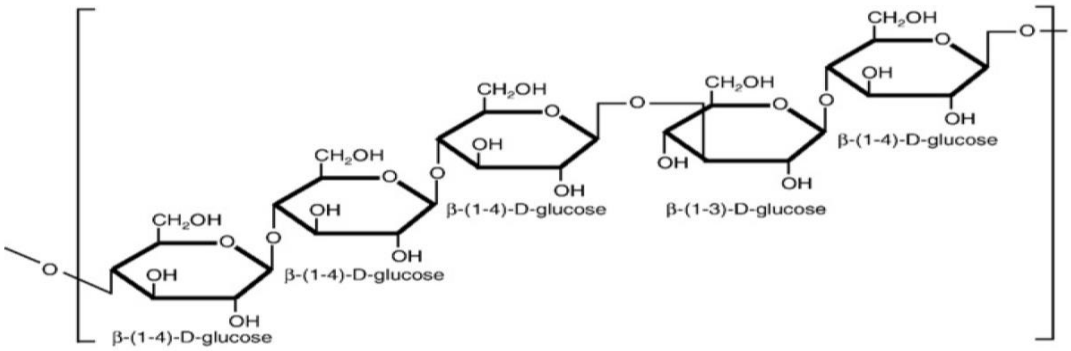


Figure 2.4. The Structure of Cereal glucan

Polymer of β-(1-4)-D-glycopyranosyl units separated by single β-(1-3)-D-glycopyranosyl units (cited from Volman et al., 2008).

Structure, molecular weight and compositional characteristic of cereal β-glucans are also probably involved into the modulation of inflammation (Wismar et al., 2010). In the study conducted by Rieder et al. (2011), cereal β-glucan with lower average molecular weight significantly stimulated the expression of IL-8 in HT-29 cells, however, the higher molecular weight β-glucan did not exhibited this effect. Samuelsen et al. (2011) verified a similar result of the higher molecular weight β-glucan from barley. Chanput et al. (2012) also found β-glucans from oat, barley and shiitake stimulated expressions of inflammation-related gene

IL-1β, IL-8, IL-10 and NF-κB in human monocytic leukemia cell line THP- 1 by different ways. These cereal crude β-glucans, and their commercial forms, also showed similar activations, but with different fold incubation values. Sahasrabudhe et al. (2016b) explained the mechanism why particulate β-glucan enhance the immune activity more than the soluble from. Particulate β-glucan could activate both Dectin-1A-TLR4 and Dectin-1B-TLR4 receptor cell lines, but the stimulation of soluble β-glucan did not appear in the Dectin-1B-TLR4 receptor cell lines.

Other ingredients such as soybean could had adverse effects on the intestinal immune response. Lectins from soybean could connect with polysaccharide chains of receptors on the intestinal membrane (Kik et al., 1989) and induce different kind of responses. Brown and Williams. (1982) found *in vitro* that soybean lectin bond to leucocyte-common antigens from B lymphocytes, not T lymphocytes. Results from a study of Dunsford et al. (1989) with animal models, exhibited that diets with high concentrations of soybean meal leaded to greater lamina propria depth in post weaning pigs. Antigenic materials in soybean seems to be the main factors being response for the detrimental results. Compared with non-antigenic soybean products, antigenic soybean diets substantially increased eosinophil density in the duodenal mucosa of early-weaned pigs (Dréau et al., 1994). In another study, heat-treated soybean proteins stimulated both B and T lymphocytes in the piglet duodenal mucosa more efficiently than ethanol-treated soybean proteins (Dréau et al., 1995). The damage of duodenal villi caused by heat-treated soyabean proteins may be the manifestation of these immune reactions.

Cereals and legumes contain polyphenols (Salunkhe et al., 1983; Fardet, 2010). Most of these polyphenols are in the coat, bran or germ, and belong to the flavonoid and tannin groups (Salunkhe et al., 1983). Although their contents is not as high as in fibrous ingredients, research findings illustrate that these polyphenols can be also considered as important bioactive compounds with an immune modulation effect.

Diet containing polyphenol-rich cereals improved the expressions of IL-2 and TNFα of the leukocyte from mouse when these leukocyte were separately incubated with Con A and LPS (Álvarez et al., 2006). Hole et al. (2009; 2012) found that NF-$_K$B activity in monocyte cell line U937-3xkB-LUC induced by LPS were differently affected by the phenolic extracts of

barley, oat, wheat and buckwheat. Polyphenols binding to fiber in whole-grain wheat were more than in refined wheat, and the consumption of whole-grain wheat was demonstrated to inhibit the expressions of inflammatory TNF-α and IL-6 while stimulated the production of anti-inflammatory IL-10 (Vitaglione et al., 2015). Bound phenolic acids accompanying the fibrous materials are mostly digested in colon, but not in stomach or small intestine (Shao and Bao, 2015). Therefore these kinds of polyphenols are supposed to exert anti-cancer and anti-inflammatory effects mostly in colon. In addition, isoflavone fraction from soybean has been demonstrated to alleviate the IL-8 secretion induced by TNFα in Caco-2 cells (Satsu et al., 2009). Results from the study of Kanamoto et al. (2011) also verified that polyphenol-rich extract of black soybean seed suppress inflammation in mice, as the gene expressions of TNFα and monocyte chemoattractant protein-1 in whited adipose tissue were down regulated. Recently, polyphenols from grapes also have attracted researcher attention (Brenes et al., 2016). Intakes of grape by-products could prevent intestinal inflammatory processes and reduce *E. coli* induced diarrhoea in weaned piglet models (Gessner et al., 2013; Fiesel et al., 2014; Verhelst et al., 2014).

Milk powder is also important ingredient for the post weaning piglets. A variety of carbohydrates, proteins, fat as well as bioactive components could be found in milk with ability to modulate directly or indirectly the intestinal immunity.

In human, bovine and goat milk, there has been describe different kind of oligosaccharides (Lara-Villoslada et al., 2006), with reported anti-inflammation or proinflammation properties. For examples, human milk oligosaccharide 2′-fucosyllactose reduced the inflammation in T84 and H4 IECs induced by invasions of *E. coli* stains by inhibiting CD14 expression (He et al., 2014). Kurakevich et al. (2013) using gene knockdown mice revealed that milk oligosaccharide sialyl (α2, 3) lactose directly stimulated MLN CD11c+ dendritic cells via Toll-like receptor 4 signaling. Moreover, human milk oligosaccharides effectively increased the basophils in PBMC and mesenteric lymph node (MLN), NK cells in PBMC, memory effector T cells in MLN than probiotic in neonatal piglets (Comstock et al., 2016). Lane et al. (2013) comparing immune responses in HT-29 cells induced by human milk oligosaccharides and bovine milk oligosaccharides found that oligosaccharides modulated the immune systems genes in similar patterns. Additionally, goat milk oligosaccharides attenuated the colonic inflammation induced by dextran sodium sulfate (DSS) in rats, and

maintained expressions of mucin genes down-regulated by DSS (Lara-Villoslada et al., 2006).

Regarding dairy proteins, excellent papers have reviewed some peptides like lactoferrin in the developments of intestine immunity (Baldi et al., 2005; Donovan, 2016). The modulation effects of milk peptides were in both directions: induction and suppression (Politis and Chronopoulou, 2008). As known, some toll-like receptor signaling pathways could be involved in immune modulations recognizing glycoproteins, peptides as well as oligosaccharides (He et al., 2016). Immune responses stimulated by these ingredients in milk could help hosts to prevent pathogen invasions (Walker et al., 2015).

2.2.3. INGREDIENTS AND ANTI-ADHESIVE ABILITIES

As it has been described above, in many cases the infections of some pathogens were firstly related with the connections between fimbriae and their receptors in the intestinal epithelium surface. Providing suitable agents to inhibit or reduce these connections is recognized as a natural method to prevent or treat microbial disease (Sharon, 2006). Ingredients, such as milk, plants or microbial products, has been described as important sources of these agents (Lane et al., 2010).

Carbohydrates, proteins and fats from milk have been verified by many authors as anti-adhesive analogues in terms of the prevention of pathogen attachment. Among them, the most highlighted bioactive components have been certain soluble oligosaccharides or glycoconjugates, especially from human milk (Bode, 2009; Hickey, 2012). Coppa et al. (2006) evidenced how oligosaccharides from human milk reduced the adhesion of diarrheogenic bacteria (*E. coli*, *Vibrio cholera*, *Salmonella fyris*) to Caco-2 cells. Caseinoglycomacropeptide (CGMP) derived from κ-casein also reduced the adhesions of verotoxigenic *Escherichia coli*, enteropathogenic *Escherichia coli, Lactobacillus pentosus*, *L. casei* and *L. acidophilus* to human HT29 cells. However the anti-adhesive ability of CGMP was also strain-depend, as adhesions of some other strains were not affected (Rhoades et al., 2005). In the tests with ETEC K88, CGMP also reduced the attachment of this bacteria to piglet mucosa *in vitro* and *in vivo* (Hermes et al., 2013), and also intestinal epithelial cells

(Gonzalez-Ortiz et al., 2013). The terminal *N*-acetylneur-aminic acid residues of CGMP probably bind with these pathogens. In the illustration by Ofek et al. (2003), these acid residues acted as the receptors of bacteria such as enterotoxigenic and enteroaggregative *E. coli* strains.

There is a summary in the following table regarding the studies about anti-adhesive properties of milk components in recent years.

In animal models, many plants or their extracts also exhibit beneficial effects in the reduction of diarrhoea and some of these effects could be related to the interference with pathogen attachment to epithelial cells. Palla et al. (2015) found in a diarrhoea model in mice that the flaxseed extract was able to reduce diarrhoeal scores. Also in mice model Leódido et al. (2017), found that feeding sulphated polysaccharide fraction from *Gracilaria intermedia* seaweed, diminished *E. coli* promoted diarrhoea. Moreover supplementing sows with seaweed extracts also reduced caecal *E. coli* numbers in the progenie 9 d post-weaning (Leonard et al., 2011). The β-glucans isolated from *Laminaria digitate* and *L. hyperborean* seaweeds reduced Enterobacteriaceae numbers in the piglet ileum and colon as yeast β-glucans from *Saccharomyces cerevisiae*, without affect numbers of *Lactobacilli* or *Bifidobacteria* (Sweeney et al., 2012). In piglets challenged with ETEC K88, intake of wheat bran diet reduced the number of *E.coli* attached to the ileal mucosa (Molist et al., 2010).

Many factors probably contribute to these results in animal models. Different authors have described anti-adhesive properties for the water soluble extracts of different vegetable ingredients. In the studies of Gonzalez-Ortiz et al. (2013; 2014), wheat bran competitively reduced the attachments of ETEC K88 to piglet mucus or IPEC-J2 cells. Soybean extracts blocked the adhesions of *E. coli*, *S. aureus*, *L. monocytogenes* and *C. sakazakii* to Caco-2 cells (Zhao and Shah, 2015). Tempe extracts of soybeans also prevented the adhesion of ETEC to intestinal cells (Roubos-Van Den Hil et al., 2010) and certain carbohydrates were deduced as the bioactive component. This hypothesis is not unreasonable or groundless. Wheat bran, some common cereal (wheat, oat, barley and rye), soybean and rapeseed are rich in soluble non-starch polysaccharides within their fiber fraction (Knudsen, 2014).

Table 2.2. Examples of milk compounds in the preventions of bacterial attachment.

Compounds	Sources	Tested tissue	Targeted bacteria	Reference
Defatted milk fat globule membrane	bovine milk	HT-29 cells	*E. coli* O157:H7	Ross et al ., 2016
Glycomacropeptide	cheese-making	HT-29 and Caco-2 cells	EHEC 12900, EPEC 0111:H2, EPEC 0125:H32	Feeney et al., 2017
Milk fat globules	raw milk and raw milk cheese	HT-29 and Caco-2 cells, mice	Enterohemorrhagic *E. coli*	Douëllou et al., 2018
Milk fat globule membrane	bovine milk	Caco-2 TC7	*Lactobacillus rhamnosus* GG	Guerin et al., 2018
Glycosylated components	Commercial dairy powders and raw buttermilk	Caco-2 cells	*Cronobacter sakazakii*	Ripollés et al., 2017
Lactoferrin	human milk	Caco-2 cells	*E. coli O157:H7, Salmonella, enterica ssp enterica heidelberg*	Barboza et al., 2012
Glycan	human milk lactoferrin	Caco-2 cells	*S. heidelberg*, *S.* Typhimurium, *S. enteritidis, L. monocytogenes*	Barboza et al., 2012
Oligosaccharides	Human milk	pIECs	*Candida albicans*	Gonia et al.,2015
Glycosaminoglycans	human milk	human intestinal cells	*E. coli, Salmonella fyris*	Coppa et al., 2016
N-glycome	Human and bovine milk	Caco-2 cells	*Salmonella* Typhimurium, *Shigella sonnei*, *S. aureus* *E.coli* O157, *Listeria monocytogenes*	Wang et al., 2017
2'-fucosyllactose and 6'-sialyllactose	human milk	Caco-2 cells	*E. coli*	Facinelli et al., 2018

Polyphenols from plants could also been involved. Actually polyphenols are one of the most well understood components with anti-adhesive attributes in the preventions of bacterial infections. Tannins isolated from cranberries has been demonstrated to have anti-adhesive activity preventing the infections of P-fimbriated *E. coli* to uroepithelial cells (Howell et al., 1998, De Llano et al., 2015). In the prevention of bacterial colonization of intestine, anti-adhesive abilities of polyphenols also have been described. Extracts from *alpinia katsumadai* with a high phenolic content reduced the adhesion of *Campylobacter jejuni* to pig small-intestine cell lines (Pogačar et al., 2011). Cranberry extract with 41% proanthocyanidins *in vivo* reduced the attachments of F4 and $F18^+$ *E. coli* to pig intestinal epithelium, and piglets challenged with $F18^+$ *E. coli* had less excretion and diarrhoea when they were supplied with this extract in feed (10 g/kg) and drinking water (1g/L) (Coddens et al., 2017). In addition, Verhelst et al. (2014) found two plant derived polyphenol extracts reducing diarrhoea caused by ETEC infection in piglets, but this result was highly related with bluntness of heat labile toxin by polyphenol extracts.

However anti-adhesive properties of some ingredients could be not only specific for pathogenic but they could also interfere with the adhesion of some beneficial bacteria. For instance, additions of barley β-glucans or barley flour has been shown to inhibit *in vitro* the attachment of some *Lactobacillus* strains to Caco-2 cells (Arena et al., 2014). Volštátová et al. (2015) also found that acid-hydrolyzed milk could reduce more than 50% the attachment of *Lactobacillus plantarum* S2 and *L.gasseri* R to HT29-MTX cells. Less intestinal *B.bifidum* 2 attachment to Caco-2 / HT29-MTX cells was also reported in the presence of human milk oligosaccharides (Musilova et al. 2017). Cellobiose, stachyose, lactulose and chitooligosaccharides also have been shown to diminish bifidobacteria and lactobacilli adhesion to HT-29 cells. As stated above, these ingredients or their matrix probably bond to adhesins or their receptors on the enterocytes. Negative effects of diet ingredients on the probiotic colonization should be also therefore considered when looking for food strategies able to promote intestinal health.

Table 2.3. Examples of plant derived extracts in the preventions of bacterial attachment.

Compounds	Source	Tested tissue	Targeted bacteria	Reference
arabinogalactan	Ribes nigrum seeds	Human gastric cells	*Helicobacter pylori*	Messing et al., 2014
Extracts	Rosemary water	HEp-2 cells	*Bacillus Pseudomonas*	Elhariry et al ., 2014
SNP	Plantain	Porcine epithelial cell-line, primary chick caecal crypts	*Salmonella* Typhimurium	Parsons et al., 2014
Extracts /residue	thyme, olive leaf / thyme post-hydrodistillation	PSI cl1 cells	*C. jejuni*	Šikić Pogačar et al., 2016
Salvianolic acid B	*Salviae miltiorrhizae*	HEC-1B cells	*Neisseria meningitidis*	Huttunen et al., 2016
Pectic oligosaccharide	Orange peel	HT-29 cells	*E. coli* O157:H7	Di et al., 2017
Pectin	kiwifruit	Caco-2 cells	*Salmonella* Typhimurium	Parkar et al., 2010
Xyloglucan-rich fraction	cranberry	human bladder epithelial cells/ HT29 cells	*E. coli* CFT073 and UTI89, *E. coli* O157:H7	Hotchkiss et al., 2015
Extracts /fractions	Cranberry juice	proanthocyanidins	*E.coli* B78	Gupta et al., 2016
A-type procyanidins metabolites (rat urine samples)	Cranberry	HT-29 cells	Uropathogenic *E. coli*	Peron et al., 2017

2.2.4. EFFECTS ON INTESTINAL MICROBIOTA

Another way by which particular components of feed ingredients could improve animal health, would be by modulating microbial community of the gastrointestinal tract. There would be two main ways for this modulation. First, by a prebiotic effect favoring the growth of beneficial bacteria, and consequently reducing potential pathogens, and secondly by direct antimicrobial effects preventing the growth of pathogenic bacteria or directly killing them.

Many dietary carbohydrates can resist the hydrolysis by endogenous enzymes in the upper small intestine. These indigested carbohydrates arriving to the proximal hindgut turn in growth substrate for mutualist bacteria that will finally produce short-chain fatty acids (SCFA) including mostly acetate, propionate and butyrate but also organic acids like formic or lactic acid in different percentages (Engfer et al., 2000; Macfarlane and Macfarlane, 2006). These fermentation products could inhibit the growth of certain potential pathogens, like certain strains of Enteropathogenic *E. coli* or *Salmonella* spp. that has been described to be sensitive to the lower pH environment favored by these SCFA (Bach Knudsen et al., 2012).

Prebiotic effects has been described for different vegetable ingredients. For example barley β-glucan has been describe to enhance the *in vitro* growth of probiotic *Lactobacillus* strains like *L. plantarum*, *L. acidophilus* or *L. fermentum* (Arena et al., 2014). In normal rats and LPS-promoted enteritis rats, both high and low molecular weight oat β-glucans increased the LAB numbers in faces (Wilczak et al., 2015). Barley also enriched the abundance of *Lactobacillus* in rat caecum accompanying a decrease of the *Bacteroides fragilis* group (Zhong et al., 2015). Bindelle et al. (2010), also described how β-glucans in cereals selectively enriched *in vitro* certain bacteria of pig feces. Soybean oligosaccharides also promoted the growth of bifidobacteria in the human faecal flora *in vitro* (Saito et al., 1992). However, not always prebiotic effects has been described. For example Pieper et al. (2008) found that less numbers of *Lactobacilli* could be counted in ileum of pigs fed high mixed-linked β-glucan and hulless barley diets, compared to the oat diets. This effect was not dependent on the amount of β-glucan indicating that probably other factors or components, such as the structures of the carbohydrates, may be involved.

Regarding potential prebiotic components in milk or milk derived ingredients, several complex oligosaccharides and proteins has been described to act as prebiotics promoting

growths of bifidobacterial species. Petschow and Talbott. (1990) found whey and casein fractions from human and bovine milk owned growth-promoting activities of *Bifidobacterium* species in infant faeces. High growth of *B. longum* biovar *infantis* strains has been described to be supported by human milk oligosaccharides (HMOs) (LoCascio et al., 2009). Human milk peptides also has shown *in vitro* to stimulate the growth of bifidobacterial strains (Liepke et al., 2002). Different human milk oligosaccharides have been described to have prebiotic properties. (Marcobal et al., 2010; LoCascio et al., 2007; Hunt et al., 2012). Further works are still needed to explore the underlying mechanisms.

But not only by prebiotic properties feed ingredients could modulate intestinal microbiota, also many phytochemicals, particularly proteins, saponins, polyphenols as well as carbohydrates, in cereals and milk also could have antibacterial activities. Encouraged results illustrated potential usages of natural ingredients as replacements of antibiotics.

Pu and Tang. (2017) showed that under treatments of certain enzymes, hydrolysis products from rice bran-protein could inhibit the growth and biofilm formation of *Listeria monocytogenes*. Protein hydrolysate from a Thailand rice (Sangyod Phatthalung) also exhibited bactericidal characters against *S. aureus*, *E. coli* and *Pesudomonas aeruginosa* (Ditsawannon et al., 2018). Moreover, defatted wheat germ specifically inhibited growths of *E. coli* and *S. enterica* (Mahmoud et al., 2015). Kim et al. (2010) discovered that wheat germ extracts rich on 2, 6-dimethoxy-1, 4-benzoquinone (DMBQ) had efficient antibacterial activities against *S. aureus* and *B. cereus*, and synthesized DMBQ also verified similar bactericidal effects. Saponins isolated from sorghum bicolor also inhibited growths of *S. aureus* (Soetan et al., 2006).

Polyphenol was also another commonly reported antibacterial compound. Polyphenols has been widely considered as prebiotics and antibiotic agents in preventing pathogen invasions (Hervert-Hernández and Goñi, 2011; Daglia, 2012). In cereal, flavonoids, tannins, and phenolic acids were in considerable amount (Kadiri et al., 2017). Phenolic acid released from oat bran after carbohydrases hydrolysis, prevented the growth of *E. coli* (Alrahmany et al., 2013). Phenolic extracts from barley varieties also prevented the growth of pathogenic *E. coli*, *S. aureus*, *Salmonella enterica* or *Micrococcus luteus* (Boubakri et al., 2017). In addition, soybean contains a variety of isoflavones and phenolic acids (Villalobos et al.,

2016), and natural phenolic extracts from defatted soybean flour had been shown to exhibit high antimicrobial activities against pathogens like *S. aureus*, *E. coli*, *L. monocytogenes* or *S. enterica*. Grape, cranberry, tea as well as other plant extracts were also important sources of bactericidal polyphenols (Leitão et al., 2005; Friedman, 2007; Cardona et al., 2013; Natarajan et al., 2017).

Many bactericidal ingredients can also be found in milk. Malkoski et al. (2001) found caseinomacropeptide from bovine milk preventing the growth of pathogenic *Streptococcus mutans*, *Porphyromonas gingivalis* and *E. coli*. Peptides produced after hydrolysis of bovine milk lactoferrin exhibited antibacterial activity against *L .monocytogenes* (Ripolles et al., 2015). But not only proteins also milk carbohydrate have showed antimicrobial activity. Some HMOs exhibited *in vitro* growth inhibition of *Acinetobacter baumannii*. The tests also showed antibiofilm activities against *S. agalactiae* and methicillin-resistant *S. aureus* strain (Ackerman et al., 2017). These milk bactericidal ingredients could help neonate preventing bacterial infections especially considering that the immune systems is not yet well established.

A third way by which dietary ingredients could also favor the establishment of beneficial bacteria in the intestine would be by favouring their attachment to intestinal epithelium and the colonization of the gastrointestinal tract. Volstatova et al. (2016) described that sheep milk significantly facilitated the adhesion of *Lactobacillus casei* FMP to co-culture Caco-2/HT29-MTX cell lines. Furthermore, adhesion-promoting activities of oligosaccharides have selective characters. According to the study of Kavanaugh et al. (2013), human milk 6' sialyllactose promoted the adhesion of *B. longum* biovar *infantis* ATCC 15697 to HT-29 cells, while 3' sialyllactose did not. More *B. longum* 15697 strains attached to HT-29 cells under the assist of the mixtures of these two oligosaccharides. Pro-adhesives properties could be due to a direct effect exert as anchors between receptors and microbial adhesins but also by indirect effects. In this regard Hedemann et al. (2005) verified in pig ileal tissue that pro-adhesives properties of some milk oligosaccharides could be caused by changes promoted in the secretions of mucins.

In summary, we have seen above how dietary ingredients, digested and absorbed in intestine, are not only providers of nutrients but they also could play a role in animal health. These

bioactive actions cover immune modulation, anti-adhesive properties, prebiotic activities and bactericidal functions. All these beneficial effects would be helpful for reducing pathogen overgrowth and preventing disease, contributing all together to boost host health. This is particularly relevant in a scenario where the risk of antibiotic resistance require new alternatives to their use that will be able to reinforce natural defences of the animal. In this regard the functionality exhibited by many feed ingredients would be of help in the reduction of antibiotics in livestock by their rational use in diet formulation.

2.3. WHAT ARE MICROBIAL EXOPOLYSACCHARIDES?

As stated at the beginning of this chapter, in this section we will review the potential of different complex molecules produced by a variety of microorganisms known as exopolysaccharides (EPS) as possible in-feed additives to be used in the piglet diets. As we will see in the following pages different properties has been attributed to this kind of compounds that turn this molecules in potential nutraceutics.

Microbes can synthesize diverse kind of polysaccharides with different biological functions according to their structure and architecture (Schmid et al., 2015; Delattre et al., 2016). One of these polysaccharides are known as exopolysaccharides (EPS) being extracellular bacterial polysaccharide, which are secreted to the immediate environment under normal physiological processes and harsh conditions (Badel et al., 2011; Schmid et al., 2015; Delattre et al., 2016). Depending on constituent monosaccharide types, EPSs are divided into homopolysaccharide (HoPS), which comprises a single monosaccharide type, and heteropolysaccharide (HePs) comprising different monosaccharides (Salazar et al., 2016). Polysaccharides are not the only components of EPSs. Some mount of proteins, glycoproteins, glycolipid and extracellular DNA also could be found with them (Flemming et al., 2007).

2.3.1. WHY SOME MICROORGANISMS PRODUCE EPS?

Both *Archebacteria* and *Eubacteria* (Grampositive and negative) can produce EPS (Badel et al., 2011). Microalgae also can secrete EPS, some of them with high yields (Flemming and Wingender, 2010). Surrounding the bacterial outside, EPS are usually considered as a protective cover guarding bacterial surfaces. Microbial EPSs also have the ability to bond with proteins and nucleic acids forming biofilms (Irie et al., 2012). Multitudinous bacteria in the biofilm could form dynamic systems and be seemed as multicellular organisms (Hall-Stoodle et al., 2004). In turn, biofilm can guard bacteria against hostile environments and antibiotic treatment. Research also found certain polysaccharides in these biofilm, not only as architecture ingredients, but also acting as a molecular signal (Badel et al., 2011).

2.3.2. HEALTH PROMOTING PROPERTIES OF MICROBIAL EPS

Multiple researches have demonstrated that EPSs synthesized by some probiotic strains are associated to a series of beneficial effects to their hosts. Most of these promoting attributes are related with intestinal health. In the following sections it will be reviewed some of these health promoting properties attributed to microbial EPS.

2.3.2.1. THE ROLE OF MICROBIAL EPS IN BIOFILMS

It is generally accepted that one of the main function of microbial EPS is to help bacteria to build biofilms. In this way bacteria can aggregate together around themselves and form biofilms (Cvitkovitch et al., 2003; López et al., 2010). Biofilms assist bacterial to resist antibiotic and unfavorable environments as more cells survival under the protection of the biofilm (López et al., 2010). Actually in human and veterinary medicine, biofilms has been considered as a relevant element in many bacterial infection (Cvitkovitch et al., 2003) and also has been associated to the resistance of some pathogens to antibiotic treatments (Stewart and William, 2001) or related to catheter-associated bacterial infections.

However despite their role in the building of microbial biofilms, it also has been described the capacity of many EPS to prevent and/or destroy the bacteria biofilm built by other microorganisms. In recent years, several works has been published demonstrating antibiofilm

properties of several bacterial EPS. In this regard EPS produced by *Lactobacillus* strains impaired the biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7 *in vitro*, nevertheless without statistically significant effect on the growth of ETEC bacteria (Kim et al., 2009). EPS produced by *Lactobacillus plantarum* inhibited biofilm forming of *S. aureus*, *K. pneumoniae* and *E. coli* (Pradeepa et al., 2016). Abid et al. (2017) also reported how EPS produced by *Leuconostoc citreum* and *L. mesenteroid,s* isolated from fermented bovine meat sausage and fermented turkey meat sausage *in vitro,* could inhibit and disturb biofilms of some pathogens like *E. coli*, *E. faecalis* or *S. aureus*. In another study, EPS produced by *L. pseudo-mesenteroides* isolated from cow milk showed similar anti-biofilm effects (Abid et al., 2018). Rosca et al. (2017) found that dextran EPS produced *in vitro* by *Weissella confusa* inhibited the biofilms formed by a *Candida albicans* strain.

From these results it can be concluded that the impact of microbial EPS on biofilms will depends on the microbial strain producing it and in the nature of the biofilm consortium in which it is produced. The scientific evidence at the moment make clear that in some cases they could had a relevant role reducing and destroying the biofilm produced by other microorganisms.

2.3.2.2. ANTIMICROBIAL PROPERTIES ASSOCIATED TO EPS PRODUCING BACTERIAL STRAINS

Results from many published works suggest that the production of EPS could be behind the antimicrobial properties described for some probiotic bacteria. In the study conducted by Li et al. (2014), EPSs produced by *Bifidobacterium bifidum* WBIN03 isolated from human feces, and *Lactobacillus plantarum* R315 from breast milk, inhibited the growths of *E. coli* O157:H7, *Salmonella* Typhimurium ATCC13311, *Bacillus cereus* ATCC14579, *Staphylococus aureus* CMCC26003 and *Cronobacter sakazakii* ATCC29544. In another study, sulphonation of EPS from *Streptococcus thermophilus* GST-6 could significantly improve its inhibitory ability against *E. coli*, *S.* Typhimurium and *Staphylococcus aureus* (Zhang et al., 2016). In the study conducted by Santos et al. (2003), an EPS-producing strain *Lactobacillus kefiranofaciens* CYC 10058 isolated from kefir prevented the growth of enteropathogenic bacteria, such as *E. coli*, *Lysteria monocytogenes*, *S.* Typhimurium, *Salmonella enteritidis, Salmonella flexneri* and *Yersinia enterocolitica.* Abushelaibi et al.

(2017) also reported most EPS-producing strains of *Streptococcus* and *Enterococcus* isolated from camel milk also showed antimicrobial against *E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*, or *S. aureus*. Neither EPS-negative strains showed any antimicrobial ability. However, one EPS-producing strain did not have antimicrobial activity against these four pathogens. Therefore, in term of antimicrobial abilities, although not all EPS or EPS-producing strains exhibited antimicrobial properties, for some of them there are clear evidences that could have a relevant role controlling the growth of other microorganisms.

2.3.2.3. ANTI-ADHESIVE ABILITIES

Pathogenic bacteria, such as *E. coli*, use their fimbriae to attach intestinal receptors as a first step to colonize the gut and cause disease (Dai et al., 2000; Jin and Zhao, 2000). Carbohydrates have been recognized as the main structures of these receptors for the bacteria recognition (Dai et al., 2000). Based on this mechanism, providing carbohydrate-related analogue molecules could be a competition-based strategy in the prevention of pathogen infections. From this point of view, EPSs secreted by microorganisms, as natural polymers of carbohydrates, also could be explored as anti-adhesive agents against pathogenic infections.

In the *in vitro* study conducted by Kšonžeková et al. (2016) two high MW D-glucan EPSs produced by *L. reuteri* DSM 17938 and L26, inhibited the adherence of ETEC to IPEC-1 cells. Results from other works also have evidenced that EPSs might be important factors in the anti-adhesive abilities of EPS-producing strains. Santos et al. (2003) found that an EPS-producing strain of *L. kefiranofaciens* CYC 10058 isolated from kefir reduced the attachment *of S.* Typhimurium to Caco-2 cells. In a recent study, *Lactobacillus plantarum* WLPL04, an EPS-producing LAB strain from human breast milk, reduced the attachments of *E. coli* O157:H7, *S.* Typhimurium ATCC 13311, and *Staphilococcus aureus* CMCC 26003 to Caco-2 cells (Jiang et al., 2016). A *Streptococcus thermophilus* strain producing EPS inhibited the attachment of *Salmonella* 657/7E to Caco-2 cells (Veljovic et al., 2017). Živković et al. (2016) also corroborated that EPS-SJ^{+}-producing strain *Lactobacillus paracasei* subsp. paracasei BGSJ2-83 impaired *E. coli* ATCC25922's interaction with Caco-2 cells, but the EPS-SJ^{-} strain did not. Moreover, in a mice model *Bifidobacterium breve* UCC2003 EPS^{+}

strain also prevented more *Citrobacter rodentium* to colonize the gut than EPS$^-$ *B. breve* strain (Fanning et al., 2012). In the study conducted by Yang et al. (2015 a) reuteran-containing diets (reuteran is a glucan) significantly impaired the colonization of ETEC to the gut of weaning piglets, although in another research reuteran did not alter the composition of gut microbiota (Yang et al., 2015b).

It is worthy to mention that anti-adhesive properties of EPS do not just affect pathogenic bacteria but also to other bacteria including potential probiotics. Ruas-Madiedo et al. (2006) found that EPS from fermented milk prevented the adherence of *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG to human intestinal mucus in a dose-dependent way, however it did not affect the adhesion of the pathogenic strains, such as *E. coli* NCTC 8603, *S*. Typhimurium ATCC 29631, even at a higher concentration of 1 mg/ml.

Although many studies evidenced that EPS-producing bacteria strains reduce the attachment of pathogenic bacteria only few of them have tested the antiadhesive properties of isolated EPS. It could be not discarded therefore that other mechanisms or compounds from the bacteria could also be involved in this interference. In this regard, according to the same study of Veljovic et al. (2017) a *Lactobacillus heveticus* strain also reduced the bacteria attachment to Caco-2 cells, but in this case this strain did not produce EPS.

2.3.2.4. PROMOTION OF GUT COLONIZATION BY PROBIOTIC BACTERIA

The attachment to the intestinal surface could help a probiotic to colonize the gut, establish and proliferate, avoiding being swept away by the digesta flow. Many factors have been supposed to be involved in probiotic adhesions (Ouwehand et al., 2003). Between them EPS, as materials surrounding the outside of probiotic bacteria, could have a relevant role.

De Palencia et al. (2009) found that the strain *Pediococcus parvulus* 2.6 from natural ciders producing 2-substituted (1, 3)-β-D-glucan had a higher adhesion to Caco-2 cells than its EPS-isogenic strain 2.8NR. In a further research (Garai-Ibabe et al., 2010), another *P. parvulus* CUPV22 also producing EPS also showed a strong adhesion ability to Caco-2 cells. When surface EPS of these bacteria were removed, by washing them, it was diminished the number of attached bacteria up to levels similar to those of an EPS-isogenic strain 2.8NR. In the study

conducted by Živković et al. (2016) EPS-SJ^{+}-producing strain *Lactobacillus paracasei* subsp. paracasei BGSJ2-83 showed a higher ability of attachment to Caco-2 cells than the EPS-SJ^{-} strain. Two *Lactobacillus rhamnous* KL37B and KL37C strains producing EPS isolated from newborn stool also showed a high attaching ability to Caco-2 cells (Górska-Frczek et al., 2011). These adhesive properties of EPS could be related to their physical properties than in many cases could increase viscosity of digesta facilitating the colonization of probiotic bacteria (Duboc and Mollet, 2001).

However, not all EPSs produced by probiotics means help for the bacteria colonization. In the research from Burns et al. (2011), a low EPS-producing strain of *Lactobacillus delbrueckii subsp. lactis* (193) attached more to HT29-MTX than the higher EPS-producing strain (193+). Compared to non-ropy derivative strains, a big-size EPS-producing *Lactobacillus paraplantarum* BGCG11 strain inhibited its attachment to epithelial intestinal cell lines (Nikolic et al., 2012). Mercan et al. (2015) found *in vitro,* with chicken gut explants, lower adhesion abilities for a high EPS-producing *L. salivarius* strains compared to the low EPS-producing counterpart. However, the conclusion of a negative relationship between EPS production and the adhesive abilities of strains could not be deduced just from these results.

From the review above we have seen that microbial EPSs can show opposite effects on the probiotic colonization depending on the probiotic strain. The mechanism of EPS interfering with bacterial adhesion probably is more complex than thought. Together to EPSs, other bacterial surface molecules, such as specific proteins, also could be involved in the probiotic adherence to the cells (Sarkar and Mandal, 2016). It could be supposed that adhesin factors probably work together or mutually with EPS in constructing biofilms and modulating adhesion. More work are still needed to explain the mechanism under the phenomenon.

2.3.2.5. EPS AS A FERMENTATION SUBSTRATE AND A POTENTIAL PREBIOTIC

Microorganisms in the large intestine can ferment carbohydrate substrates. As the major productions of this fermentation, SCFA are important energy sources for colonocytes and have been demonstrated to have beneficial effects for the gut and other organs (Cummings and MacFarlane, 1997). Many studies have reported the potentiality of microbial EPSs as fermentation substrates for the intestinal microbiota promoting the SCFA production and

modulating the intestinal microbiota. From this point of view EPS could be therefore also considered as potential prebiotics.

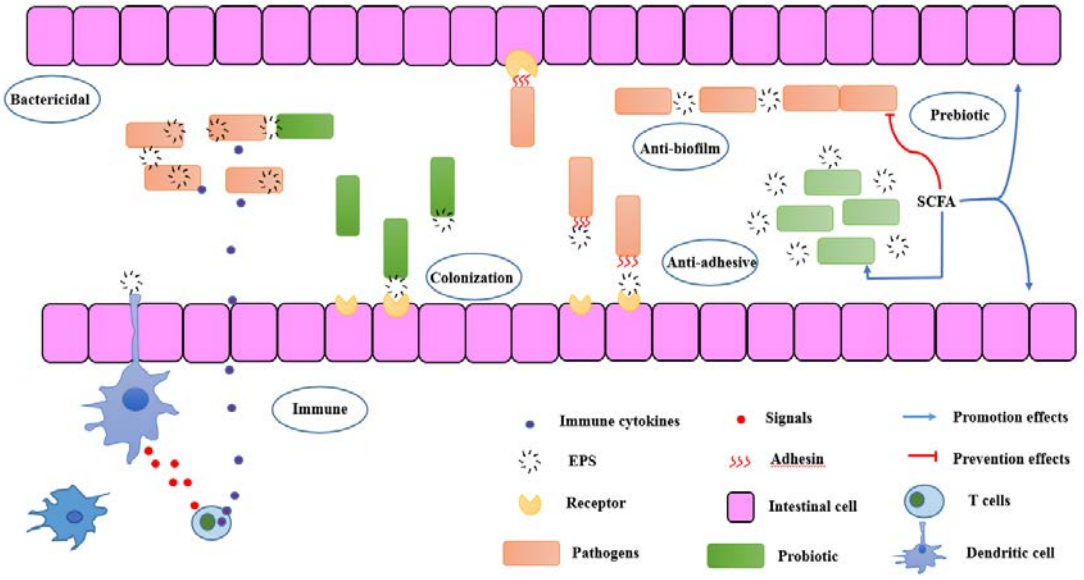


Figure 2.5. Functions of EPS in term of intestinal healthy.

Purified EPS isolated from the fungus Fomitopsis castaneus Imaz has been desmonstrated to enhanced SCFA production in *in vitro* (Guo and Chi, 2017). Zhang et al. (2017) reported that mice fed EPS produced by a *Lactobacillus plantarum* YW11 strain showed more than two fold increase in their SCFA concentration in faeces. In another research with western diet, Lindström et al. (2012) tested four commercial EPSs: scleroglucan, xanthan, gellan and native dextran in LDL receptor knock-out mice. Results showed that scleroglucan, xanthan and dextran increased caecal SCFA contents significantly. These results suggests that intestinal bacteria could be using selectively EPSs as fermentable substances.

In this regard *in vitro* studies demonstrated that levan-type EPS produced by *L sanfranciscensis* was able to stimulate the growth of *Bifidobacteria* and brought a growth trend of *Lactobacilli* (Bello et al., 2001). In mice models, kefiran, an EPS from kefir, significantly increased the percentage of *Bifidobacteria* in faecal samples at the 21st day after

the administration, but there was no significant difference on the number of *Lactobacillus* (Hamet et al., 2016). Furthermore, Mårtensson et al. (2005) observed in an study with human voluntaries how an oat-based fermented product with an EPS-producing strain (*Pediococcus damnosus* 2.6) significantly increased total bacterial and *Bifidobacterium* ssp counts in faces compared to the standard oat-based fermented product. From these results seemed that the microbial glucan produced by the strain *P. damnosus* 2.6 was acting as an effective prebiotic.

The prebiotic effect also could rely on the doses of EPS administered. The study conducted by Li et al. (2014) found that EPS from *Bifidobacterium bifidum* WBIN03 promoted changes in the intestinal microbiota of mice depending on the dose given. Whereas *Bacteroidales* sp. / *Lactobacillus* sp. were promoted in mice taking a low-dose of EPS, *L. johnsonii, L. animalis* and *L. reuteri* were the main intestinal strains in the high-dose treatment.

Despite promising prebiotic properties reported for the microbial EPSs, negative effects on the microbiota balance have also been found. In a test with mice, intake of purified EPS from the *P. parvulus* 2.6 strain leaded to lower cecal microbial diversity and a decreased *Bifidobacterial* population (Lindström et al., 2013).

As review above, results of EPSs as potential prebiotics are variable. Endogenous or exogenous factors, such as molecular structures, methods of EPS production or limitation of *in vivo/in vitro* models could explain part of this variability and should be considered for future researches.

2.3.2.6. ANTIOXIDANT PROPERTIES

During the cellular metabolism, reactive oxygen species (ROS) are constantly generated (Birben et al., 2012; Amir Aslani and Ghobadi, 2016). In the normal physiologic condition, excess ROS can be scavenged by antioxidant defences (Halliwell. 1991). When the balance of ROS and antioxidant defences is broken, more ROS can damage all cellular organism and cause serious diseases to human (Halliwell. 1991; Duarte and Lunec, 2005). Therefore, antioxidants play an essential role in the organism and are vital ingredients promoting health (Benbrook. 2005).

Some studies have described EPS-producing bacteria or isolated microbial EPS to have antioxidant properties. Camel cheese cultured with EPS-producing strain (*Lactobacillus*

plantarum) showed higher antioxidant activities during the storage (AI-Dhaheri et al., 2017). According to the study conducted by Luang-In and Deeseenthum. (2016) with 24 EPS-producing strains of *Bacillus* spp. isolated from milk kefir in Thailand, they demonstrated, in 9 of these strains, a decline in their antioxidant activities when EPS were destroyed or removed. In an "in vivo" study using a colitis model in rats, it was demonstrated a higher antioxidant enzyme activity after the administration of two EPS-producing *L.delbrueckii subsp. bulgaricus* strains compared to the control group. Moreover, between strains, it was found a higher level of antioxidant activity for the high EPS-producing strain compared to the low EPS-producing strain. From these studies, it seemed that EPS would be the main responsible for the antioxidants properties of the probiotic strains.

Some other research directly verified EPS as the antioxidants. Tang et al. (2017) reported three EPSs isolated from fermented milk of *Lactobacillus delbrueckii* spp.*bulgaricus* SRFM-1, mainly composed of galactose and glucose, to have good antioxidant properties. Moreover some EPS produced from bacteria isolated from human also have showed antioxidant property. In this regard, Li et al. (2014) observed how EPSs produced by *Bifidobacterium bifidum* WBIN03, from human feces, and *Lactobacillus plantarum* R315, from breast milk, had strong antioxidant activities. According to the hypothesis of enhancing intestinal barrier function by these antioxidant properties (Albillos et al., 2002), these EPS-producing bacteria, with antioxidant activities, were proposed to be selected to improve intestinal health.

2.3.2.7. EPS AND IMMUNE RESPONSE

Intestinal cells are able to recognize different microbial structures by a set of particular receptors like those know as patter recognition receptors (PRRs) (Peterson and Artis, 2014). These receptors play a crucial role in the proper function of the immune system (Suchecka et al., 2017) and many of them are able to recognize carbohydrates or more complex glycomolecules (Jin and Zhao, 2000). It could be expected therefore that microbial EPS could exert certain modulation of the immune system and recent research verify that some EPS are able to modulate the immunity response.

Jiang et al. (2016) describe how an EPS-producing LAB strain from human breast milk (*Lactobacillus plantarum* WLPL04) was able to impair the mRNA expression level of interleukin 6 (IL-6), IL-8 and TNF-α induced by *Salmonella* on Caco-2 cells. Similar results were also observed in another study conducted by Kšonžeková et al. (2016). Two high MW D-glucan EPSs produced by *L. reuteri* DSM 17938 and L26 inhibited the gene expressions of proinflammatory cytokine IL-1β and IL-6 induced by ETEC infection. According to another study, human peripheral blood mononuclear cells incubated with *Propionibacterium freudenreichii* producing surface β-glucan EPS, secreted less anti-inflammatory IL-10 cytokine than cells incubated with knockout mutants (Deutsch et al., 2012).

Some results from animal models also described the immune modulation of EPS. Providing mice with EPS produced by *L. fermentum* reduced IL-6 and enhanced s-IgA in the small intestine and also helped to prevent *Salmonella* infection (Ale et al., 2016). Another study also tested EPS produced by *Lactobacillus kefiranofaciens* with mice (Vinderola et al., 2006). More IgA+ cells appeared in both small and large intestine lamina propria of mice fed EPS. The number of IgG+ cells also increased in the large intestine lamina propria, but not in the small intestine. Expressions of IL-4, IL-6, IL10 and TNF also increased during seven days in the large intestine, but most of them did not changed in the small intestine with exception for IL-6 at the fifth and seventh days, and IL-10 at the fifth day. It seemed that gut mucosal responded differently between small and large intestine.

Other studies also have found modulation of splenocyte response *in vitro*. EPSs from *Lactobacillus rhamnosus* KF5 significantly stimulated *in vitro* splenocyte proliferation (Shao et al., 2014) and in the work of Fanning et al (2012), *Bifidobacterium breve* UCC2003 EPS^+ strain also modulate splenocyte response to produce less proinflammatory cytokines IFN-ϒ, TNF-α and IL-12 compared with EPS^- strains. Regarding *in vivo* response, less number of B-cells were found in mice spleens treated with the EPS^+ strain, and the evasion of B-cell was beneficial for the long-term colonization of the probiotic strain. In another research with mice, cell wall components from *Bifidobacteria* stimulated splenocytes proliferation, cytokines, TNF-ϒ and IL-10 productions. However, EPS from these *Bifidobacteria* did not have these characters (Amrouche et al., 2006).

As reviewed above, some EPS or EPS-producing strains has been demonstrated to have immunomodulatory properties and this field of research is attracting attention in the recent times. Results however could had been mediated, not only by a direct effect of EPS on intestinal receptors, but also indirectly by prebiotic changes in other microbial populations (*in vivo* studies) or by other microbial components in the ropy strains (*in vitro* studies). More research is needed to verify their specific role in the immune modulation.

Summarizing in this chapter, we gave a simple description of some potential health promoting properties of microbial EPS, especially on the intestine. EPSs can be really complex in nature and diverge in their structure and functions. This could the reason for so many potential functions attributed to these compounds. As reviewed, EPS excreted by bacteria showed antimicrobial, anti-biofilm and antioxidant properties. They also could connect with cellular surface receptors, interfering with bacteria adhesins, probiotic colonization and modulating immune response. They could be also important as prebiotics beings selectively fermented in the intestine. But not only these beneficial effects has been attributed to EPS, they also has been demonstrated to have a role in DNA repair (Morifuji et al., 2017), to contribute to prevent virus infection (Nagai et al., 2011), cancer (Purohit et al., 2009), to lowering cholesterol levels (Miranda-Nantes et al., 2011; Ghoneim et al., 2016) or help to reduce poisoning heavy metals (Feng et al., 2012). Particular chemical structure, molecular weights of EPS and doses may be the essential factors for these bioactivities. Although in most of studies mechanisms under biofunctions were not clearly illuminated, the achievements pointed out the possibility of EPS as functional ingredients in food/feed production.

2.4. REFERENCES

Abid, Y., Casillo, A., Gharsallah, H., Joulak, I., Lanzetta, R., Corsaro, M. M., Attia, H., Azabou, S., 2018. Production and structural characterization of exopolysaccharides from newly isolated probiotic lactic acid bacteria. Int. J. Biol. Macromol., 108, 719-728.

Abushelaibi, A., Al-Mahadin, S., El-Tarabily, K., Shah, N. P., Ayyash, M., 2017. Characterization of potential probiotic lactic acid bacteria isolated from camel milk. LWT-Food Science and Technology, 79, 316-325.

Ackerman, D. L., Craft, K. M., Doster, R. S., Weitkamp, J. H., Aronoff, D. M., Gaddy, J. A., Townsend, S. D., 2017. Antimicrobial and Antibiofilm Activity of Human Milk Oligosaccharides against *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter baumannii*. ACS Infect Dis, 4, 315-324.

Albillos, A., de la Hera, A., 2002. Multifactorial gut barrier failure in cirrhosis and bacterial translocation: working out the role of probiotics and antioxidants. Journal of hepatology, 37, 523-526.

Al-Dhaheri, A. S., Al-Hemeiri, R., Kizhakkayil, J., Al-Nabulsi, A., Abushelaibi, A., Shah, N. P., Ayyash, M., 2017. Health-promoting benefits of low-fat akawi cheese made by exopolysaccharide-producing probiotic *Lactobacillus plantarum* isolated from camel milk. J. Dairy Sci., 100, 7771-7779.

Ale, E. C., Perezlindo, M. J., Burns, P., Tabacman, E., Reinheimer, J. A., Binetti, A. G., 2016. Exopolysaccharide from *Lactobacillus fermentum* Lf2 and its functional characterization as a yogurt additive. J. Dairy Sci., 83, 487-492.

Alrahmany, R., Avis, T. J., Tsopmo, A., 2013. Treatment of oat bran with carbohydrases increases soluble phenolic acid content and influences antioxidant and antimicrobial activities. Food Res. Int., 52, 568-574.

Álvarez, P., Alvarado, C., Mathieu, F., Jiménez, L., and De La Fuente, M., 2006. Diet supplementation for 5 weeks with polyphenol-rich cereals improves several functions and the redox state of mouse leucocytes. Eur J Nutr, 45, 428–438.

Amrouche, T., Boutin, Y., Prioult, G., Fliss, I., 2006. Effects of bifidobacterial cytoplasm, cell wall and exopolysaccharide on mouse lymphocyte proliferation and cytokine production. Int. Dairy J., 16, 70-80.

Arena, M. P., Russo, P., Capozzi, V., López, P., Fiocco, D., Spano, G., 2014. Probiotic abilities of riboflavin-overproducing *Lactobacillus* strains: a novel promising application of probiotics. Appl. Microbiol. Biotechnol., 98, 7569-7581.

Arena, M. P., Russo, P., Capozzi, V., Rascon, A., Felis, G. E., Spano, G., Fiocco, D., 2016. Combinations of cereal β-glucans and probiotics can enhance the anti-inflammatory activity on host cells by a synergistic effect. J Funct Foods, 23, 12-23.

Aronson, M., Medalia, O., Schori, L., Mirelman, D., Sharon, N., Ofek, I., 1979. Prevention of colonization of the urinary-tract by blocking bacterial adherence with methyl-alpha-D-mannopyranoside. Israel J. Med. Sci., 15, 88.

Aslani, B. A., Ghobadi, S., 2016. Studies on oxidants and antioxidants with a brief glance at their relevance to the immune system. Life sciences, 146, 163-173.

Bach Knudsen, K. E., Hedemann, M. S., Lærke, H. N., 2012. The role of carbohydrates in intestinal health of pigs. Anim. Feed Sci. Technol., 173, 41–53.

Badel, S., Bernardi, T., Michaud, P., 2011. New perspectives for *Lactobacilli* exopolysaccharides. Biotechnol. Adv., 29, 54-66.

Baldi, A., Ioannis, P., Chiara, P., Eleonora, F., Roubini, C., Vittorio, D., 2005. Biological effects of milk proteins and their peptides with emphasis on those related to the gastrointestinal ecosystem. J. Dairy Sci., 72, 66–72.

Barboza, M., Pinzon, J., Wickramasinghe, S., Froehlich, J.W., Moeller, I., Smilowitz, J.T., Ruhaak, L.R., Huang, J., Lönnerdal, B., German, J.B., Medrano, J.F., Weimer, B.C., Lebrilla, C.B., 2012. Glycosylation of Human Milk Lactoferrin Exhibits Dynamic Changes During Early Lactation Enhancing Its Role in Pathogenic Bacteria-Host Interactions. Mol. Cell Proteomics., 11, M111.015248.

Baümler, A.J., Sperandio, V., 2016. Interactions between the microbiota and pathogenic bacteria in the gut. Nature, 535, 85–93.

Bello, F.D., Walter, J., Hertel, C., Hammes, W.P., 2001. In vitro study of Prebiotic Properties of Levan-type Exopolysaccharides from *Lactobacilli* and Non-digestible Carbohydrates Using Denaturing Gradient Gel Electrophoresis. Syst. Appl. Microbiol., 24, 232–237.

Belotserkovsky, I., Brunner, K., Pinaud, L., Rouvinski, A., Dellarole, M., Baron, B., Dubey, G., Samassa, F., Parsot, C., Sansonetti, P., Phalipon A., 2018. Glycan-Glycan Interaction Determines Shigella Tropism toward Human T Lymphocytes. MBio, 9, e02309-17.

Benbrook, C. M., 2005. Elevating antioxidant levels in food through organic farming and food processing. Organic Center.

Bindelle, J., Pieper, R., Leterme, P., Rossnagel, B., Van Kessel, A.G., 2010. Changes in intestinal microbial ecophysiology as related to the carbohydrate composition of barleys and oats cultivars in an in vitro model of the pig gastrointestinal tract. Livest Sci, 133, 151–153.

Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Organ J, 5, 9.

Bode, L., 2009. Human milk oligosaccharides: Prebiotics and beyond. Nutr. Rev., 67, S183-S191.

Boubakri, H., Jdey, A., Taamalli, A., Taamalli, W., Jebara, M., Brini, F., Riciputi Y., Pasini F., Christian M., Verardo, V., 2017. Phenolic composition as measured by liquid chromatography/mass spectrometry and biological properties of Tunisian barley. Int J Food Prop, 20, 1783-1797.

Boudry, G., Lallès, J.P., Malbert, C.H., Bobillier, E., Sève, B., 2002. Diet-related adaptation of the small intestine at weaning in pigs is functional rather than structural. J. Pediatr. Gastroenterol. Nutr., 34, 180–187.

Brenes, A., Viveros, A., Chamorro, S., Arija, I., 2016. Use of polyphenol-rich grape by-products in monogastric nutrition. A review. Anim. Feed Sci. Technol., 211, 1–17.

Brown, W. R., Williams, A. F., 1982. Lymphocyte cell surface glycoproteins which bind to soybean and peanut lectins. Immunology, 46, 713.

Brunsgaard, G., 1998. Effects of Cereal Type and Feed Particle Size on Morphological Characteristics, Epithelial Cell Proliferation, and Lectin Binding Patterns in the Large Intestine of Pigs. J. Anim. Sci., 76, 2787–2798.

Burns, P., Vinderola, G., Reinheimer, J., Cuesta, I., de los Reyes-Gavilán, C.G., Ruas-Madiedo, P., 2011. Technological characterization and survival of the exopolysaccharide-producing strain *Lactobacillus delbrueckii* subsp. *lactis* 193 and its bile-resistant derivative 193+ in simulated gastric and intestinal juices. J. Dairy Res., 78, 357–364.

Campbell, J.M., Crenshaw, J.D., Polo, J., 2013. The biological stress of early weaned piglets. J Anim Sci Biotechnol, 4, 19.

Canny, G.O., McCormick, B.A., 2008. Bacteria in the intestine, helpful residents or enemies from within? Infect. Immun. 76, 3360–3373.

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., Queipo-Ortuño, M. I., 2013. Benefits of polyphenols on gut microbiota and implications in human health. J. Nutr. Biochem., 24, 1415-1422.

Chagnot, C., Listrat, A., Astruc, T., and Desvaux, M., 2012. Bacterial adhesion to animal tissues: Protein determinants for recognition of extracellular matrix components. Cell. Microbiol., 14, 1687–1696.

Chanput, W., Reitsma, M., Kleinjans, L., Mes, J. J., Savelkoul, H. F. J., Wichers, H. J., 2012. β-Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr Food Res, 56, 822–833.

Coddens, A., Loos, M., Vanrompay, D., Remon, J.P., Cox, E., 2017. Cranberry extract inhibits *in vitro* adhesion of F4 and $F18^+$ *Escherichia coli* to pig intestinal epithelium and reduces *in vivo* excretion of pigs orally challenged with $F18^+$ verotoxigenic *E. coli*. Vet. Microbiol., 202, 64–71.

Comstock, S.S., Li, M., Wang, M., Monaco, M.H., Kuhlenschmidt, T.B., Kuhlenschmidt, M.S., Donovan, S.M., 2017. Dietary Human Milk Oligosaccharides but Not Prebiotic Oligosaccharides Increase Circulating Natural Killer Cell and Mesenteric Lymph Node

Memory T Cell Populations in Noninfected and Rotavirus-Infected Neonatal Piglets. J. Nutr., 147, 1041–1047.

Coppa, G. V., Zampini, L., Galeazzi, T., Facinelli, B., Ferrante, L., Capretti, R., Orazio, G., 2006. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrhoeal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatr. Res., 59, 377.

Coppa, G.V., Facinelli, B., Magi, G., Marini, E., Zampini, L., Mantovani, V., Galeazzi, T., Padella, L., Marchesiello, R.L., Santoro, L., Coscia, A., Peila, C., Volpi, N., Gabrielli, O., 2016. Human milk glycosaminoglycans inhibit in vitro the adhesion of *Escherichia coli* and *Salmonella fyris* to human intestinal cells. Pediatr. Res., 79, 603–607.

Corbí, A. L., Fernández de Palencia, P., 2010. Naturally occurring 2-substituted (1, 3)-β-D-glucan producing *Lactobacillus suebicus* and *Pediococcus parvulus* strains with potential utility in the production of functional foods. Bioresour. Technol., 101, 9254-9263.

Cozens, D., Read, R. C., 2012. Anti-adhesion methods as novel therapeutics for bacterial infections. Expert Rev. Anti-Infe., 10, 1457-1468.

Cummings, J. H., Macfarlane, G. T., 1997. Collaborative JPEN-Clinical Nutrition Scientific Publications Role of intestinal bacteria in nutrient metabolism. JPEN J Parenter Enteral Nutr, 21, 357-365.

Cummings, J.H., MacFarlane, G.T., 1997. Role of intestinal bacteria in nutrient metabolism. JPEN J Parenter Enteral Nutr, 16, 3–11.

Cvitkovitch, D. G., Li, Y. H., Ellen, R. P., 2003. Quorum sensing and biofilm formation in Streptococcal infections. J. Clin. Invest., 112, 1626-1632.

Daglia, M., 2012. Polyphenols as antimicrobial agents. Curr. Opin. Biotechnol., 23, 174-181.

Dai, D., Nanthkumar, N. N., Newburg, D. S., Walker, W. A., 2000. Role of oligosaccharides and glycoconjugates in intestinal host defense. J. Pediatr. Gastroenterol. Nutr., 30, S23-S33.

Day, C.J., Tran, E.N., Semchenko, E.A., Tram, G., Hartley-Tassell, L.E., Ng, P.S.K., King, R.M., Ulanovsky, R., McAtamney, S., Apicella, M.A., Tiralongo, J., Morona, R., Korolik,

V., Jennings, M.P., 2015. Glycan:glycan interactions: High affinity biomolecular interactions that can mediate binding of pathogenic bacteria to host cells. PNAS, 112, E7266–E7275.

De Llano, D.G., Esteban-Fernández, A., Sánchez-Patán, F., Martín-Álvarez, P.J., Moreno-Arribas, M.V., and Bartolomé, B., 2015. Anti-adhesive activity of cranberry phenolic compounds and their microbial-derived metabolites against uropathogenic *Escherichia coli* in bladder epithelial cell cultures. Int J Mol Sci,16, 12119–12130.

de Oliveira, J.T.A., Pusztai, A., Grant, G., 1988. Changes in organs and tissues induced by feeding of purified kidney bean (Phaseolus vulgaris) lectins. Nutr. Res., 8, 943–947.

de Palencia, P. F., Werning, M. L., Sierra-Filardi, E., Dueñas, M. T., Irastorza, A., Corbí, A. L., López, P., 2009. Probiotic properties of the 2-substituted (1, 3)-β-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. Appl. Environ. Microbiol., 75, 4887-4891.

Delattre, C., Pierre, G., Laroche, C., Michaud, P., 2016. Production, extraction and characterization of microalgal and cyanobacterial exopolysaccharides. Biotechnol. Adv., 34, 1159-1179.

Deutsch, S. M., Parayre, S., Bouchoux, A., Guyomarc'h, F., Dewulf, J., Dols-Lafargue, M., Foligné, B., 2012. Contribution of surface β-glucan polysaccharide to physicochemical and immunomodulatory properties of *Propionibacterium freudenreichii*. Appl. Environ. Microbiol., AEM-07027.

Di, R., Vakkalanka, M.S., Onumpai, C., Chau, H.K., White, A., Rastall, R.A., Yam, K., Hotchkiss, A.T., 2017. Pectic oligosaccharide structure-function relationships: Prebiotics, inhibitors of *Escherichia coli* O157:H7 adhesion and reduction of Shiga toxin cytotoxicity in HT29 cells. Food Chem., 227, 245–254.

Ditsawanon, T., Yingchutrakul, Y., Roytrakul, S. Antibacterial activity of protein hydrolysates from Thai rice grain. http://www.scisoc.or.th/BMBThailand/images/BMB2018/S2-P-29.pdf

Donovan, S.M., 2016. The Role of Lactoferrin in Gastrointestinal and Immune Development and Function: A Preclinical Perspective. J Pediatr, 173, S16–S28.

Douëllou, T., Galia, W., Kerangart, S., Marchal, T., Milhau, N., Bastien, R., Bouvier, M., Buff, S., Montel, M.C., Sergentet-Thevenot, D., 2018. Milk fat globules hamper adhesion of enterohemorrhagic *Escherichia coli* to enterocytes: *In vitro* and *in vivo* evidence. Front Microbiol, 9.

Dréau, D., Lallès, J.P., Philouze-Romé, V., Toullec, R., Salmon, H., 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. J. Anim. Sci., 72, 2090–2098.

Dréau, D., Lallès, J.P., Toullec, R., Salmon, H., 1995. B and T lymphocytes are enhanced in the gut of piglets fed heat-treated soyabean proteins. Vet. Immunol. Immunopath., 47, 69–79.

Duarte, T. L., Lunec, J., 2005. When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radic. Res., 39, 671-686.

Duboc, P., Mollet, B., 2001. Applications of exopolysaccharides in the dairy industry. Int. Dairy J., 11(9), 759-768.

Dunsford, B.R., Knabe, D.A., Haensly, W.E., 1989. Effect of dietary soybean meal on the microscopic anatomy of the small intestine in the early-weaned pig. J. Anim. Sci., 67, 1855–1863.

Engfer, M.B., Stahl, B., Finke, B., Sawatzki, G., Daniel, H., 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am. J. Clin. Nutr., 71, 1589–1596.

Facinelli, B., Marini, E., Magi, G., Zampini, L., Santoro, L., Catassi, C., Monachesi, C., Gabrielli, O., Coppa, G.V., 2018. Breast milk oligosaccharides: effects of 2′-fucosyllactose and 6′-sialyllactose on the adhesion of *Escherichia coli* and *Salmonella fyris* to Caco-2 cells. J Matern Fetal Med, 1–3.

Fanning, S., Hall, L. J., Cronin, M., Zomer, A., MacSharry, J., Goulding, D., van Sinderen, D., 2012. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. PNAS, 109, 2108-2113.

Fardet, A., 2010. New hypotheses for the health-protective mechanisms of whole-grain cereals: What is beyond fibre? Nutr Res Rev, 23, 65–134.

Feeney, S., Ryan, J., Kilcoyne, M., Joshi, L., Hickey, R., 2017. Glycomacropeptide Reduces Intestinal Epithelial Cell Barrier Dysfunction and Adhesion of Entero-Hemorrhagic and Entero-Pathogenic *Escherichia coli in Vitro*. Foods, 6, 93.

Feng, M., Chen, X., Li, C., Nurgul, R., Dong, M., 2012. Isolation and identification of an Exopolysaccharide-Producing lactic acid bacterium strain from Chinese paocai and biosorption of Pb (II) by its exopolysaccharide. J. Food Sci., *77*, T111-T117.

Fiesel, A., Gessner, D.K., Most, E., Eder, K., 2014. Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs. BMC Vet. Res., 10, 196.

Finlay, B.B., Cossart, P., 1997. Exploitation of mammalian host cell functions by bacterial pathogens. Science, 276, 718–725.

Flemming, H. C., Neu, T. R., Wozniak, D. J., 2007. The EPS matrix: the "house of biofilm cells". J Bacteriol, 189, 7945-7947.

Flemming, H. C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol., 8, 623.

Friedman, M., 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. Mol Nutr Food Res, 51, 116-134.

Gabius, H.J., Manning, J.C., Kopitz, J., André, S., Kaltner, H., 2016. Sweet complementarity: The functional pairing of glycans with lectins. Cell. Mol. Life Sci., 73, 1989–2016.

Garai-Ibabe, G., Dueñas, M.T., Irastorza, A., Sierra-filardi, E., Werning, M.L., López, P.,

Garside, P., Millington, O., Smith, K.M., 2004. The anatomy of mucosal immune responses. Ann N Y Acad Sci, 1029, 9–15.

Gessner, D.K., Fiesel, A., Most, E., Dinges, J., Wen, G., Ringseis, R., Eder, K., 2013. Supplementation of a grape seed and grape marc meal extract decreases activities of the oxidative stress-responsive transcription factors NF-κB and Nrf2 in the duodenal mucosa of pigs. Acta Vet. Scand., 55, 18.

Ghoneim, M.A.M., Hassan, A.I., Mahmoud, M.G., Asker, M.S., 2016. Effect of polysaccharide from Bacillus subtilis sp. on cardiovascular diseases and atherogenic indices in diabetic rats. BMC Complement Altern Med, 16, 112. doi:10.1186/s12906-016-1093-1

Gonia, S., Tuepker, M., Heisel, T., Autran, C., Bode, L., Gale, C.A., 2015. Human Milk Oligosaccharides Inhibit *Candida albicans* Invasion of Human Premature Intestinal Epithelial Cells. J. Nutr., 145, 1992–1998.

González de Llano, D., Esteban-Fernández, A., Sánchez-Patán, F., Martínlvarez, P. J., Moreno-Arribas, M., Bartolomé, B., 2015. Anti-adhesive activity of cranberry phenolic compounds and their microbial-derived metabolites against uropathogenic *Escherichia coli* in bladder epithelial cell cultures. Int J Mol Sci, 16, 12119-12130.

Górska-Frączek, S., Sandström, C., Kenne, L., Rybka, J., Strus, M., Heczko, P., Gamian, A., 2011. Structural studies of the exopolysaccharide consisting of a nonasaccharide repeating unit isolated from *Lactobacillus rhamnosus* KL37B. Carbohydr. Res., 346, 2926-2932.

Guarino, A., Wudy, A., Basile, F., Ruberto, E., Buccigrossi, V., 2012. Composition and roles of intestinal microbiota in children. J. Matern. Fetal Neonatal Med., 25: 63–66.

Guerin, J., Soligot, C., Burgain, J., Huguet, M., Francius, G., El-Kirat-Chatel, S., Gomand, F., Lebeer, S., Le Roux, Y., Borges, F., Scher J., Gaiani C., 2018. Adhesive interactions between milk fat globule membrane and Lactobacillus rhamnosus GG inhibit bacterial attachment to Caco-2 TC7 intestinal cell. Colloids Surf B Biointerfaces, 167, 44–53.

Guo, W., Chi, Y., 2017. Purification and fermentation characteristics of exopolysaccharide from Fomitopsis castaneus Imaz. Int. J. Biol. Macromol., 105, 213–218.

Gupta, P., Song, B., Neto, C., Camesano, T. A., 2016. Atomic force microscopy-guided fractionation reveals the influence of cranberry phytochemicals on adhesion of *Escherichia coli*. Food & Funct, 7, 2655-2666.

Halliwell, B., 1991. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med, 91, S14-S22.

Hall-Stoodley, L., Costerton, J. W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol., 2, 95.

Hamet, M. F., Medrano, M., Perez, P. F., Abraham, A. G., 2016. Oral administration of kefiran exerts a bifidogenic effect on BALB/c mice intestinal microbiota. Benef Microbes, 7, 237-246.

He, Y., Lawlor, N. T., Newburg, D. S., 2016. Human Milk Components Modulate Toll-Like Receptor-Mediated Inflammation. Adv Nutr, 7, 102–111.

He, Y.Y., Liu, S.B., Kling, D.E., Leone, S., Lawlor, N.T., Huang, Y., Feinberg, S.B., Hill, D.R., Newburg, D.S., 2016. The human milk oligosaccharide 2′-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. Gut, 65, 33–46.

Hedemann, M. S., Eskildsen, M., Lærke, H. N., Pedersen, C., Lindberg, J. E., Laurinen, P., Knudsen, K. B., 2006. Intestinal morphology and enzymatic activity in newly weaned pigs fed contrasting fiber concentrations and fiber properties. J. Anim. Sci., 84, 1375-1386.

Hedemann, M. S., Mikkelsen, L. L., Naughton, P. J., Jensen, B. B., 2005. Effect of feed particle size and feed processing on morphological characteristics in the small and large intestine of pigs and on adhesion of *Salmonella enterica serovar* Typhimurium DT12 in the ileum in vitro. J. Anim. Sci., 83, 1554-1562.

Hermes, R. G., Molist, F., Francisco Pérez, J., De Segura, A.G., Ywazaki, M., Davin, R., Nofrarías, M., Korhonen, T.K., Virkola, R., Martín-Orúe, S.M., 2013. Casein glycomacropeptide in the diet may reduce *Escherichia coli* attachment to the intestinal mucosa and increase the intestinal *lactobacilli* of early weaned piglets after an enterotoxigenic *E. coli* K88 challenge. Br. J. Nutr., 109, 1001–1012.

Hermes, R.G., Manzanilla, E.G., Martín-Orúe, S.M., Pérez, J.F., and Klasing, K.C., 2011. Influence of dietary ingredients on in vitro inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). Comp. Immunol. Microbiol. Infect. Dis., 34, 479–488.

Hervert-Hernandez, D., Goñi, I., 2011. Dietary polyphenols and human gut microbiota: a review. Food Rev Int, 27, 154-169.

Hickey, R.M., 2012. The role of oligosaccharides from human milk and other sources in prevention of pathogen adhesion. Int. Dairy J., 22, 141–146.

Hill, D.A., Artis, D., 2010. Intestinal Bacteria and the Regulation of Immune Cell Homeostasis. Annu. Rev. Immunol., 28, 623–667.

Hole, A.S., Grimmer, S., Naterstad, K., Jensen, M.R., Paur, I., Johansen, S.G., Balstad, T.R., Blomhoff, R., Sahlstrom, S., 2009. Activation and inhibition of nuclear factor kappa B activity by cereal extracts: Role of dietary phenolic acids. J. Agric. Food Chem., 57, 9481–9488.

Hotchkiss, A.T., Nuñez, A., Strahan, G.D., Chau, H.K., White, A.K., Marais, J.P.J., Hom, K., Vakkalanka, M.S., Di, R., Yam, K.L., Khoo, C., 2015. Cranberry Xyloglucan Structure and Inhibition of *Escherichia coli* Adhesion to Epithelial Cells. J. Agric. Food Chem. 63, 5622–5633.

Howell, A.B., Vorsa, N., Der Marderosian, A., Foo, L.Y., 1998. Inhibition of the adherence of P-fimbriated Escherichia coli to uroepithelial-cell surfaces by proanthocyanidin extracts from cranberries. N Engl J Med, 339, 1085–1086.

Hunt, K.M., Preuss, J., Nissan, C., Davlin, C.A., Williams, J.E., Shafii, B., Richardson, A.D., McGuire, M.K., Bode, L., McGuire, M.A., 2012. Human milk oligosaccharides promote the growth of staphylococci. Appl. Environ. Microbiol., 78, 4763–4770.

Huttunen, S., Toivanen, M., Liu, C., Tikkanen-Kaukanen, C., 2016. Novel anti-infective potential of salvianolic acid B against human serious pathogen *Neisseria meningitidis*. BMC Res Notes, 9.

Irie, Y., Borlee, B. R., O'Connor, J. R., Hill, P. J., Harwood, C. S., Wozniak, D. J., Parsek, M. R., 2012. Self-produced exopolysaccharide is a signal that stimulates biofilm formation in Pseudomonas aeruginosa. PNAS, 109, 20632-20636.

Jansman A J M., 2016. Health and functions of the gastrointestinal tract in pigs: effects of functional ingredients and feed and ingredient processing. J. Anim. Sci., 94, 12-21.

Jiang, M., Zhang, F., Wan, C., Xiong, Y., Shah, N. P., Wei, H., Tao, X., 2016. Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk. J. Dairy Sci., 99, 1736-1746.

Jin, L. Z., Zhao, X., 2000. Intestinal receptors for adhesive fimbriae of enterotoxigenic *Escherichia coli* (ETEC) K88 in swine–a review. Appl. Microbiol. Biotechnol., 54, 311-318.

Jones, G.W., Isaacson, R.E., 1982. Proteinaceous bacterial adhesins and their receptors. Crit. Rev. Microbiol., 10, 229–260.

Juge, N., 2012. Microbial adhesins to gastrointestinal mucus. Trends Microbiol., 20, 30–39.

Jung, C., Hugot, J. P., Barreau, F., 2010. Peyer's patches: the immune sensors of the intestine. Int J Inflam, 2010.

Kadiri, O., 2017. A review on the status of the phenolic compounds and antioxidant capacity of the flour: Effects of cereal processing. Int J Food Prop, 20, S798-S809.

Kanamoto, Y., Yamashita, Y., Nanba, F., Yoshida, T., Tsuda, T., Fukuda, I., Nakamura-Tsuruta, S., Ashida, H., 2011. A black soybean seed coat extract prevents obesity and glucose intolerance by up-regulating uncoupling proteins and down-regulating inflammatory cytokines in high-fat diet-fed mice. J. Agric. Food Chem. 59, 8985–8993.

Kato, K., Ishiwa, A., 2015. The Role of Carbohydrates in Infection Strategies of Enteric Pathogens. Trop Med Health, 43, 41–52.

Kavanaugh, D. W., O'Callaghan, J., Buttó, L. F., Slattery, H., Lane, J., Clyne, M., Hickey, R. M., 2013. Exposure of Bifidobacterium longum subsp. infantis to milk oligosaccharides increases adhesion to epithelial cells and induces a substantial transcriptional response. PLoS One, 8, e67224.

Kelly, C.G., Younson, J.S., 2000. Anti-adhesive strategies in the prevention of infectious disease at mucosal surfaces. Expert Opin Investig Drugs, 9, 1711–1721.

Kenny, B., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A., Finlay, B.B., 1997. *Enteropathogenic* E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell, 91, 511–520.

Kik, M. J. L., Rojer, J. M., Mouwen, J. M. V. M., Koninkx, J. F. J. G., Van Dijk, J. E., Van der Hage, M. H., 1989. The interaction between plant lectins and the small intestinal epithelium: a primary cause of intestinal disturbance. Vet Q, 11, 108-115.

Kim, M. H., Jo, S. H., Ha, K. S., Song, J. H., Jang, H. D., Kwon, Y. I., 2010. Antimicrobial activities of 1, 4-benzoquinones and wheat germ extract. J Microbiol Biotechnol, 20, 1204-1209.

Kim, Y., oh, S., Kim, S.H., 2009. Released exopolysaccharide (r-EPS) produced from probiotic bacteria reduce biofilm formation of enterohemorrhagic *Escherichia coli* O157:H7. Biochem. Biophys. Res. Commun., 379, 324–329.

Klemm, P., Schembri, M. A.,2000. Bacterial adhesins: function and structure. Int. J. Med. Microbiol., 290, 27-35.

Kline, K.A., Fälker, S., Dahlberg, S., Normark, S., Henriques-Normark, B., 2009. Bacterial Adhesins in Host-Microbe Interactions. Cell Host Microbe, 5, 580–592.

Knudsen, K.E.B., 2014. Fiber and nonstarch polysaccharide content and variation in common crops used in broiler diets. In Poultry Science (Oxford University Press), 2380–2393.

Krachler, A.M., Orth, K., 2013. Targeting the bacteria-host interface strategies in anti-adhesion therapy. Virulence, 4, 284–294.

Kšonžeková, P., Bystrický, P., Vlčková, S., Pätoprstý, V., Pulzová, L., Mudroňová, D., Kubasková T., Csank T., Tkáčiková, Ľ., 2016. Exopolysaccharides of *Lactobacillus reuteri*: their influence on adherence of E. coli to epithelial cells and inflammatory response. Carbohydr Polym, 141, 10-19.

Kuehn, M.J., Kesty, N.C., 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. Genes Dev., 19, 2645–2655.

Kurakevich, E., Hennet, T., Hausmann, M., Rogler, G., and Borsig, L., 2013. Milk oligosaccharide sialyl(2,3)lactose activates intestinal CD11c+ cells through TLR4. PNAS, 110, 17444–17449.

Lane, J. a, O'Callaghan, J., Carrington, S.D., Hickey, R.M., 2013. Transcriptional response of HT-29 intestinal epithelial cells to human and bovine milk oligosaccharides. The Br. J. Nutr. 110, 2127–2137.

Lane, J.A., Mehra, R.K., Carrington, S.D., Hickey, R.M., 2010. The food glycome: A source of protection against pathogen colonization in the gastrointestinal tract. Int. J. Food Microbiol., 142, 1–13.

Lara-Villoslada, F., Debras, E., Nieto, A., Concha, A., Gálvez, J., López-Huertas, E., Boza, J., Obled, C., and Xaus, J., 2006. Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis. Clin Nutr, 25, 477–488.

Leitão, D. P., Polizello, A. C. M., Ito, I. Y., Spadaro, A. C. C., 2005. Antibacterial screening of anthocyanic and proanthocyanic fractions from cranberry juice. J Med Food, 8, 36-40.

Leódido, A. C. M., Costa, L. E., Araújo, T. S., Costa, D. S., Sousa, N. A., Souza, L. K., Souza, F.B.M., Filho, M.D.S., Vasconclos, D.F.P., Silva, F.R.P., Nogueira, K.M., Araujo, A.R., Barros, F.C.N., Freitas, A.L.P., Nogueira, K. M., 2017. Anti-diarrhoeal therapeutic potential and safety assessment of sulphated polysaccharide fraction from Gracilaria intermedia seaweed in mice. Int. J. Biol. Macromol., 97, 34-45.

Leonard, S. G., Sweeney, T., Bahar, B., Lynch, B. P., O'Doherty, J. V., 2011. Effect of dietary seaweed extracts and fish oil supplementation in sows on performance, intestinal microflora, intestinal morphology, volatile fatty acid concentrations and immune status of weaned pigs. Br. J. Nutr., 105, 549-560.

Li, S., Chen, T., Xu, F., Dong, S., Xu, H., Xiong, Y., Wei, H., 2014. The beneficial effect of exopolysaccharides from *Bifidobacterium bifidum* WBIN03 on microbial diversity in mouse intestine. J. Sci. Food Agric., 94, 256-264.

Li, S., Huang, R., Shah, N. P., Tao, X., Xiong, Y., Wei, H., 2014. Antioxidant and antibacterial activities of exopolysaccharides from *Bifidobacterium bifidum* WBIN03 and Lactobacillus plantarum R315. J. Dairy Sci., 97, 7334-7343.

Liepke, C., Adermann, K., Raida, M., Mägert, H.J., Forssmann, W.G., Zucht, H.D., 2002. Human milk provides peptides highly stimulating the growth of bifidobacteria. Eur. J. Biochem., 269, 712–718.

Lindström, C., Holst, O., Hellstrand, P., Öste, R., Andersson, K.E., 2012. Evaluation of commercial microbial hydrocolloids concerning their effects on plasma lipids and caecal formation of SCFA in mice. Food Hydrocoll, 28, 367–372.

Lindström, C., Xu, J., Öste, R., Holst, O., Molin, G., 2013. Oral administration of live exopolysaccharide-producing *Pediococcus parvulus*, but not purified exopolysaccharide suppressed *Enterobacteriaceae* without affecting bacterial diversity in caecum of mice. Appl. Environ. Microbiol., AEM-01456.

LoCascio, R.G., Ninonuevo, M.R., Freeman, S.L., Sela, D.A., Grimm, R., Lebrilla, C.B., Mills, D.A., and German, J.B., 2007. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J. Agric. Food Chem., 55, 8914–8919.

LoCascio, R.G., Niñonuevo, M.R., Kronewitter, S.R., Freeman, S.L., German, J.B., Lebrilla, C.B., Mills, D.A., 2009. A versatile and scalable strategy for glycoprofiling bifidobacterial consumption of human milk oligosaccharides. Microb Biotechnol, 2, 333–342.

López, D., Vlamakis, H., Kolter, R., 2010. Biofilms. Cold Spring Harbor perspectives in biology, a000398.

Luang-In, V., Deeseenthum, S., 2016. Exopolysaccharide-producing isolates from Thai milk kefir and their antioxidant activities. LWT-Food Sci. Technol., 73, 592-601.

Macfarlane, S., Macfarlane, G. T., 2006. Composition and metabolic activities of bacterial biofilms colonizing food residues in the human gut. Appl. Environ. Microbiol., 72, 6204-6211.

Mahmoud, A. A., Mohdaly, A. A., Elneairy, N. A., 2015. Wheat germ: an overview on nutritional value, antioxidant potential and antibacterial characteristics. Food Nutr Sci, 6, 265.

Maldonado-Gomez, M.X., Lee, H., Barile, D., Lu, M., Hutkins, R.W., 2015. Adherence inhibition of enteric pathogens to epithelial cells by bovine colostrum fractions. Int. Dairy J., 40, 24–32.

Malkoski, M., Dashper, S.G., O'Brien-Simpson, N.M., Talbo, G.H., Macris, M., Cross, K.J., Reynolds, E.C., 2001. Kappacin, a novel antibacterial peptide from bovine milk. Antimicrob Agents Ch, 45, 2309–2315.

Mandlik, A., Swierczynski, A., Das, A., Ton-That, H., 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol., 16, 33–40.

Marcobal, A., Barboza, M., Froehlich, J. W., Block, D. E., German, J. B., Lebrilla, C. B., Mills, D. A. (2010). Consumption of human milk oligosaccharides by gut-related microbes. J. Agric. Food Chem., 58, 5334-5340.

Mårtensson, O., Biörklund, M., Lambo, A.M., Dueñas-Chasco, M., Irastorza, A., Holst, O., Norin, E., Welling, G., Öste, R., Önning, G., 2005. Fermented, ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. Nutr Res, 25, 429–442. doi:10.1016/j.nutres.2005.03.004

Mathew, A.G., Cissell, R., Liamthong, S., 2007. Antibiotic Resistance in Bacteria Associated with Food Animals: A United States Perspective of Livestock Production. Foodborne Path. Dis. 4, 115–133.

Mekbungwan, A.,Yamauchi, K., 2004. Growth performance and histological intestinal alterations in piglets fed dietary raw and heated pigeon pea seed meal. Histol. Histopathol., 19, 381–389.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., Cossart, P., 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell, 84, 923–932.

Mercan, E., İspirli, H., Sert, D., Yılmaz, M. T., Dertli, E., 2015. Impact of exopolysaccharide production on functional properties of some *Lactobacillus salivarius* strains. Arch. Microbiol., 197, 1041-1049.

Messing, J., Niehues, M., Shevtsova, A., Borén, T., Hensel, A., 2014. Antiadhesive properties of arabinogalactan protein from ribes nigrum seeds against bacterial adhesion of *Helicobacter pylori*. Molecules, 19, 3696–3717.

Miranda-Nantes, C.C.B.O., Fonseca, E.A.I., Zaia, C.T.B.V., Dekker, R.F.H., Khaper, N., Castro, I.A., Barbosa, A.M., 2011. Hypoglycemic and hypocholesterolemic effects of botryosphaeran from *Botryosphaeria rhodina* MAMB-05 in diabetes-induced and hyperlipidemia conditions in rats. Mycobiology, 39, 187–193. doi:10.5941/MYCO.2011.39.3.187

Molist, F., Gómez de Segura, A., Pérez, J.F., Bhandari, S.K., Krause, D.O., Nyachoti, C.M., 2010. Effect of wheat bran on the health and performance of weaned pigs challenged with *Escherichia coli* K88+. Livest. Sci. 133, 214–217.

Molist, F., Hermes, R.G., De Segura, A.G., Martí-N-Orúe, S.M., Gasa, J., Manzanilla, E.G., Pérez, J.F., 2011. Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. Br. J. Nutr. 105, 1592–1600.

Montagne, L., Pluske, J.R., and Hampson, D.J., 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol., 108, 95–117.

Morifuji, M., Kitade, M., Fukasawa, T., Yamaji, T., Ichihashi, M., 2017. Exopolysaccharides isolated from milk fermented with lactic acid bacteria prevent ultraviolet-induced skin damage in hairless mice. Int J Mol Sci, 18, 146.

Mowat, A.M.I., 2003. Anatomical basis of tolerance and immunity to intestinal antigens. Nat. Rev. Immunol., 3, 331–341.

Mudgil, D., Barak, S., 2013. Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: A review. Int. J. Biol. Macromol., 61, 1–6.

Musilova, S., Modrackova, N., Doskocil, I., Svejstil, R., and Rada, V., 2017. Influence of human milk oligosaccharides on adherence of bifidobacteria and clostridia to cell lines. Acta Microbiol Immunol Hung, 1–8.

Nagai, T., Makino, S., Ikegami, S., Itoh, H., Yamada, H., 2011. Effects of oral administration of yogurt fermented with *Lactobacillus delbrueckii ssp. bulgaricus* OLL1073R-1 and its exopolysaccharides against influenza virus infection in mice. Int. Immunopharmacol., 11, 2246-2250.

Natarajan, S. B., Hwang, J. W., Kim, Y. S., Kim, E. K., Park, P. J., 2017. Ocular promoting activity of grape polyphenols—A review. Environ Toxicol Phar, 50, 83-90.

Nicholson, J.K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., Pettersson, S., 2012. Host-gut microbiota metabolic interactions. Science, 336, 1262–1267.

Nikolic, M., López, P., Strahinic, I., Suárez, A., Kojic, M., Fernández-García, M., Topisirovic, L., Golic, N., Ruas-Madiedo, P., 2012. Characterisation of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics. Int. J. Food Microbiol., 158, 155-162.

Nougayrède, J.-P., Fernandes, P.J., Donnenberg, M.S., 2003. Adhesion of enteropathogenic *Escherichia coli* to host cells. Cell. Microbiol., 5, 359–372.

Ofek, I., Hasty, D. L., Sharon, N., 2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. FEMS Immunol. Med. Microbiol. 38, 181-191.

Ohlsen, K., Oelschlaeger, T.A., Hacker, J., and Khan, A.S., 2009. Carbohydrate Receptors of Bacterial Adhesins: Implications and Reflections. Top Curr Chem., 288, 109–120.

Ouwehand, A.C., Salminen, S., 2003. In vitro Adhesion Assays for Probiotics and their in vivo Relevance: A Review. Microb. Ecol. Health Dis., 15, 175–184.

Palla, A. H., Khan, N. A., Bashir, S., Iqbal, J., Gilani, A. H., 2015. Pharmacological basis for the medicinal use of *Linum usitatissimum* (Flaxseed) in infectious and non-infectious diarrhoea. J Ethnopharmacol, 160, 61-68.

Parkar, S.G., Redgate, E.L., Wibisono, R., Luo, X., Koh, E.T.H., Schröder, R., 2010. Gut health benefits of kiwifruit pectins: Comparison with commercial functional polysaccharides. J Funct Foods, 2, 210–218.

Parsons, B.N., Wigley, P., Simpson, H.L., Williams, J.M., Humphrey, S., Salisbury, A.M., Watson, A.J.M., Fry, S.C., O'Brien, D., Roberts, C.L., O'Kennedy, N., Keita, A.V., Söderholm, J.D., Rhodes, J.M., Campbell, B.J., 2014. Dietary supplementation with soluble plantain non-starch polysaccharides inhibits intestinal invasion of *Salmonella typhimurium* in the chicken. PLoS ONE 9.

Patel, S., Mathivanan, N., Goyal, A., 2017. Bacterial adhesins, the pathogenic weapons to trick host defense arsenal. Biomed. Pharmacother., 93, 763–771.

Peron, G., Pellizzaro, A., Brun, P., Schievano, E., Mammi, S., Sut, S., Castagliuolo, I., Dall'Acqua, S., 2017. Antiadhesive Activity and Metabolomics Analysis of Rat Urine after Cranberry (Vaccinium macrocarpon Aiton) Administration. J. Agric. Food Chem., 65, 5657–5667.

Peterson, L. W., Artis, D., 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nature Reviews Immunology, 14, 141.

Petschow, B. W., Talbott, R. D., 1990. Growth promotion of Bifidobacterium species by whey and casein fractions from human and bovine milk. J. Clin. Microbiol., 28, 287-292.

Piel, C., Montagne, L., Sève, B., and Lallès, J.-P., 2005. Increasing digesta viscosity using carboxymethylcellulose in weaned piglets stimulates ileal goblet cell numbers and maturation. J. Nutr., 135, 86–91.

Pieper, R., Janczyk, P., Zeyner, A., Smidt, H., Guiard, V., Souffrant, W. B., 2008. Ecophysiology of the developing total bacterial and *Lactobacillus* communities in the terminal small intestine of weaning piglets. Microbial ecology, 56, 474-483.

Pizarro-Cerdá, J., Cossart, P., 2006. Bacterial adhesion and entry into host cells. Cell, 124, 715–727.

Pogačar, M.Š., Klančnik, A., Bucar, F., Langerholc, T., Možina, S.S., 2015. Alpinia katsumadai extracts inhibit adhesion and invasion of *Campylobacter jejuni* in animal and human foetal small intestine cell lines. Phytother Res, 29, 1585–1589.

Politis, I., Chronopoulou, R., 2008. Milk peptides and immune response in the neonate. Adv. Exp. Med. Biol., 606, 253–269.

Pradeepa., Shetty, A.D., Matthews, K., Hegde, A.R., Akshatha, B., Mathias, A.B., Mutalik, S., Vidya, S.M., 2016. Multidrug resistant pathogenic bacterial biofilm inhibition by *Lactobacillus plantarum* exopolysaccharide. Bioactive Carbohydrates and Dietary Fibre, 8, 7–14.

Pu, C., Tang, W., 2017. The antibacterial and antibiofilm efficacies of a liposomal peptide originating from rice bran protein against Listeria monocytogenes. Food & Funct, 8, 4159-4169.

Purohit, D. H., Hassan, A. N., Bhatia, E., Zhang, X., Dwivedi, C., 2009. Rheological, sensorial, and chemopreventive properties of milk fermented with exopolysaccharide-producing lactic cultures. J. Dairy Sci., 92, 847-856.

Puupponen-Pimiä, R. A. M. A., Aura, A. M., Oksman-Caldentey, K. M., Myllärinen, P., Saarela, M., Mattila-Sandholm, T., Poutanen, A. K., 2002. Development of functional ingredients for gut health. Trends Food Sci Tech., 13, 3-11.

Rhoades, J.R., Gibson, G.R., Formentin, K., Beer, M., Greenberg, N., Rastall, R. a, 2005. Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. J. Dairy Sci., 88, 3455–3459.

Rieder, A., Grimmer, S., Kolset, S.O., Michaelsen, T.E., Knutsen, S.H. Cereal β-glucan preparations of different weight average molecular weights induce variable cytokine secretion in human intestinal epithelial cell lines. Food Chem, 2011, 128, 1037–1043.

Ripolles, D., Harouna, S., Parrón, J. A., Calvo, M., Pérez, M. D., Carramiñana, J. J., Sánchez, L., 2015. Antibacterial activity of bovine milk lactoferrin and its hydrolysates prepared with pepsin, chymosin and microbial rennet against foodborne pathogen *Listeria monocytogenes*. Int. Dairy J., 45, 15-22.

Ripollés, D., Harouna, S., Parrón, J.A., Arenales, I., Calvo, M., Pérez, M.D., Sánchez, L., 2017. Inhibition of *Cronobacter sakazakii* Adhesion to Caco-2 Cells by Commercial Dairy Powders and Raw Buttermilk. J. Agric. Food Chem., 65, 1043–1050.

Rojo, J., Morales, J.C., and Penades, S., 2002. Carbohydrate-carbohydrate interactions in biological and model systems. Top Curr Chem, 218, 45–92.

Rosca, I., Petrovici, A. R., Peptanariu, D., Nicolescu, A., Dodi, G., Avadanei, M., Ivadanei, IC., Bostanaru, AC., Mares, M., Ciolacu, D., 2018. Biosynthesis of dextran by *Weissella confusa* and its In vitro functional characteristics. Int. J. Biol. Macromol., 107, 1765-1772.c

Rosner, J.L., 2014. Ten Times More Microbial Cells than Body Cells in Humans? Microbe, 9.

Ross, S.A., Lane, J.A., Kilcoyne, M., Joshi, L., Hickey, R.M., 2016. Defatted bovine milk fat globule membrane inhibits association of enterohaemorrhagic *Escherichia coli* O157:H7 with human HT-29 cells. Int. Dairy J., 59, 36–43.

Roubos-Van Den Hil, P.J., Schols, H.A., Rob Nout, M.J., Zwietering, M.H., Gruppen, H., 2010. First characterization of bioactive components in soybean tempe that protect human and animal intestinal cells against enterotoxigenic *Escherichia coli* (ETEC) infection. J. Agric. Food Chem., 58, 7649–7656.

Ruas-Madiedo, P., Gueimonde, M., Margolles, A., de los Reyes-Gavilán, C.G., Salminen, S., 2006. Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. J. Food Prot., 69, 2011–2015.

Sahasrabudhe, N.M., Tian, L., van den Berg, M., Bruggeman, G., Bruininx, E., Schols, H.A., Faas, M.M., de Vos, P., 2016a. Endo-glucanase digestion of oat β-Glucan enhances Dectin-1 activation in human dendritic cells. J Funct Foods, 21, 104–112.

Sahasrabudhe, N. M., Dokter-Fokkens, J., de Vos, P., 2016b. Particulate β-glucans synergistically activate TLR4 and Dectin-1 in human dendritic cells. Mol Nutr Food Res, 60, 2514–2522.

Saito, Y., Takano, T., Rowland, I., 1992. Effects of soybean oligosaccharides on the human gut microflora in in vitro culture. Microb. Ecol. Health Dis., 5, 105-110.

Salazar, N., Gueimonde, M., de los Reyes-Gavilan, C. G., Ruas-Madiedo, P., 2016. Exopolysaccharides produced by lactic acid bacteria and bifidobacteria as fermentable substrates by the intestinal microbiota. Crit Rev Food Sci Nutr, 56, 1440-1453.

Salunkhe, D.K., Jadhav, S.J., Kadam, S.S., Chavan, J.K., 1983. Chemical, biochemical, and biological significance of polyphenols in cereals and legumes. Crit Rev Food Sci Nutr, 17, 277–305.

Samuelsen, A. B., Rieder, A., Grimmer, S., Michaelsen, T. E., Knutsen, S. H., 2011. Immunomodulatory activity of dietary fiber: Arabinoxylan and mixed-linked beta-glucan isolated from barley show modest activities in vitro. Int J Mol Sci, 12, 570–587.

Santos, A., San Mauro, M., Sanchez, A., Torres, J. M., Marquina, D., 2003. The antimicrobial properties of different strains of *Lactobacillus* spp. isolated from kefir. Syst. Appl. Microbiol., 26, 434-437.

Sarkar, A., Mandal, S., 2016. Bifidobacteria—Insight into clinical outcomes and mechanisms of its probiotic action. Microbiol Res, 192, 159-171.

Satsu, H., Hyun, J. S., Shin, H. S., Shimizu, M., 2009. Suppressive effect of an isoflavone fraction on tumor necrosis factor-α-induced interleukin-8 production in human intestinal epithelial Caco-2 cells. J. Nutr. Sci. Vitaminol., 55, 442-446.

Schmid, J., Sieber, V., Rehm, B., 2015. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Front Microbiol, 6, 496.

Shao, L., Wu, Z., Zhang, H., Chen, W., Ai, L., Guo, B., 2014. Partial characterization and immunostimulatory activity of exopolysaccharides from *Lactobacillus rhamnosus* KF5. Carbohydr Polym, 107, 51–56.

Shao, Y., Bao, J., 2015. Polyphenols in whole rice grain: Genetic diversity and health benefits. Food Chem, 180, 86-97.

Sharon, N., 2006. Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta Gen Subj, 1760, 527–537.

Sharon, N., Lis, H., 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology, 14, 53R-62R.

Shinkai, H., Toki, D., Okumura, N., Takenouchi, T., Kitani, H., Uenishi, H., 2016. Polymorphisms of the immune-modulating receptor dectin-1 in pigs: their functional influence and distribution in pig populations. Immunogenetics, 68, 275-284.

Šikić Pogačar, M., Klančnik, A., Bucar, F., Langerholc, T., Smole Možina, S., 2016. Anti-adhesion activity of thyme (*Thymus vulgaris L.*) extract, thyme post-distillation waste, and olive (*Olea europea L.*) leaf extract against Campylobacter jejuni on polystyrene and intestine epithelial cells. J. Sci. Food Agric., 96, 2723-2730.

Soetan k O., Oyekunle, M. A., Aiyelaagbe, O. O., Fafunso, M. A., 2006. Evaluation of the antimicrobial activity of saponins extract of Sorghum bicolor *L. Moench*. Afr. J. Biotechnol., 5(23).

Solanki, V., Tiwari, M., Tiwari, V., 2018. Host-bacteria interaction and adhesin study for development of therapeutics. Int. J. Biol. Macromol., 112, 54–64.

Sonck, E., Stuyven, E., Goddeeris, B., Cox, E., 2009. Identification of the Porcine C-type lectin dectin-1. Vet. Immunol. Immunopathol., 130, 131–134.

Soto, G.E., Hultgren, S.J., 1999. Bacterial adhesins: Common themes and variations in architecture and assembly. J. Bacteriol., 181, 1059–1071.

Stein, H. H., Lagos, L. V., Casas, G. A., 2016. Nutritional value of feed ingredients of plant origin fed to pigs. Anim. Feed. Sci. Tech, 218, 33-69.

Stewart, P. S., Costerton, J. W., 2001. Antibiotic resistance of bacteria in biofilms. The lancet, 358, 135-138.

Suchecka, D., Błaszczyk, K., Harasym, J., Gudej, S., Wilczak, J., Gromadzka-Ostrowska, J., 2017. Impact of purified oat 1-3, 1-4-β-d-glucan of different molecular weight on alleviation of inflammation parameters during gastritis. J Funct Foods, 28, 11-18.

Suchecka, D., Gromadzka-Ostrowska, J., Żyła, E., Harasym, J. P., Oczkowski, M., 2017. Selected physiological activities and health promoting properties of cereal beta-glucans. A review. J. Anim. Feed Sci, 26, 183-191.

Suchecka, D., Harasym, J. P., Wilczak, J., Gajewska, M., Oczkowski, M., Gudej, S., Blaszczyk, D.K., Filip, R., Gromadzka-Ostrowska, J., 2015. Antioxidative and anti-inflammatory effects of high beta-glucan concentration purified aqueous extract from oat in experimental model of LPS-induced chronic enteritis. J Funct Foods, 14, 244-254.

Sun, P., Li, D., Li, Z., Dong, B., Wang, F., 2008. Effects of glycinin on IgE-mediated increase of mast cell numbers and histamine release in the small intestine. J. Nutr. Biochem., 19, 627–633.

Sun, X., Gänzle, M., Wu, J., 2017. Identification and characterization of glycopeptides from egg protein ovomucin with anti-agglutinating activity against porcine K88 enterotoxigenic *Escherichia coli* Strains. J. Sci. Food Agric., 65, 777-783.

Sweeney, T., Collins, C. B., Reilly, P., Pierce, K. M., Ryan, M., O'doherty, J. V., 2012. Effect of purified β-glucans derived from *Laminaria digitata*, *Laminaria hyperborea* and *Saccharomyces* cerevisiae on piglet performance, selected bacterial populations, volatile fatty acids and pro-inflammatory cytokines in the gastrointestinal tract of pigs. Br. J. Nutr., 108, 1226-1234.

Tada, R., Ikeda, F., Aoki, K., Yoshikawa, M., Kato, Y., Adachi, Y., Tanioka, A., Ishibashi, K. ichi, Tsubaki, K., Ohno, N., 2009. Barley-derived β-d-glucan induces immunostimulation via a dectin-1-mediated pathway. Immunol. Lett., 123, 144–148.

Tang, W., Dong, M., Wang, W., Han, S., Rui, X., Chen, X., Jiang, M., Zhang, Q., Wu, J., Li, W., 2017. Structural characterization and antioxidant property of released exopolysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* SRFM-1. Carbohydr Polym, 173, 654-664.

Torrallardona, D., Andrés-Elias, N., López-Soria, S., Badiola, I., Cerdà-Cuéllar, M., 2012. Effect of feeding different cereal-based diets on the performance and gut health of weaned piglets with or without previous access to creep feed during lactation. J. Anim. Sci., 90, 31–33.

Nizet, V., Varki, A., Aebi, M., 2017. Microbial lectins: Hemagglutinins, adhesins, and toxins. Cold Spring Harbor Laboratory Press, 3rd edition.

Veljović, K., Dinić, M., Lukić, J., Mihajlović, S., Tolinački, M., Živković, M., Begović, J., Mrvaljević, I., Golić, N., Terzić-Vidojević, A., 2017. Promotion of Early Gut Colonization by Probiotic Intervention on Microbiota Diversity in Pregnant Sows. Front Microbiol, 8, 2028.

Verhelst, R., Schroyen, M., Buys, N., Niewold, T., 2014. Dietary polyphenols reduce diarrhoea in enterotoxigenic *Escherichia coli* (ETEC) infected post-weaning piglets. Livest Sci, 160, 138–140.

Villalobos, M. D. C., Serradilla, M. J., Martín, A., Ordiales, E., Ruiz-Moyano, S., Córdoba, M. D. G., 2016. Antioxidant and antimicrobial activity of natural phenolic extract from defatted soybean flour by-product for stone fruit postharvest application. J. Sci. Food Agric., 96, 2116-2124.

Vinderola, G., Perdigón, G., Duarte, J., Farnworth, E., Matar, C., 2006. Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine, 36, 254-260.

Vitaglione, P., Mennella, I., Ferracane, R., Rivellese, A.A., Giacco, R., Ercolini, D., Gibbons, S.M., La Storia, A., Gilbert, J.A., Jonnalagadda, S., Thielecke, F., Gallo, Ma., Scalfi, L., Fogliano, V., 2015. Whole-grain wheat consumption reduces inflammation in a randomized controlled trial on overweight and obese subjects with unhealthy dietary and lifestyle behaviors: Role of polyphenols bound to cereal dietary fiber. Am. J. Clin. Nutr., 101, 251–261.

Volman, J. J., Ramakers, J. D., Plat, J., 2008. Dietary modulation of immune function by β-glucans. Physiol. Behav., 94, 276–284.

Volštátová, T., Havlík, J., Doskočil, I., Geigerová, M., Rada, V., 2015. Effect of hydrolyzed milk on the adhesion of lactobacilli to intestinal cells. Scientia agriculturae bohemica, 46, 21-25.

Volstatova, T., Havlik, J., Potuckova, M., Geigerova, M., 2016. Milk digesta and milk protein fractions influence the adherence of *Lactobacillus gasseri* R and *Lactobacillus casei* FMP to human cultured cells. Food & Funct, 7, 3531-3538.

Walker, W.A., and Iyengar, R.S., 2015. Breast milk, microbiota, and intestinal immune homeostasis. Pediatr. Res., 77, 220–228.

Wang, W.L., Wang, W., Du, Y.M., Wu, H., Yu, X.B., Ye, K.P., Li, C.B., Jung, Y.S., Qian, Y.J., Voglmeir, J., Liu, L., 2017. Comparison of anti-pathogenic activities of the human and bovine milk N-glycome: Fucosylation is a key factor. Food Chem, 235, 167–174.

Wang, Y., Gänzle, M. G., Schwab, C., 2010. Exopolysaccharide synthesized by Lactobacillus reuteri decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes. Appl. Environ. Microb.,76, 4863-4866.

Watarai, M., Funato, S., Sasakawa, C., 1996. Interaction of Ipa proteins of *Shigella* flexneri with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. J. Exp. Med., 183, 991–999.

Wilczak, J., Błaszczyk, K., Kamola, D., Gajewska, M., Harasym, J. P., Jałosińska, M., Gudej, S., Suchecka, D., Oczkowski, M., Gromadzka-Ostrowska, J., 2015. The effect of low or high molecular weight oat beta-glucans on the inflammatory and oxidative stress status in the colon of rats with LPS-induced enteritis. Food & Funct, 6, 590-603.

Wismar, R., Brix, S., Lærke, H. N, Frøkiær, H., 2011. Comparative analysis of a large panel of non-starch polysaccharides reveals structures with selective regulatory properties in dendritic cells. Mol Nutr Food Res, 55, 443–454.

World Health Organization, 2015. Global action plan on antimicrobial resistance. WHO Press 1–28.

Wyss, D.F., Wagner, G., 1996. The structural role of sugars in glycoproteins. Curr. Opin. Biotechnol., 7, 409–416.

Yang, S., Höti, N., Yang, W., Liu, Y., Chen, L., Li, S., Zhang, H., 2017. Simultaneous analyses of N-linked and O-linked glycans of ovarian cancer cells using solid-phase chemoenzymatic method. Clin Proteomics, 14, 3.

Yang, Y., Galle, S., Le, M.H.A., Zijlstra, R.T., Gänzle, M.G., 2015a. Feed fermentation with reuteran- and levan-producing Lactobacillus reuteri reduces colonization of weanling pigs by enterotoxigenic *Escherichia coli*. Appl. Environ. Microbiol., 81, 5743–5752.

Yang, Y., Zhao, X., Le, M.H.A., Zijlstra, R.T., Gänzle, M.G., 2015b. Reutericyclin producing *Lactobacillus reuteri* modulates development of fecal microbiota in weanling pigs. Front Microbiol, 6, 762.

Yin, Y., Fatufe, A. A., Blachier, F., 2011. Soya bean meal and its extensive use in livestock feeding and nutrition. In Soybean and Nutrition. InTech.

Zárate, G., Sáez, G.D., and Pérez Chaia, A., 2017. Dairy propionibacteria prevent the proliferative effect of plant lectins on SW480 cells and protect the metabolic activity of the intestinal microbiota in vitro. Anaerobe 44, 58–65.

Zhang, J., Cao, Y., Wang, J., Guo, X., Zheng, Y., Zhao, W., Mei, X., Guo, T., Yang, Z., 2016. Physicochemical characteristics and bioactivities of the exopolysaccharide and its sulphated polymer from *Streptococcus thermophilus* GST-6. Carbohydr Polym, 146, 368–375.

Zhang, J., Zhao, X., Jiang, Y., Zhao, W., Guo, T., Cao, Y., Teng, J., Hao, X., Zhao, J., Yang, Z., 2017. Antioxidant status and gut microbiota change in an aging mouse model as influenced by exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibetan kefir. J. Dairy Sci., 100, 6025-6041.

Zhao, D., Shah, N.P., 2015. Tea and soybean extracts in combination with milk fermentation inhibit growth and enterocyte adherence of selected foodborne pathogens. Food Chem, 180, 306–316.

Zhong, Y., Marungruang, N., Fåk, F., Nyman, M., 2015. Effects of two whole-grain barley varieties on caecal SCFA, gut microbiota and plasma inflammatory markers in rats consuming low-and high-fat diets. Br. J. Nutr., 113, 1558-1570.

Zhu, Y., González-Ortiz, G., Solà-Oriol, D., López-Colom, P., Martín-Orúe, S. M., 2018 a. Screening of the ability of natural feed ingredients commonly used in pig diets to interfere with the attachment of ETEC K88 (F4) to intestinal epithelial cells. Anim. Feed Sci. Technol., 242, 111-119.

Zhu, Y., González-Ortiz, G., Jiménez-Díaz, R., Pérez-Trujillo, M., Parella, T., López-Colom, P., Orúe, S. M. M. (2018 b). Exopolysaccharides (EPSs) obtained from green olive brines

could reduce the adhesion of enterotoxigenic *E. coli* (ETEC) K88 to porcine intestinal epithelial cells. Food & Funct, 9, 3884-3894.

Živković, M., Miljković, M. S., Ruas-Madiedo, P., Markelić, M. B., Veljović, K., Tolinački, M., Soković, S., Golić, N., 2016. EPS-SJ exopolisaccharide produced by the strain *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 is involved in adhesion to epithelial intestinal cells and decrease on *E. coli* association to Caco-2 cells. Front Microbiol, 7, 286.

3. OBJECTIVE AND EXPERIMENTAL DESIGN

In the last years, new biological properties of feed/food ingredients, beyond their nutrient value, have attracted interest from the scientific community. Within them, the ability of some feed/food derived molecules to interfere in the adhesion of pathogenic microorganism to the host epithelium has deserved specific attention. The general hypothesis of this Thesis consider that a rational use of feed/food ingredients in livestock diets, could help to reduce the incidence of intestinal disorders by providing molecular compounds with anti-adhesive properties. This is particularly relevant in the post-weaning period in piglets when the incidence of diarrhoea is high and also the use of prophylactic and therapeutic antibiotics. New feeding strategies aimed to improve the natural resistance of the animal to disease would be of great help to reduce the development of bacterial antibiotic resistances.

Our research group (Animal Nutrition, Management and Welfare group) has been working from some years ago at exploring different feed strategies to fight intestinal pathogens and particularly screening different functional ingredients (Funded public projects: AGL2007-60851/GAN; AGL: 2009-07328/GAN and AGL 2012-31294). The research project of this Thesis would give continuity to this line of research being framed in the project: AGL 2012-31294: *Two new strategies to improve the adaptation of piglets to weaning: the optimizations of Zn status and the EPS from fermentation of green olives*.

The main objectives in this Thesis are the following:

A. To screen the potential of common feed ingredients of piglet diets to interfere in the attachment of Enterotoxigenic *Escherichia coli* (ETEC) K88 (F4) to the intestinal epithelial cells of pigs (IPEC-J2 cells).

B. To characterize the chemical structure of the microbial exopolysaccharides (EPSs) produced during industrial fermentation of table green olives.

C. To assess the anti-adhesive properties of the EPSs isolated from different industrial olive fermenters in the attachment of ETEC K88 (F4) to IPEC-J2 cells.

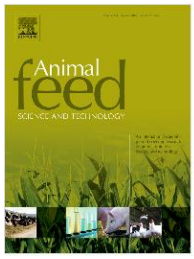
D. To evaluate the effects of different starters (different combinations of *Candida boidinii* and *Lactobacillus pentosus* strains) to control the fermentations of green olives and to improve the anti-adhesive properties of their brines EPSs.

According to these four objectives, three trials were done. Results of these trials will be included in Chapters 4 to 6.

Trial 1: (Zhu et al., 2018a). Screening of the ability of natural feed ingredients commonly used in pig diets to interfere with the attachment of ETEC K88 (F4) to intestinal epithelial cells. The soluble extracts of natural feed ingredients were tested *in vitro* to evaluate their abilities to specifically attach ETEC K88 and to inhibit its adhesion to IPEC-J2 cells. To evaluate these activities different miniaturized in vitro models were used.

Trial 2: (Zhu et al., 2018b). Exopolysaccharides (EPSs) obtained from green olive brines could reduce the adhesion of enterotoxigenic *E.coli* (ETEC) K88 to porcine intestinal epithelial cells. This trial first aimed to characterize the chemical composition and molecular structure of EPSs isolated from olive brines by HPLC and nuclear magnetic resonance (NMR) spectroscopy. Secondly it was evaluated the anti-adhesive properties of different microbial exopolysaccharides (EPSs) isolated from different industrial olive fermenters. For this purpose a set of different miniaturized *in vitro* models were used with ETEC K88 and IPEC-J2 cells.

Trial 3: Starters in the fermentation of green olives can affect functional properties of brine EPS in the prevention of ETEC K88 attachment to IPEC-J2 cells. This trail aimed to evaluate the potential of using different microbial starters to modulate olive fermentation and to improve the anti-adhesive properties of brine EPSs. For that, one strain of *C. boidinii* and four strains of *L. pentosus* strains were involved in a 2 × 5 design giving to ten different starter-treatments. The physicochemical characteristics of the fermentation process were monitored. The anti-adhesive abilities of the different EPSs isolated from the brines were also assessed with similar *in vitro* approaches than in previous trials.



4. SCREENING OF THE ABILITY OF NATURAL FEED INGREDIENTS COMMONLY USED IN PIG DIETS TO INTERFERE WITH THE ATTACHMENT OF ETEC K88 TO INTESTINAL EPITHELIAL CELLS

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5. EXOPOLYSACCHARIDES FROM OLIVE BRINES COULD REDUCE THE ADHESION OF ETEC K88 TO INTESTINAL EPITHELIAL CELLS

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6. STARTERS IN THE FERMENTATION OF GREEN OLIVES CAN AFFECT FUNCTIONAL PROPERTIES OF BRINE EPS IN THE PREVENTION OF ETEC K88 ATTACHMENT TO IPEC-J2 CELLS

Unpublished

6.1. ABSRACT

In this study, a strain of *Candida boidinii* and four *Lactobacillus pentosus* strains were used as starters in green-table olive fermentation (10 treatments in a 2 x 5 design). A total of 20 exopolysaccharides (EPS) samples were isolated from the brines (2 fermentors per treatment) and tested by different miniaturized *in vitro* models using porcine intestinal epithelial cells (IPEC-J2). *In vitro* studies included an adhesion test (AT) aimed to evaluate the ability of EPS to attach specifically ETEC K88 and three different approaches to evaluate its interference on adhesion of the pathogen to IPEC-J2 cells: competition (CT), exclusion (ET) and displacement tests (CT). AT demonstrated that most EPS samples could attach specifically ETEC K88 ($P<0.05$). Regarding the studies with IPEC-J2 cells, CT (ability to block fimbria) showed consistent results for the mutant strain *L. pentosus* 119-14MT when used alone as starter (not combined with *C. boidinii* TOMC-Y13). The corresponding wild-type strain *L. pentosus* 119WT also showed a trend although of lower magnitude. ET (ability to block receptors in the cells) showed consistent reductions for the wild-type strain *L. pentosus* 119WT when used not combined. Regarding *C. boidinii* TOMC-Y13 it was the only starter that significantly showed positive results for AT, CT and ET when used alone. DT failed to demonstrated ability of any EPS samples to remove the pathogen once attached. According to these results, *L. pentosus* 119WT and specially *L. pentosus* 119-14MT seem to be the most effective starters improving the antiadhesive properties of brine EPS, but not when combined with *C. boidinii* TOMC-Y13.

6.2. INTRODUCTION

In Mediterranean regions green-table olives, which are also called Spanish or Sevillian style, is a particularly important industry. During the year 2017-2018, the production of table olives in the world was estimated at 2.95 million tons, and Spain produced about 521,500 tons (17.68 %) (Data from International Olive Oil Council IOOC, 2017.11).

Green-table olives is a fermented food. For their manufacturing, oleurpein in olive fruit is firstly eliminated by a diluted lye solution (sodium hydroxide), and after water wash, green olives are covered with brine in suitable containers, and then the fermentation is carried out spontaneously for several months (Brenes et al., 1998; Rejano et al., 2010). During this time, environmental conditions in the fermenters evolve distinguishing three different stages regarding pH (Lucena-Padrós et al., 2014). During the fermentation, hundreds of bacteria and yeast have been identified by molecular techniques to participate in the process (Lucena-Padrós et al., 2014). Lactic acid bacteria (LAB) have been considered main bacteria responsible for the fermentation by translating sugars into lactic acid (Benítez-Cabello et al., 2015). Yeast also play important roles during the fermentation as they can provide LAB with vitamins, amino acids and purines, and improve LAB growth (Arroyo-López et al., 2008). Actually many of these microbial species has been described to grow on polymicrobial biofilms covering biotics (skin of the olives) and abiotic (fermenter walls) surfaces (Dominguez-Manzano et al., 2012; Grounta et al., 2015).

The predominant LAB forming biofilms on olive surfaces is *Lactobacillus pentosus* although many other yeast species has been also described to coexist in the same biofilms (Arroyo-López et al., 2012). In particular works of Leon-Romero et al. (2016) describe co-aggregative interaction between different strains of *L. pentosus* and *Candida boidinii* isolated from natural green olive fermentations when they were co-cultured together *in vitro*. The same authors suggest that, considering that the proper fermentation of Spanish-style table olives relies on the development of *L. pentosus*-yeast species biofilms in the skins of fruits, the right selection of strains to be used as starter cultures could improve the quality and safety of this food product.

To build biofilms, microbial communities surround themselves by a matrix of exopolysaccharide material (EPSs) and extracellular DNA. This matrix allow bacteria to share nutrients, communicate ant protect themselves from harmful factors in the environment (Stoodley et al., 2002). However together to these, biofilms matrix could also have other still unknown biological functions. Previous works of our group firstly described how EPS isolated from olive brines had abilities to interfere with adhesion of enterotoxigenic *Escherichia coli* (ETEC) K88 to the porcine intestinal mucus and intestinal porcine epithelial

cells (IPEC-J2) (González-Ortiz et al., 2013; 2014). In following researches, it was compared the activity of EPSs isolated from up to 5 different industrial olive fermenters, confirming their biological functions against ETEC K88 but also evidencing variability in the intensity of the anti-adhesive abilities depending on the fermentation batch (Zhu et al., 2018). Variability of results suggests that naturally expected variations in the fermentative communities and environmental conditions in the fermenters could lead to compositional changes in biofilm matrix and EPS functionality. Actually previous research of Leon-Romero et al. (2016) corroborated *in vitro* that the biofilm formation is a complex mechanism, probably affected by changes in environmental conditions that occurs in the natural ecosystem provided by the olive fermentations, and that biofilm formation can be favored by a specific mate of yeast and *L. pentosus* strains. Until now, there is no research regarding the possible effects of using particular olive fermentation starters on the anti-adhesive properties of the EPSs produced by biofilms on olive surfaces. It is therefore hypothesized that using defined starter cultures of *L. pentosus* and *C. boidinii* it could be possible to improve the biofunctions of the microbial EPSs isolated from green-olive brines.

In this study, four preselected *L. pentosus* strains (with different capacity to produce biofilms) were combined or not with *C. boidinii* TOMC-Y13, giving ten possible starters (2 × 5) that were used in 20 pilot fermenters. EPSs isolated from olive brines after 60 days of fermentation, were evaluated in different miniaturized *in vitro* models for their ability to attach ETEC K88 and to interfere in its adhesion to IPEC-J2 cells.

6.3. MATERIAL AND METHODS

6.3.1. OLIVE FERMEANTATION

Manzanilla-variety fruits (4.05 ± 0.49 g size) were obtained during the 2015/2016 season at the green ripening stage from JOLCA, S.A. (Huévar del Aljarafe, Seville, Spain), and transported to the laboratory, where they were debittered according to the Spanish style (lye treatment with 2.3% NaOH). After washing (12 h) to remove excess alkali, the fruits were

brined in polyethylene fermenters with a capacity for 5.2 kg of fruits and 3.4 L of brine (10% NaCl). After 2 days to reach equilibrium, CO_2 was bubbled into fermenters to adjust the initial pH below 6.0. Then, 20 pilot fermenters (2 per treatment) were subjected to 10 different treatments according to a factorial design 5 x 2 (no LAB or one of four *L. pentosus* strains x inoculated or not with *C. boidinii*). According to this design two fermenters were not inoculated and left to ferment spontaneously and the other 18 received one of the 9 different microbial starters (2 fermenters/starter). Initial starter doses per fermenter were adjusted to approximately 7 log10 CFU mL−1 (final concentration) for each of the following *L. pentosus* strains: 119WT, 119-14MT, 13B4WT, 13B4-13MT and to 5 log10 CFU mL−1 for the *C. boidinii* TOMC-Y13 strain (final concentration). The 119WT and 13B4WT strains of *L. pentosus* were previously isolated from diverse table olive processing brines. They were based on their technological and probiotic potential (León-Romero, 2014 PhD Thesis). Non-coagregative spontaneous mutants 119-14MT and 13B4-13MT were obtained as previously described (Furukawa et al., 2012. Biosci. Biotechnol. Biochem 76(2), 326-330). *C. boidinii* TOMC-Y13 strain was selected in basis to their technological and inter-species co-aggregative properties (León-Romero, 2014, PhD Thesis). All these microbial strains belong to the Table Olive Microorganisms Collection (TOMC) of Instituto de la Grasa (CSIC, Seville, Spain).

6.3.2. MONITORING OF THE FERMENTATION

Physiochemical characters of pH, titratable acidity (TA) (expressed as lactic acid, g/100 mL) and combined acidity (CA) (expressed as undissociated organic salts, Eq/L) were tested at different period (0, 30, 60 days) according to the methods described by Garrido Fernandez et al. (1997). The yeast and LAB populations adhered to olive surface or in the fermentation brines were also tested as the description in the study from Benítez-Cabello et al. (2016).

Briefly microbial populations adhered to fruits were isolated by homogenizing clean and previous washed olives for 1 min at 300 rpm in a stomacher model Seward 400 (Seward Medical, Ltd., West Sussex, England). Suspensions of the samples were then plated onto solid selective culture media. Enterobacteriaceae were counted on Crystal Violet Neutral-Red Bile Glucose (VRBD) agar (Merck, Darmstadt, Germany), lactobacilli were spread onto de Man Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, Hampshire, England)

supplemented with 0.02% (wt/vol) sodium azide (Sigma, St. Luis, MI, USA), and yeasts were grown on yeast-malt-peptone-glucose medium (YM) agar (Difco, Becton and Dickinson Company, Sparks, MD, USA) supplemented with oxytetracycline and gentamicin sulfate (0.005%, wt/vol) as selective agents. Counts were expressed as log10 CFU/g.

6.3.3. EPS ISOLATION

The extraction of EPSs from olive-fermentation brines obtained after 60 days of incubation was carried out using the method described by Sánchez et al. (2006) with some modifications. Briefly, brines were centrifuged at 10,000 x g at 4℃ for 30 min to get rid of suspended solids and bacteria. Then, 10% w/v trichloracetic acid (TCA) was added and stirred for 30 min at RT followed by centrifugation to remove the pellet. Two volumes of 96% cold ethanol (4℃) were used to precipitate the EPSs at 4℃ overnight. After this, the precipitated EPSs were recovered by centrifugation, re-suspended in ultrapure water and dialysed in dialysis tubes of a 10 kDa-14 kDa molecular-mass cutoff (Medicell Membrane Ltd.; UK) for 48h at 4℃. Dialysates were subsequently lyophilized and stored at RT until use.

6.3.4. MINIATURIZED *IN VITRO* MODELS

6.3.4.1. PREPARATION OF EPS SOLUTIONS FOR *IN VITRO* STUDIES

Each lyophilized EPS sample was suspended in PBS at a solid-to-liquid ratio of 1:10 (w/v). These suspensions were vortexed and sonicated (J. P. Selecta; Spain) alternately three times for 30s each and then centrifuged at 460 x g for 5min (Mikro 220R, Hettich Zentrifugen). All supernatants were stored at -20℃ until used. Casein glycomacropeptide (CGMP) (Arla Foods, S. A.) was also included as a control in the different tests. Solutions of CGMP to be used in the tests were obtained following the same procedure.

6.3.4.2. *Escherichia coli* STRAINS

In this research, two different strains of *E. coli* were used. ETEC K88 was generously provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo), which was isolated from a colibacillosis outbreak in Spain,[30] with serotype (O149:K91;H10(K-88)/LT-I/STb). The other strain was a non-fimbriated *E. coli* (NF-*E. coli*)

(F4-, F6-, F18-, LT1-, ST2+, Stx2e-) that was kindly donated by the Departament de Sanitat i d'Anatomia Animals of the Universitat Autonoma de Barcelona, and was isolated from the faeces of a post-weaning piglet. The ETEC K88 strain was grown in unshaken Luria broth (LB) at 37ºC for 24h, and the non-fimbrated strain was cultured in shaking LB. Finally, cultured bacteria were centrifuged (1,700 x g, 10 min., 20ºC) and the cell pellet was re-suspended in PBS buffer and adjusted to an optical density (OD) of 1 at 650 nm. For the adhesion test, adjusted suspensions were used directly (OD=1); for the *in vitro* test with IPEC-J2 cells, a dilution of 1/100 times of this suspension was performed to reach a final concentration of 6.5-7 log CFU/ml in order to optimize the bacteria/cells ratio.

6.3.4.3. CELL-CULTURE GROWTH

The IEPC-J2 cells (epithelial cells isolated from the jejunum of the neonatal piglet), kindly donated by Dr. Antony Blikslager, from the College of Veterinary Medicine (North Carolina State University), were cultured in Dulbecco´s Modified Eagle Medium (DMEM)/Ham´s F-12 (GIBCO®, Ref.no.:31331-028, Life Technologies; Spain), with insulin, transferrin, selenium and ethanolamine added as ITS solution (GIBCO®, Ref.no.:41400-045, Life Technologies; Spain). Cells were maintained in an incubator at 37ºC with an atmosphere of 5% CO_2. Cells were used between passages 95 and 105 and were routinely tested to be free of mycoplasma contamination. For the *in vitro* tests (competition, exclusion and displacement tests described below), 2×10^4 cells were seeded into 96-well flat-bottom plates (Nunclon Delta Surface, Ref.no.: 167008, Thermo Scientific; Denmark) in a 200 µl volume. After 24h culture in an atmosphere of 5% CO_2 at 37ºC, cell confluence was confirmed under the microscope by a technician specifically trained for this, and then cells were washed once with PBS. Two-hundred-microlitre CO_2-independent medium (GIBCO®, Ref.no.: 18045-054, Life Technologies; Spain) were added into each well and cells were left in the incubator (37ºC for 24h without CO_2) until use.

6.3.4.4. ADHESION TEST (AT)

Adhesion test was implemented as described previously (González-Ortizet al., 2014). Briefly, 300 µl of the PBS soluble extracts (1:100 w/v) obtained from the different EPSs and CGMP (described above), were incubated into 96-well high-binding polystyrene

microtitration plates (Microlon F plate 655 092; Greiner Bio-One BV) at 4℃ overnight. After removing nonbinding material by sterile PBS wash, wells were incubated with 1% bovine serum albumin and 0.5% sodium azide in PBS (w/v) at 4℃ for 1h to block non-specific adhesion sites. Following twice washes with sterile PBS again, 300 µl of the bacteria suspensions (ETEC K88 or NF-*E. coli*) were incubated for 30 min at room temperature with the 96-well plate. Wells were washed three times with sterile PBS to remove the non-attached bacteria. Three hundred milliliter sterile Luria broth were added, and the sigmoidal growth of bacteria was measured in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37℃ for 12 h at a wavelength of 650 nm at 10-minute intervals (Becker et al., 2007). All the readings were taken in two independent assays and in triplicate per trial.

6.3.4.5. MINIATURIZED ASSAYS WITH IPEC-J2 CELLS

Miniaturized assays were performed as the previous descriptions by González-Ortiz et al. (2013) and Salcedo et al. (2013). A dilution of 1/100 times of suspension (OD=1) of *E. coli* was performed in order to optimize the ratio bacteria/cells.

Competition test (CT)

Solubilized EPS samples (1:100 w/v) were gently mixed with an equal volume of each *E. coli* strain suspension. These mixtures were immediately added to confluent monolayers of IPEC-J2 in 200 µl volume and were incubated with the IPEC-J2 cells at 37℃ for 30 min to allow non-blocked bacteria to adhere to cells. Wells were washed once by gently pipetting with sterile PBS to remove the non-adhered bacteria, but without disturbing the cell monolayer.

Exclusion test (ET)

One hundred microliter of solubilized EPS (1:100 w/v) was gently mixed with equal volume of PBS. After this dilution, mixtures were immediately added to confluent monolayers of IPEC-J2 in 200 µl volume. IPEC-J2 cells were incubated with mixtures at 37℃ for 30 min to allow EPS to adhere to cells. Wells were gently washed twice with sterile PBS to remove the non-adhered EPS, but without disturbing the cell monolayer. One hundred microliter of *E. coli* culture was then mixed with 100 µl PBS, and IPEC-J2 cells were co-incubated with

this mixture again at 37ºC for 30 min. Wells were washed twice by gently pipetting with sterile PBS to remove the non-adhered bacteria, but without disturbing the cell monolayer.

Displacement test (DT)

One hundred microliter *E. coli* culture was mixed with 100 µl PBS, and then were co-incubated with IPEC-J2 cells at 37ºC for 30 min. After washed twice with PBS solutions, IPEC-J2 cells were incubated with 200 µl EPS (0.5%) at 37ºC for 30 min. Wells were then washed twice as previously described.

The following steps were the same for the previous three tests. Two hundred microliter of CO_2-independent medium was added to allow for the growth of the adhered bacteria and to keep cells alive. Plates were covered by a film (VWR, Cat No.6094-064) and monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37ºC for 12 h at a wavelength of 650 nm at 10-minute intervals. All the readings were taken in two independent assays and in triplicate per trial.

6.3.4.6. ANALYSIS OF OD DATA

The OD650 data from the tests were processed by non-linear regression analysis using the non-linear P-NLIN procedure (Gauss-Newton method) through SAS 9.2 (SAS Inc.; Cary, NC, USA) following the equations described by Becker and Galletti (2008). From the time at which the bacterial growth reached an OD650 nm of 0.05 it was defined the $t_{OD=0.05}$ value (in hours).

The final $t_{OD=0.05}$ values were translated into colony forming units (CFU) by correlations previously defined between $t_{OD=0.05}$ values and initial number of bacterial seeded in microplate wells. Cultivated ETEC K88 and NF-*E. coli* strains were serially diluted in LB medium and the CFU/ml determined by plate counting. At the same time, 300 µl per well of each dilution and bacteria were added into microtitration plates in three replicates. Plates monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation) at 37ºC for 18h, as previously described. Fitted equations were: $y = -1.682x + 13.916$ (R^2=0.989) for ETEC K88 and $y = -1.084x + 9.364$ (R^2=0.975) for NF-*E.coli*, where "y" corresponds to $t_{OD=0.05}$ and "x" to the log of CFU per well.

Data from *in vitro* tests were also expressed as the **Δ** t observed between the $t_{OD=0.05}$ values registered for each EPS sample and its PBS control included in a same assay (**Δ** t = $t_{OD=0.05}$ (h) of EPS sample - $t_{OD=0.05}$ (h) of PBS control). In AT, lower negative **Δ** t value reflects the ability of EPS to attach more bacteria. In the other three tests with IPEC-J2 cells, a higher positive **Δ** t value means higher anti-adhesive effects with less attached bacteria to the cells.

6.3.4.7. STATISTICAL ANALYSES

Significant differences between treatments were determined by a linear model with two-way analysis of variance (ANOVA) with the R v.3.3 free software. Values are presented as means ± SEM. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons.

6.4. RESULTS

6.4. 1. PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF FERMENTATION

Table 6.1. shows changes in pH, titratable acidity (TA) and combined acidity (CA) or the fermenters at day 0, 30 and 60. pH decreased during the process being the main decrease observed between day 0 and day 30 (from 5.2± 0.13 to 4.3 ± 0.08). Last 30 days of fermentation pH values only changed slightly from 4.3 ± 0.08 to 4.3 ± 0.08. Regarding the effect of the starters it was found a trend for an increased pH at day 60 when *C. boidinii* was included ($P = 0.06$). There were also found differences related to the different *L. pentosus* strains, corresponding the highest value to the mutant type 119-14 and the lowest to the mutant type 13B4 ($P = 0.06$).

Titratable acidity and combined acidity were clearly increased after the first 30 days of incubation but slightly afterwards. After 60 days, and according to pH values, TA was decreased when *C. boidinii* was included in the starters ($P = 0.07$) but no significant changes were found related to the different *L. pentosus* strains. CA only showed significant

differences related to the treatments at day 0 when we compare fermenters that included or not *C. boidinii* as starters.

Table 6.2. shows microbiological changes registered in the fermentors as plate counts of lactobacilli and total yeast (log CFU/ml) both in the liquid media and in the olive surfaces. Total numbers of yeast did not shown a consistent pattern along the time of incubation and they increased or decreased depending on the treatment. In any case, counts of total yeast were not high and ranged values between 1.30 and 4.15 log CFU/ml for liquid brines and 0 and 3.51 log CFU/ml for olive surfaces. Regarding lactobacilli they were clearly increased in the olive surfaces after 30 days of incubation and decreased afterwards (5.4 ± 0.80, 8.1 ± 0.60 and 6.2 ± 0.27 log CFU/mL for days 0, 30 and 60 respectively). In the liquid brine lactobacilli showed a trend to decrease with time (8.6 ± 1.64, 7.7 ± 0.38 and 6.4 ± 0.40 log CFU/mL for days 0, 30 and 60 respectively).

Regarding the effects of the starters on microbial communities of the fermenters, significant changes were registered in the number of yeast attached to the olive surfaces after 60 days of incubation, being this effect of different magnitude depending on the *L. pentosus* strain (*P* interaction = 0.03). The number of total yeast cells was higher when *C. boidinii* was combined with *L. pentosus* 119-14MT and *L. pentosus* 13B4 WT compared to *L. pentosus* 119WT and *L. pentosus* 13B4 MT. Also it deserves to be mentioned the decreased counts of lactobacilli registered at day 0 when *C. boidinii* were included in the starters (*P* = 0.01).

Table 6.1. Effects of microbial starters on the physicochemical characteristics of the fermentation of table green-olives.

		C. boidinii **TOMC-Y13**		***L. pentosus*** **strain**						***P*****-Value**		
	day	**+**	**-**	**119 WT**	**119-14MT**	**13B4**	**13B4-13MT**	**N**	**RSE**	**C**	**L**	**C × L**
pH	0	5.21	5.21	5.15	5.29	5.22	5.16	5.23	0.11	0.89	0.44	0.08
	30	4.31	4.36	4.34	4.41	4.33	4.28	4.31	0.07	0.13	0.24	0.51
	60	4.29	4.23	4.29	4.31	4.24	4.18	4.29	0.06	0.06	0.06	0.09
TA	0	0.14	0.14	0.15	0.10	0.17	0.15	0.14	0.04	0.64	0.28	0.32
(g/100ml)	30	0.78	0.71	0.87	0.63	0.71	0.77	0.76	0.13	0.27	0.19	0.38
	60	0.83	0.91	0.88	0.79	0.87	0.94	0.88	0.08	0.07	0.23	0.65
CA	0	0.11^{a}	0.12^{b}	0.12	0.10	0.11	0.11	0.12	0.01	0.03	0.36	0.12
(Eq/L)	30	0.15	0.15	0.16	0.15	0.15	0.15	0.15	0.01	0.76	0.60	0.40
	60	0.15	0.16	0.16	0.15	0.15	0.16	0.16	0.01	0.25	0.68	0.13

Different starters resulted from a 2 x 5 design combining or not a *Candida boidinii* TOMC-Y13 strain with four strains of *Lactobacilli pentosus.* Treatments also included one spontaneous fermentation (with no starter addition). Variables included pH, titratable acidity (TA) and combined acidity (CA) of the brine at day 0, 30 and 60 of fermentation.

Mean values were from two independent *in vitro* assays (in triplicate each).
MT & WT. Mutant Type and Wild Type strains, characterized by a different amount of production of EPS (higher in MT).
RSE: Residual standard error.
C: *Candida boidinii* TOMC-Y13.
L: *Lactobacillus pentosus.*
Different superscript letters within a row mean a significant difference ($P < 0.05$) among treatments.

Table 6.2. Effects of different starters on the microbiological fermentation of table green-olives.

			C. boidinii TOMC-Y13		*L. pentosus* strain						*P*-Value		
		day	+	-	119 WT	119-14MT	13B4	13B4-13MT	N	RSE	C	L	C × L
Yeast	**olive**	0	1.53	0.98	2.26	0.57	1.08	1.55	1.33	0.56	0.36	0.76	0.29
		30	0.37	0.68	ND	0.65	ND	1.04	0.93	1.06	0.54	0.51	0.88
		60	1.92[a]	0.66[b]	0.71	2.36	1.36	0.46	1.57	0.83	0.01	0.06	0.03
	brine	0	3.22	3.52	3.81	2.83	3.39	3.59	3.22	0.74	0.39	0.44	0.38
		30	3.54	3.20	2.32	3.49	3.75	3.52	3.76	1.05	0.48	0.33	0.29
		60	1.90	2.04	1.74	2.00	2.04	2.02	2.04	0.34	0.40	0.70	0.20
LAB	**olive**	0	5.54	5.34	5.90	5.47	5.02	6.08	4.96	0.73	0.59	0.30	0.27
		30	8.18	8.07	8.26	8.10	8.33	7.43	8.50	0.48	0.62	0.07	0.17
		60	6.23	6.20	6.28	6.27	5.96	6.06	6.50	0.24	0.74	0.06	0.82
	brine	0	7.54[a]	9.55[b]	7.62	8.86	8.35	8.56	9.34	1.38	0.01	0.53	0.59
		30	7.71	7.70	7.80	7.84	7.56	7.60	7.72	0.47	0.93	0.89	0.87
		60	6.48	6.23	6.31	6.34	6.49	6.37	6.28	0.47	0.27	0.98	0.68

Different starters resulted from a 2 x 5 design combining or not a *Candida boidinii* TOMC-Y13 strain with four strains of *Lactobacillus pentosus.* Treatments also included one spontaneous fermentation (with no starter addition). Variables included total plate counts of lactobacilli and total yeast, on olive surfaces or liquid brines (log CFU/mL) at day 0, 30 and 60 of fermentation.

Mean values (log CFU per well ± SD) were from two independent *in vitro* assays (in triplicate each). MT & WT. Mutant Type and Wild Type strains, characterized by a different amount of production of EPS (higher in MT). RSE: Residual standard error.

C: *Candida boidinii* TOMC-Y13. L: *Lactobacillus pentosus.* LAB: lactic acid bacteria.

Different superscript letters within a row mean a significant difference ($P < 0.05$) among treatments. ND. Not determined.

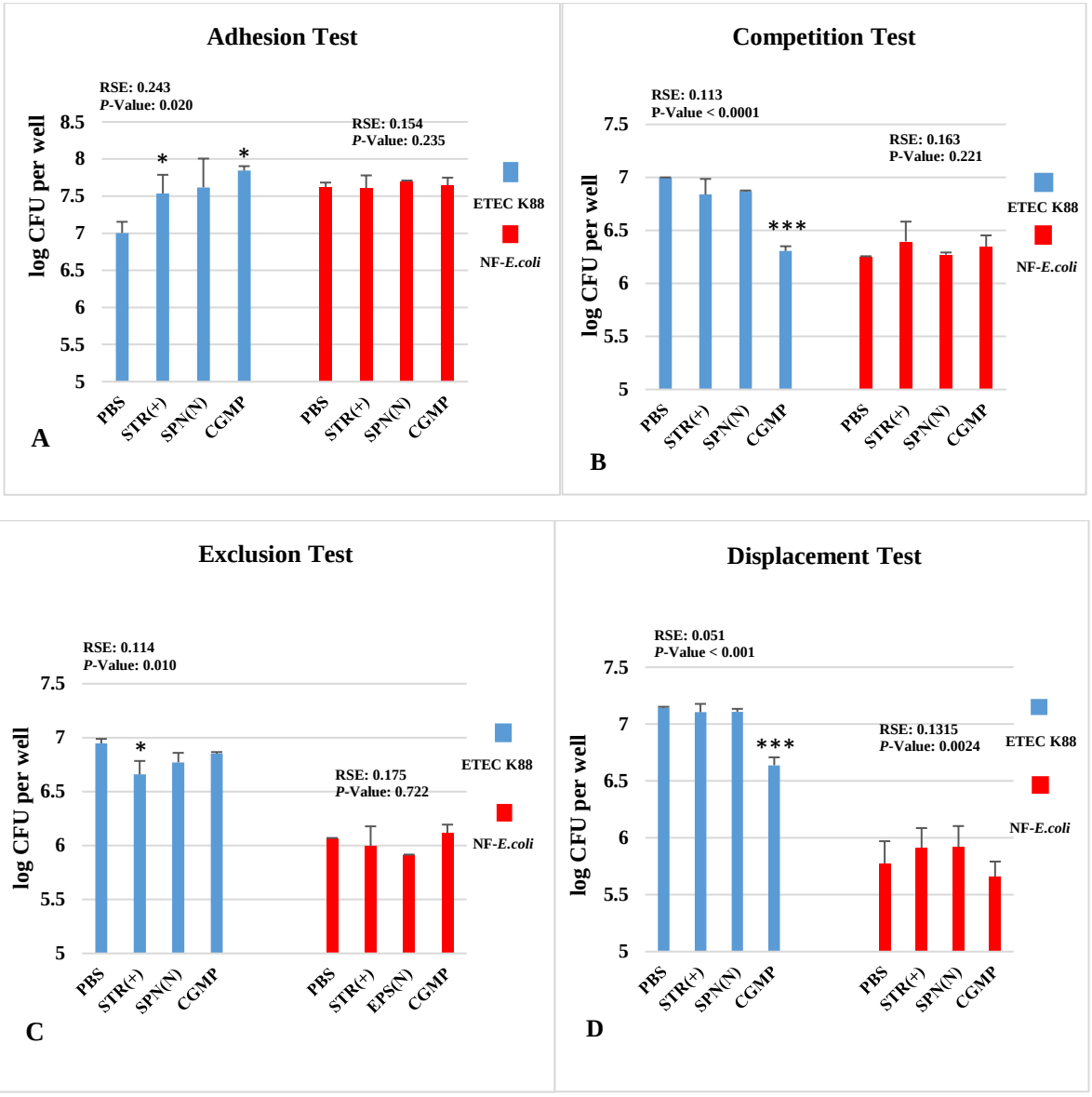

Figure 6.1. Ability of exopolysaccharides (EPSs) isolated from green-olive brines to attach ETEC K88 and/or interfere its adhesion to IPEC-J2 cells.

Number of bacteria (log CFU per well) that attached to coated wells (Adhesion Test) or IPEC-J2 cells (Competition, Exclusion and Displacement Tests). Mean values were from two independent *in vitro* assays (in triplicate each) considered as the experimental units. PBS was included as the negative control and casein glycomacropeptide (CGMP)

as the positive control. For details in preparation of samples see material and methods section. Superscript asterisks of treatments mean significant differences when compared to negative control (PBS): *: *P* <0.05; **: *P* <0.01; *: *P* <0.001. ETEC K88: Enterotoxigenic *Escherichia coli* K88. NF-*E.coli*: Non fimbriated *E. coli*. STR (+): EPS isolated from the green olives fermented with different starters (n=18). SPN (N): EPS isolated from green olives fermented spontaneously (n=2). RSE: Residual standard error.**

6.4.2. ABILITY OF EPS TO ATTACH ETEC K88 AND TO INTERFERE ITS ADHESION TO IPEC-J2 CELLS

Figure 6.1. shows the ability of EPS isolated from green-olive brines to attach and interfere with the adhesion of ETEC K88 to IPEC-J2 cells. Data are expressed as log CFU/well for the different miniaturized *in vitro* models and CGMP and PBS were included as positive and negative control respectively. Results clearly shown how EPS isolated from green olive brines have ability to specifically attach ETEC K88 in a similar degree than CGMP. Regarding the impairment of adhesion of ETEC K88 to IPEC-J2 cells, olive brine EPS reduce numerically the number of adhered bacteria compared to PBS in the CT although differences did not reach the statistical significance ($P = 0.26$). In the ET, EPS obtained from fermenters including starters did reduce significantly the number of adhered ETE K88 cells ($P = 0.02$) but no effect was found in the DT. As expected, results obtained with NF-*E.coli* revealed no significant difference between EPS treatments.

Regarding the effect of the different starters on the functionality of the isolated EPS, Table 6.3. shows the results obtained from the different miniaturized models. Results from the AT could not evidence differences due to the starter in the ability of the EPS to attach specifically ETEC K88 ($P <0.05$). Regarding the studies with IPEC-J2 cells, CT (ability to block fimbria) showed improved results for mutant strain 119-14MT ($P = 0.04$), effect that was specially manifested when used not combined with *C. boidinii* TOMC-Y13 (P interaction = 0.05; see Figure 6.2.). The decreased activity when combined with *C. boidinii* TOMC-Y13 also was seen for the wild type strain (119WT) although with lower $\Delta t_{OD=0.05}$ mean values (Figure 6.2.). The ET (ability to block receptors in the cells) showed for all the starter treatments

reductions in $\Delta t_{OD=0.05}$ of similar magnitude to those reported in the CT for the strain 119-14MT when not combined with *C. boidinii* TOMC-Y13. The DT failed to demonstrated ability of any EPS samples to remove the pathogen once attached regardless of the starter treatment.

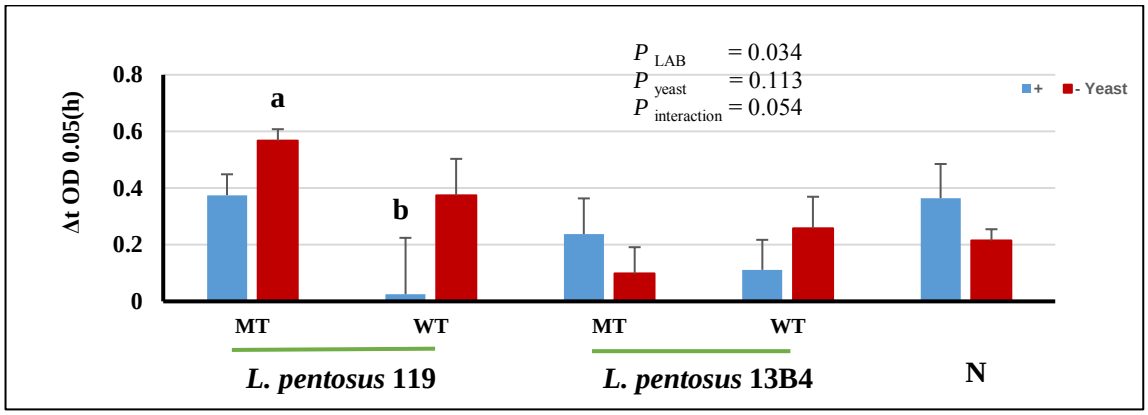

Figure 6.2. Effects of different starters on the ability of the EPS isolated from green-olive brines to reduce the adhesion to IPEC-J2 cells.

Results from *in vitro* competition test (test ability to block fimbria). Different starters resulted from a 2 x 5 design combining or not a *Candida boidinii* TOMC-Y13 strain with four strains of *Lactobacillus pentosus*. Treatments also included one spontaneous fermentation with no starter. Data are expressed as $\Delta t_{OD=0.05}$ values related to the control-PBS included in each assay. Mean values came from two independent *in vitro* assays (in triplicate each). For details see material and methods section. MT & WT. Mutant Type and Wild Type strains, characterized by a different amount of production of EPS (higher in MT). Different superscript letters mean a significant difference ($P < 0.05$) among treatments.

Table 6.3. Effects of different starters on the ability of the EPS isolated from green-olive brines to attach ETEC K88 and interfere its adhesion to IPEC-J2 cells.

		C. boidinii TOMC-Y13		*L. pentosus* strain						*P*-value		
Bacteria	**Test**	**+**	**-**	**119WT**	**119-14MT**	**13B4**	**13B4-13MT**	**N**	**RSE**	**C**	**L**	**C × L**
ETEC K88	AT	-0.87	-0.95	-1.10	-0.86	-0.82	-0.93	-0.84	0.48	0.68	0.91	0.60
	CT	0.22	0.30	0.20^{ab}	0.47^{a}	0.19^{b}	0.17^{b}	0.29^{ab}	0.09	0.11	0.03	0.05
	ET	0.47	0.46	0.57	0.44	0.53	0.41	0.37	0.29	0.97	0.85	0.78
	DT	0.08	0.06	0.10	0.04	0.03	0.05	0.12	0.06	0.57	0.30	0.25

Different starters resulted from a 2 x 5 design combining or not a *Candida boidinii* TOMC-Y13 strain with 4 strains of *Lactobacillus pentosus*. Treatments also included one spontaneous fermentation with no starter. Miniaturized *in vitro* models included: Adhesion Tests (AT), Competition Test (CT), Exclusion Test (ET) and Displacement Test (DT). Data are expressed as $\Delta t_{OD=0.05}$ values related to the control-PBS included in each assay. For details see material and methods section.

ETEC K88: Enterotoxigenic *Escherichia coli* K88. NF-*E.coli*: Non fimbriated *E. coli*. RSE: Residual standard error. C: *Candida boidinii* TOMC-Y13. L: *Lactobacillus pentosus.* Mean values (log CFU per well ± SD) were from two independent *in vitro* assays (in triplicate each). MT & WT. Mutant Type and Wild Type strains, characterized by a different amount of production of EPS (higher in MT). Different superscript letters within a row mean a significant difference ($P < 0.05$) among treatments.

6.5. DISCUSSION

Carbohydrate related structures of receptors on cell surface are the main biofunctional sites for ETEC K88 adhesin recognition (Jin and Zhao, 2000). Several researches have verified that natural ingredients or extracts containing carbohydrates acts as fimbriae or receptor analogues in prevention of bacterial infections (Ofek et al., 2003). In our previous studies, EPS isolated from green olive brines could reduce adhesion of ETEC K88 to porcine intestinal mucus and IPEC-J2 cells (González-Ortiz et al., 2013; 2014). Further research confirmed the anti-adhesive abilities of EPSs from 5 different olive fermenters with variable intensity in the anti-adhesive activity depending on the fermentation batch (Zhu et al., 2018). Those results suggested that differences in the microbial consortia involved in the fermentation of green olives could lead to changes in the composition and structure of the produced EPS and therefore in their activity.

During the olive fermentation, hundreds of bacteria and yeast have been identified to be involved in the different fermentation stages (Lucena-Padrós et al., 2014). Among these microorganisms, *Lactobacillus pentosus*, together with yeast populations are considered the main responsible, but not the only, to synthesized EPSs. Leon-Romero et al. (2016) in their work demonstrated *in vitro* how co-cultures of *L. pentosus* strains and *C. boidinii* were responsible to synthesize the strongest biofilms and that the biofilm formed by *C. boidinii* was stimulated in the absence of cell-cell contact with *L. pentosus*. Authors conclude that biomass and properties of biofilm depends on specific mates of *C. boidinii* and *L. pentosus* and that more than one mechanism might be implicated in the biofilm formation.

Considering these previous results, in this study we wanted to test how different combinations of *L. pentosus* strains, differing in their ability to produce EPS, and the yeast *C. boidinii*, introduced as starters in the fermentation of olives, could induce changes in the biofilm formation and characteristics and properties of EPS produced.

6.5.1. PHYSICOCHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF FERMENTATION

Titratable acidity (TA) and combined acidity (CA) can be used to calculate combined, free, and corrected total acidity (Leal-Sánchez et al., 2003). For all treatments, CA and TA increased significantly in the first 30 days and slowly in the next 30 days in a similar way. Different olive starters did not influence contents of TA or CA in the 30^{th} or 60^{th} day.

In accordance with changes of TA and CA, pH values in brines significantly declines in the first 30 days, and in the following 30 days there was no huge change. In the studies conducted by Blana et al. (2014) and Dalla Rosa et al. (2016) authors also found similar phenomena. Moreover, different *L. pentosus* strains or *C. boidinii* showed trends to influence the pH values at the 60^{th} day (Both $p = 0.06$). Previous research also verified how different olive starters could induce changes in pH values during the fermentation process, especially in the terminal periods (Blana et al., 2014; Dalla Rosa et al., 2016). The reason was probably related to different metabolism of bacteria. For example an increase in the growth of LAB bacteria, responsible to translated sugars into lactic acid, could be associated to decreases in the pH (Benítez-Cabello et al., 2015). Actually in our study the inclusion of *C. boidinii* in the fermentors was associated to a decrease in the numbers of LAB associated to the green olive surfaces (P =0.01) and to an increase of pH at day 60.

We should bear in mind that microorganims do not exist separately but coexist in more or less complex ecosystems. In this regard it is known that on olive surfaces, *Lactobacillus pentosus* and yeast species coexisted in biofilms (Arroyo-López et al., 2012) and that yeast can improve LAB growth by providing vitamins, amino acids and purines (Arroyo-López et al., 2008). However, from our results it seems that the introduction of *C. boidinii* in the starters diminished the growth of LAB in olive surface biofilms. Here it can be remind the results of León-Romero et al. (2016) that could verified *in vitro* the complexity of interactions between different strains of *L. pentosus* and *C. boidinii* in the formation of biofilm. We could hypothesize that in our study the introduction of yeast in the starters would have led to specific changes in the fermentation process and interactions in the biofilm formation that finally had competitively favored the growth of yeast compared to LAB on olive surfaces.

6.5.2. INFLUENCES OF FERMENTATION STARTERS ON EPS INTERFERING WITH ETEC K88 ADHESION

Confirming previous observed results (Zhu et al., 2018), EPS obtained from the starter-controlled fermenters significantly increase the number of attached bacteria in the AT and also reduced the number of ETEC K88 attached to IPEC-J2 cells in the ET (Figure 6.2.). These results confirm the ability of these complex carbohydrates to interfere in the adhesin-receptor recognition. However we were not able to detect, in this case, significant changes in the CT or DT tests. Differences in the nature of EPS between studies and also in the scale of the fermenters (pilot or industrial) could be behind these differences.

Interestingly we could find some differences in the blocking activity of the isolated EPS related to the starter used, confirming the initial hypothesis. Different *L. pentosus* starters influenced the ability of the EPSs to block fimbria in the CT being impaired when 119WT and 119-14MT strains were used combining with *C. boidinii* TOMC-Y13. From the tested combinations of starters the best results were found for 119-14MT when it was not combined with *C. boidinii* TOMC-Y13 and the lowest activity for 119-14WT when combined with the yeast. These results confirm the potential of using defined starters to improve the antiadhesive properties of olive brines EPS. Using as starter, the mutant type strain of *L. pentosus* as 119-14MT, able to produce a higher amount of EPS, appears therefore as potential strategy to control olive fermentation for the production of EPS with higher ant adhesive properties.

Multiply factors could account for preventions of bacteria adhesion by EPSs. Physicochemical and / or structural characteristics of microbial EPSs have been considered as vital factors in the probiotic and enteropathogenic adhesion to human intestinal mucus (Ruas-Madiedo et al., 2006). These characteristics have been demonstrated to be easily influenced by culture conditions that can determine the type and concentration of EPS produced (Sánchez et al., 2006). Even the same bacteria strain has been demonstrated to be capable of synthesizing different EPSs under similar conditions (van Geel-Schutten et al., 1999). In our study changes promoted in the fermentation process by the use of particular starters would have affected the kind of EPS produced and therefore their functional properties. Specifically, results obtained in the CT evaluating the ability of EPS samples to

competitively bond with ETEC K88 fimbriae, suggest that the mechanism involved in this change of activity is an increase in the recognition of bacterial fimbria by isolated EPS when 119-14MT strains is used. The reduction of activity observed for this strain, when used in combination with *C. boidinii* TOMC-Y13, would suggest that this consortium could modify the way by which *L. pentosus* colonize the olives and form biofilms. Changes in the attachment of yeast and/or LAB to olive surfaces could had modulated the fermentation conditions and therefore the amount, and characteristics of the EPS produced. Actually, attending to the number of yeast found in the olive surfaces after 60 days of fermentation, it was registered a relevant increase with this starter combination fact that would support this hypothesis.

6.6. CONCLUSIONS

In this study, we gave a first slight that the different olive starters have influences on the anti-adhesive abilities of EPS produced during the green table olive fermentation. Data from *in vitro* models exhibited that EPSs samples from different olive starters could attach ETEC K88 or inhibit the bacteria attachment to IPEC-J2 cells by combining with the fimbrae or blocking bacteria receptors on the cell surface. Lactic acid bacteria 119-14MT strain could be a potential olive starter as a probiotic with a higher ability of connection with ETEC K88. The combination with *C.boidinii* TOMC-Y13 exhibited a decreasing trend of anti-adhesive ability of EPS. Although the mechanism are still not well understood, results from this study gave a first description about the anti-adhesive properties of EPS probably affected by olive fermentation starters.

6.7. References.

Arroyo-López, F. N., Querol, A., Bautista-Gallego, J., Garrido-Fernández, A., 2008. Role of yeasts in table olive production. Int. J. Food Microbiol. 128, 189-196.

Arroyo-López, F.N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., Garrido-Fernández, A., Jiménez-Díaz, R., 2012. Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. Food Microbiol 32, 295–301.

Becker, P. M., Galletti, S., Roubos-van den Hil, P. J., Van Wikselaar, P. G., 2007. Validation of growth as measurand for bacterial adhesion to food and feed ingredients. J. Appl. Microbiol. 103, 2686-2696.

Becker, P. M., Galletti, S., 2008. Food and feed components for gut health-promoting adhesion of E. coli and Salmonella enterica. J. Sci. Food Agric. 88, 2026-2035.

Benítez-Cabello, A., Romero-Gil, V., Rodríguez-Gómez, F., Garrido-Fernández, A., Jiménez-Díaz, R., Arroyo-López, F. N., 2015. Evaluation and identification of poly-microbial biofilms on natural green Gordal table olives. Antonie Van Leeuwenhoek 108, 597-610.

Benítez-Cabello, A., Bautista-Gallego, J., Garrido-Fernández, A., Rantsiou, K., Cocolin, L., Jiménez-Díaz, R., Arroyo-López, F. N., 2016. RT-PCR–DGGE analysis to elucidate the dominant bacterial species of industrial Spanish-style green table olive fermentations. Front. Microbiol 7, 1291.

Blana, V. A., Grounta, A., Tassou, C. C., Nychas, G. J. E., Panagou, E. Z., 2014. Inoculated fermentation of green olives with potential probiotic Lactobacillus pentosus and Lactobacillus plantarum starter cultures isolated from industrially fermented olives. Food Microbiol 38, 208-218.

Brenes, M., de Castro, A., 1998. Transformation of oleuropein and its hydrolysis products during Spanish-style green olive processing. J. Sci. Food Agric. 77, 353-358.

Dalla Rosa, A., da Silveira, S. M., Coutinho, E. F., Steffens, C., Cansian, R. L., Rigo, E., Backes, G. T., 2016. Green olive fermentation using spontaneous and Lactobacillus plantarum cultures. J Verbrauch Lebensm, 11, 249-257.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F. N., Garrido-Fernández, A., Jiménez-Díaz, R., 2012. Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. Int. J. Food Microbiol. 157, 230-238.

Furukawa, S., Nojima, N., Nozaka, S., Hirayama, S., Satoh, A., Ogihara, H., Morinaga, Y., 2012. Mutants of Lactobacillus plantarum ML11-11 deficient in co-aggregation with yeast exhibited reduced activities of mixed-species biofilm formation. Biosci, Biotechnol, Biochem. 76, 326-330.

Garrido-Fernandez, A., Fernández Díez, M. J., Adams, M. R., 1997. Physical and chemical characteristics of the olive fruit. Table olives. Chapman and Hall, London, United Kingdom, 67-109.

Gonzalez-Ortiz, G., Hermes, R. G., Jimenez-Diaz, R., Perez, J. F., Martin-Orue, S. M., 2013. Screening of extracts from natural feed ingredients for their ability to reduce enterotoxigenic Escherichia coli (ETEC) K88 adhesion to porcine intestinal epithelial cell-line IPEC-J2. Vet. Microbiol. 167, 494-499.

González-Ortiz, G., Pérez, J. F., Hermes, R. G., Molist, F., Jiménez-Díaz, R., Martín-Orúe, S. M., 2014. Screening the ability of natural feed ingredients to interfere with the adherence of enterotoxigenic Escherichia coli (ETEC) K88 to the porcine intestinal mucus. Brit. J. Nutr. 111, 633-642.

Grounta, A., Doulgeraki, A. I., Panagou, E. Z., 2015. Quantification and characterization of microbial biofilm community attached on the surface of fermentation vessels used in green table olive processing. Int. J. Food Microbiol. 203, 41-48.

International Olive Oil Council (IOOC), 2017. November http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures

Jin, L. Z., Zhao, X., 2000. Intestinal receptors for adhesive fimbriae of enterotoxigenic Escherichia coli (ETEC) K88 in swine–a review. Appl. Microbiol. Biotechnol. 54, 311-318.

Leal-Sánchez, M.V., Ruiz-Barba, J.L., Sánchez, A.H., Rejano, L., Jiménez-Díaz, R., Garrido, A., 2003. Fermentation profile and optimization of green olive fermentation using Lactobacillus plantarum LPCO10 as a starter culture. Food Microbiol. 20, 421–430.

León-Romero, Á., Domínguez-Manzano, J., Garrido-Fernández, A., Arroyo-López, F. N., Jiménez-Díaz, R., 2016. Formation of in vitro mixed-species biofilms by Lactobacillus pentosus and yeasts isolated from Spanish-style green table olive fermentations. Appl. Environ. Microb. 82, 689-695.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J. L., 2014. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol. 42, 154-165.

Ofek, I., Hasty, D. L., Sharon, N., 2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. FEMS Immunol. Med. Microbiol. 38, 181-191.

Rejano, L., Montaño, A., Casado, F. J., Sánchez, A. H., de Castro, A., 2010. Table olives: varieties and variations. Olives and olive oil in health and disease prevention. 5-15.

Romero, A.M.L., 2014. Selección de cepas de lactobacillus pentosus con propiedades potencialemente probióticas, aisladas de fermentaciones de aceitunas: estudio de sus propiedades tecnológicas. Tesis doctoral, Universidad de Sevilla.

Ruas-Madiedo, P., Gueimonde, M., Margolles, A., de los REYES-GAVILÁN, C. G., Salminen, S., 2006. Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. J. Food Protect. 69, 2011-2015.

Salcedo, J., Barbera, R., Matencio, E., Alegría, A., Lagarda, M. J., 2013. Gangliosides and sialic acid effects upon newborn pathogenic bacteria adhesion: an in vitro study. Food Chem. 136, 726-734.

Sánchez, J. I., Martínez, B., Guillén, R., Jiménez-Díaz, R., Rodríguez, A., 2006. Culture conditions determine the balance between two different exopolysaccharides produced by Lactobacillus pentosus LPS26. Appl. Environ. Microb. 72, 7495-7502.

Stoodley, P., Sauer, K., Davies, D. G., Costerton, J. W., 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. 56, 187-209.

Van Geel-Schutten, G.H., Faber, E.J., Smit, E., Bonting, K., Smith, M.R., Ten Brink, B., Kamerling, J.P., Vliegenthart, J.F.G., Dijkhuizen, L., 1999. Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the Lactobacillus reuteri wild-type strain and by mutant strains. Appl. Environ. Microb. 65, 3008–3014.

Zhu, Y., González-Ortiz, G., Jiménez-Díaz, R., Pérez-Trujillo, M., Parella, T., López-Colom, P., Martín-Orúe, S. M., 2018. Exopolysaccharides (EPSs) obtained from green olive brines could reduce the adhesion of enterotoxigenic E. coli (ETEC) K88 to porcine intestinal epithelial cells. Food Funct. 9, 3884-3894.

7. GENERAL DISCUSSION

The aim of this Thesis was to screen and evaluate new functional ingredients with anti-adhesive properties as new tools to reduce the incidence of post-weaning diarrhoea caused by ETEC K88 and reduce the use of antibiotics. As has been described in previous chapters, we tested, in different *in vitro* trials, the ability of some common macro-ingredients used in the formulation of piglets' diets and also some specific complex-carbohydrates, particularly microbial EPS produced during the industrial fermentation of table-green olives. Studies also include the evaluation of different starters to modulate the olive fermentation process and in this way the anti-adhesive abilities of the microbial EPSs produced during the fermentation. In the following pages we make a general discussion of these results pointing out the possible mechanisms of action under these anti-adhesive attributes and also to analyze their potential use as non-antibiotic alternatives. Finally we also add a critical view of the *in vitro* methodologies with some discussion regarding possible complementary *in vivo* models.

7.1. MACRO-INGREDIENTS WITH FUNCTIONAL PROPERTIES TO REDUCE THE ATTACHMENT OF PATHOGEN BACTERIA

As well known, interactions between bacteria and hosts are vital during the pathogen infection. Several studies have verified protein-carbohydrate, protein-protein and carbohydrate-carbohydrate as the main interaction ways (Sharon and Lis, 2004; Chagnot et al., 2012; Day et al., 2015). Blocking bacteria adhesins or their receptors on cell surface probably inhibit pathogen attachments. In this way, providing carbohydrate or protein related agents is hoped to be a new natural method in the prevention of diseases, and these natural agents would not cause bacteria resistance like antibiotics (Cozens and Read, 2012).

Previous works of our group found that wheat bran, locust bean and CGMP reduced *in vitro* the attachment of ETEC K88 to IPEC-J2 cells (Hermes et al., 2011; Gonzalez-Ortiz et al., 2013). In the Chapter 4 of this Thesis, other common macro-ingredients of piglet diets were also tested using the same *in vitro* methodology. Among these; cereals like oat, rye, wheat (P =0.05) and their brans significantly reduced the attachments of ETEC K88 to IPEC-J2 cells.

Results from our studies show therefore how some macro-ingredients, particularly some cereals and their by-products, could prevented invasion of intestinal cells by ETEC K88 by inhibiting its attachments to the cells. Many bioactive materials from these cereal and their

brans probably could had contributed to the anti-adhesive abilities. Among them, proteins could be one of these potential molecules, moreover considering that the protein content of these cereals was around 10% (Table 1. in Chapter 4). In this sense results from the one-dimension SDS-PAGE analysis showed different patterns among different cereal brans (Chapter 4, Figure.1.) but quite simpler pattern for rice bran that could be the reason for the failure of this ingredient to prevent the attachment of ETEC K88 to IPEC-J2 cells. Some previous works have verified the anti-adhesive property of vegetable or cereal proteins. Peptides from pea seed bond with *Helicobacter pylori* adhesins (Niehues et al., 2010). Previous studies of our group (Gonzalez-Ortiz et al., 2014) also proposed some proteins from wheat to be involved in the observed results, particularly Globulin 3 (66 k Da) is suggested to interfere in the adhesion of F4 fimbria to IPEC-J2 cells.

Some previous studies have also showed that sugars linking with proteins could also be involved in the interference of connections between bacteria and intestinal cells. In this regard quite a lot of literature can be found with carbohydrates from milk. Shahriar et al. (2006) found how carbohydrate residues in lactadherin were the dependent factors for preventing the attachment of F4-positive ETEC to porcine intestinal villi. In our study the positive control, CGMP, is also a glycoprotein, and its glycosylation sites are probably offering receptor analogs to the bacteria (Grange et al., 2002; Rhoades et al., 2005). Results from the displacement test showed that this ingredient can also detach ETEC K88 once attached to IPEC-J2 cells. These results would suggest that ETEC K88 fimbriae would have a higher affinity for CGMP than for the intestinal cells. In the Exclusion Test, the previous incubation of cells with CGMP did not lead to decreased number of bacteria attached. The main reason probably was that CGMP do not cover receptors on cell surface but only interact with ETEC K88 adhesins.

Lactose also has been describe as the milk carbohydrate structure recognized by adhesins from different K88 (F4) variants (Moonens et al., 2015). Despite this, our results were not able to demonstrate inhibition of adhesion of ETEC K88 to IPEC-J2 cells with sweet whey powder reported as highly rich in lactose (63-75%). In terms of oligosaccharides, oligosaccharide and glycosaminoglycans from human milk own antiadhesive properties against pathogens like *E. coli* and *Salmonella* (Coppa et al., 2006; 2016).

More information also can be found in the literature regarding plant carbohydrates. Becker et al. (2009) found fiber and starch from pea could bind with ETEC K88. Glucomannan hydrolysates from konjac (a plant) and mannose also showed anti-adhesive abilities in *E. coli* attaching to check epithelial cells (Al-Ghazzewi and Tester, 2014). Similar anti-adhesive functions were also found with xyloglucan from cranberry and soluble fiber from plantain (Roberts et al., 2013; Hotchkiss et al., 2015). Especially, xyloglucan, as a hemicellulose, could be found in the primary cells of all higher plants (Fry, 1989). Carbohydrates are also the main components of tested cereal (83%-88%, Table 1. in Chapter 4) and brans. Soluble characters of these carbohydrates are common in cereal grains (Knudsen, 2014).

Moreover, other molecules in these cereal or brans also could not be discarded. Cereal are also sources of polyphenols in considerable content (Manach et al., 2004). Results from certain assays have confirmed polyphenols also owned anti-adhesive properties against pathogen adhesion (Lee et al., 2009; Verhelst et al., 2010; González de Llano et al., 2015). As state above, it could be hypothesized that soluble proteins, carbohydrates or even polyphenols from wheat, oat, rye and their brans would offer receptor analogues to ETEC K88 interfering with its attachment to IPEC-J2 cells.

Many factors should be considered in the formulation of pig diets with the final aim of improving animal health, especially those factors that could compromise the economic profitability of the production. Some cereal grains, such as wheat, oat and rye, are primarily produced for human consumption and therefore they are not as commonly used in animal feeding as maize or barley. The use of wheat bran as one of the main by-products of the flour industry is quite common in animal feeding, no so much other kind of brans. Despite this, some grains or their co-products have been explored for feed formulation. Wheat is used for livestock in Canada, Northern European, and Australia (Stein et al., 2016). Cereal brans (mostly wheat) are wildly used as fiber-rich ingredients in pig diets (Jarrett et al., 2018). Previous study showed that growing pig diet containing 10% (w/w) wheat bran could maintain similar gain: feed ratio to diets including coarse ground corn or sugar beet pulp diet as dietary fibre (Anguita et al., 2007). Full fat rice bran also has been described to contain considerable concentration of digestible and metabolizable energy comparable to wheat and corn (Shi et al., 2015; Stein et al., 2016). Oat bran is easily fermented in large intestine with high butyrate production promoting intestinal health (Bach Knudsen et al., 1993). However,

certain tested cereal and their brans are not yet enough explored for pig diets. Our results may help to explore new formulations of diets especially for weaning piglets. Particularly, different combinations of these ingredients are worthy of attentions.

When extracting conclusion regarding macro-ingredients from our *in vitro* miniaturized models it is important to be aware of the limitation of these methods. Huge differences exist between the simplicity of a small coated well in a plastic plate and the complex environment that represents the intestine. In our model, soluble extracts only interact with the intestinal cells and the tested bacteria, but in the animal multiple interactions could affect bacterial adhesions. Between them: other dietary components, other bacteria microbiota and other host components like the secretion of mucins (Hedemann et al., 2005) or the mucosa architecture (Brunsgaard,1998). Moreover in the *in vitro* models we are only testing the soluble fractions but not the insoluble components of the ingredients such as insoluble fiber that has been also reported to be relevant of the gut health (Montagne et al., 2003). Also the possible peptic or enzymatic digestion of some of these soluble compounds in the stomach and foregut is not taken in consideration. Therefore before recommending the use of these ingredients in piglet diets, more studies would be needed such as identifying bioactive molecules and their resistance to endogenous digestion and verifying their use *in vivo* under natural challenging conditions or experimental models of colibacillosis.

7.2. MICROBIAL EPS OBTAINED FROM THE INDUSTRIAL OLIVE FERMENTATION AS A POTENTIAL ANTIDIARRHOEIC FEED ADDITIVE

The anti-adhesive properties of EPS produced during table green olive fermentation will be specifically discussed in the following text.

In the second trial of this Thesis, five EPSs were isolated from different olive fermentation brines and tested in miniature assays. In CT, no EPS sample significantly reduced ETEC K88 attachments to IPEC-J2 cells as the study conducted by Gonzalez-Ortiz et al. (2014), but positive results appeared in AT, ET and DT among EPSs samples (Figure 3.,.5.,.6.). Especially, EPS2 could bind with ETEC K88, block bacteria receptors on the surface of IPEC-J2 cell and also significantly remove attached bacteria from cells. In addition the other

four EPSs samples, successfully detached ETEC K88 from cells in the DT suggesting a higher affinity of the ETEC K88 adhesins for EPSs than for IPEC-J2 cell receptors. Variances of results among EPS samples tested in this study shows that certain factors affect the anti-adhesive abilities of EPS samples. As hundreds of bacteria and yeast take part in the olive fermentation process (Lucena-Padrós et al., 2014) variations in the microbial consortium can be expected with variation in environmental and fermentation conditions. Bacteria such as *Lactobacillus pentosus* and yeast has been reported to build biofilms and produce EPS on the olive epidermis (Domínguez-Manzano et al., 2012; Arroyo-López et al., 2012), from this point of view it seems plausible to modulate the microbial communities by the use of defined fermentation starters.

In the third trial we tested therefore up to 10 different starters in an attempt to see differences in the characteristics of the EPS produced. Relevant results could be found in AT, CT and ET for the different isolated EPS, however no starter showed significant effects in DT contrary to trial 2. Comparison of results between Trial 2 and 3 can be found in Table 7.1. Differences between studies can be due to the complexity of the interactions and also to differences between industrial (Trial 2) and lab-scale (Trial 3) fermenters. In any case, from both trials, the potential of microbial EPS to interact with bacteria-cell recognition was evidenced.

As previously described, interactions between bacteria adhesins and their receptors are specific. Carbohydrate structures of receptors offers adhesion sites for many bacteria (Sharon, 2006). To K88 adhesin, carbohydrate-related receptors has been reported (Grange et al., 2002). In particular, galactose (Gal) and glucose (Glu) residues has been related to microbial adhesion. Payne et al. (1993) verified the vital role of β-linked Gal residues in the connections of glycoproteins and glycolipids with K88 adhesin. A terminal Gal β-linked to the *N*-acetylhexosamine assisted all K88 adhesin variants binding to glycosphingolipids (Grange et al., 2002). Moonens et al. (2015) found terminal Gal in the lactosyl unit was the carbohydrate site of two short amino acid from $F4_{ad}$ ($K88_{ad}$). The terminal β-linked Gal from egg white glycopeptides was supposed as one of binding sites for $K88_{ac}$ fimbriae (Sun et al., 2017). Regarding bacteria EPS, Glu-containing EPSs also interfered with the attachments of

Table 7.1. Summary of results from Trial 2 and Trial 3 about properties of EPS samples interfering with ETEC K88 adhesion

	Trial 2[A]					Trial 3[B]									
						C. boidinii TOMC-Y13					-				
	EPS1	EPS2	EPS3	EPS4	EPS5	119WT	119-14MT	13B4	13B4-13MT	N	119WT	119-14MT	13B4	13B4-13MT	N
AT		*			**	**	**	**	**	**	*	**	***	*	*
CT									*		*	**			
ET		*		*		*				*	*		*		
DT	**	**	**	**	**										

[A] : Data represent results of EPS samples from 5 different industrial fermenters.

[B] : Data represent results from the fermentor with most significant effects from the two included for each starter combination. Asterisks of treatments mean significant differences when compared to negative control (PBS): *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

E.coli strains to porcine erythrocytes (Wang et al., 2010). In this Thesis, as both sugars in all five EPS samples from Trail 2 were in high percentages, EPSs probably connected with ETEC K88 fimbriae with their Gal and Glu residue sites (Character 5, table 1.)

As previously shown, microbiota participating in green olive fermentation did not exist separately, but in complex ecosystems. Yeast improve LAB growth by providing vitamins, amino acids and purines (Arroyo-López et al., 2008). Arroyo-López et al. (2012b) found that *Lactobacillus pentosus* and yeast species coexisted in the same biofilms on olive surfaces. Results from the study of Leon-Romero et al. (2016) also verified co-aggregative interaction between different strains of *L. pentosus* and *C. boidinii* isolated from natural green olive fermentations when they were co-cultured together *in vitro*. Authors further explained that co-cultured *L. pentosus* and *C. boidinii* facilitated to form biofilms, and biomass in biofilm varied among different mates of *C. boidinii* and *L. pentosus.* Based on these results, it was easy to understand in this Thesis, why CFU of yeast and LAB on olive surfaces on the 60th day of fermentation seemed to have been affected by the *L. pentosus* starters and *C. boidinii* TOMC-Y13. Selective connections between yeast and LAB probably were regulated by these starters, and different biofilms were formed by these attached bacteria on olive surface partly released into olive brines. In addition, attached bacteria, especially LAB, could help to translate sugar into lactic acid inducing changes of pH in brines on the 60^{th} day. Culture conditions of EPS-producing bacteria has been describe to influence the type and concentration of EPS (Sánchez et al., 2006; Laws et al., 2008). As a conclusion it could be deduced that the use of defined starters in the industrial fermentation of green olives can improve the anti-adhesive properties of the EPSs produced. Results from CT showed that *L. pentosus* 199 14MT strain, used as starter, is capable to increase anti-adhesive properties of brine isolated EPSs. This improvement is however lost if the strain is combined with *C. boidinii* TOMC-Y13.

Our study help to move one step forward exploring bio-functions of green table olives. However there is still a long way to go for the industrial application of these results. In Mediterranean regions green table olive is a particularly important industry with million of tons produced each year (IOOC, 2017.11). As previously described, LAB and yeast together embedded themselves in biofilms on surface of green table olive (Arroyo-López et al.,

2012b). Green table olive have been therefore proposed as a good vehicle for probiotics, as dairy productions, especially for people have milk allergies. This is not just a hypothesis, studies conducted by Lavermicocca et al (2005) detected *L. paracasei* IMPC2.1 from faeces of volunteers after oral intake of table olives highly colonized by this bacteria on surface. We could therefore hypothesize that modulating the fermentation of olives with starters of high EPS producing LAB strains could therefore help to reduce diarrhoea caused by *E.coli* in humans by consumption of green table olive. However for this application there are still lots of questions that need to be answered first. For example to determine the chemical nature of EPS on olive biofilms and to confirm its stability along shelf life of the product. Not necessarily the EPS isolated and tested on this Thesis should be the same to those found on the olive surfaces ready to eat. On the other hand, it should be also evaluated the effective intake of olive need to promoted the claimed effect.

Other possible industrial application would be the use of the isolated EPS obtained from the brines in human or animal diets. This is actually the application initially stated in this Thesis. In the industrial fermentation process of olives, huge amounts of brines are finally wasted. Utilizing EPS from these brines as feed additive could therefore will give added value to this by-product. However before any industrial application of these EPSs it would be needed to find an economically effective way to isolate EPS from brines at large scale. In our study, we isolate EPS based on precipitation of EPS with alcohols but this methodology probably could not be translated to a large scale in an economic and environmental viable way. Results from our studies also indicated that not all EPSs from olive brines owned the same anti-adhesive properties. In this sense it would also be a must to standardize and define the fermentation conditions of the olives for the standardization of the EPS produced.

As well known, fermentation conditions and bacteria strains greatly influenced types of EPSs (Badel et al., 2011). Fermentation conditions such as pH, temperature, medium in bioreactors are easily controlled through industrial technology, so producing targeted EPSs in bioreactors also could be regarded as a potential strategy to produce antidiarrhoeal EPS decoupled from the olive industry. Considerable information is available nowadays from productions of some commercial EPSs (Patel et al., 2011). For the production of these EPS in bioreactors it would be needed to define many parameters that undoubtedly represent many technical challenges

(Seviour et al., 2011). Efforts would be needed, especially in selection of cheaper substrates, development of higher yielding strains and optimizing fermentation conditions (Poli et al., 2011).

7.3. LIMITATIONS OF MINIATURIZED IN VITRO TESTS AND ALTERNATIVE IN VIVO MODELS

In the last section of this chapter we also would like to add some discussion regarding the methods of study we dispose when we want to test new anti-diarrheic in-feed additives that have not get a large-scale production. Scarce amount of experimental product can therefore impose practical restrictions to test them in large animals as pigs.

Miniaturized *in vitro* models, like those used in this Thesis, offers undoubtedly an inestimable tool to test defined modes of action of a new additive and require a very small amount of material. They are therefore very useful to screen easily a large number of products in a cost-effective way. However *in vitro* trials can not reflect real environments of animal intestine in term of physiology and physical processes. When an additive or a nutraceutic is given to an animal, or to a person, it can exert lots of effects with interactions with other components of the digesta, the immune system or the intestinal microbiota. As we have reviewed before, functional ingredients could reduce diarrhoea by different ways like immune modulation, prebiotic effects, bactericidal functions or anti-adhesive properties, between other. Miniature assays in this Thesis were designed to test exclusively anti-adhesive properties and preclude important factors like the presence of the intestinal microbiota or the immune response of the animal. *In vitro* results can evidence the anti-adhesive characters of EPSs samples with IPEC-J2 cells, but obviously effects could be different when given to an animal.

The use of animal models of disease appears therefore as an indispensable way to test the efficacy of a new antidiarrhoeic additive. The use of experimental models of post-weaning colibacilosis in piglets has been used successfully used in our research group to test different feed additives and probiotics (Hermes et al., 2011; Barba-Vidal et al., 2017. However to test in-feed experimental additives in piglets require a large amount of product considering that a pig could consume up to 5-6 kg of feed during the first three weeks post-weaning.

Table 7.2. Parameters to be optimized in the implementation of ETEC diarrhoea models in mice

		Assay 1	Assay 2	Assay 3
Parameters to be optimized	**Bacterial Strain**	ETEC K88, B41	ETEC K88, B41	ETEC B41
	Bacterial dose	10^9, 10^{10} CFU/ml	10^{11} CFU/ml	10^{11} CFU/ml
	Weaning weight	15, 20 g	10 g	10 g
	Previous treatment			cimetidine
	Bacterial phase			4 h, 12 h inoculation
Parameters of responses	**Body weight changes**			
	Diarrhoea scores	In faeces and colon	In faeces and colon	In faeces and small intestine
	Microbiology			
	Water & feed intake			
	Cecal water content			
	Histology scores			

Background in grey colour in the table means parameters executed or examined.

Alternatively other animal models like mice or rat models could turn as an alternative when we dispose of limited amount of experimental product as in this Thesis for EPS.

Actually during the development of this research we tried to implement a mice model of post-weaning colibacillosis but we did not have success. In three different assays with Hsd: ICR (CD-1®) mice we tried to define the optimum parameters to get a clinical course of post-weaning diarrhoea. Parameters tested in the different assays are shown in Table 7.2. We tested different ETEC strains (K88 and B41), different growth conditions for the inocula (4 or 12 h of incubation), different oral doses (10^9-10^{11} CFU), different days post-weaning (20, 15 or 10 gr. of weight) and the use or not of cimetidine previous to the oral inoculation. To monitorize the response of the animals we registered changes in body weight, feed and water intake, diarrhoea scores in faces and digesta, microbial count of coliforms, cecal water content and histology scores (after necropsy).

We were not able to reproduce successfully the disease in any of the assays as the animals showed to be quite resistant to the pathogens. No significant changes were registered in the cecal water content or in the histological scores in any of the assays. Only in one of the studies (assay 1) the strain ETEC B41 showed to promote a slight increase in the fecal score of some animals. In the assay 2 this strains also promoted moderate reductions in body weight gains two days post-challenge, suggesting that this mice strain could be more susceptible to B41 than to K88. Promoting an early weaning (just at 10 gr. of live weight vs. 20 gr.) did not either induced diarrhoea after the challenge although animals with lower weights presented an increase in cecal coliforms numbers after the inoculation (less than 0.5 log units).

Some successful experimental models of colibacillosis can be found in the literature (Ritchie, 2015), however in those models authors use protocols incompatible with the test of in–feed additives, as the use of antibiotic-induced disbiosis (Aguilera et al., 2015), infant mice (Goldhar et al., 1986) or other highly virulent strains of *E. coli* like EPEC, EHEC or VTEC strains (Toledo et al., 2011). In summary we were not able to induce diarrhoea even using the most extreme conditions showing the Hsd: ICR (CD-1®) mice strain a high natural resistance to ETEC oral challenge.

Alternatively other animal models, like small intestinal segment perfusion model in piglet (Chen et al., 2014), could be also be consider for testing low amounts of experimental additives. However the implement of these methods is complex and was out of the scope of this Thesis.

In summary in this work we have demonstrated that the water soluble fractions of some macro–ingredients, and also the microbial EPS isolated from olive brines, exhibit anti-adhesive properties against ETEC K88. These results suggest that the inclusion of these ingredients in the piglets' diets could help to reduce the incidence of post-weaning diarrhoea and the use antibiotic at this early stage. We found water-soluble extracts from wheat, oat, rye and their brans capable of reducing attachments of ETEC K88 to IPEC-J2 cells. Specific EPS, isolated from brines obtained from industrial fermentation of green olives also interfere with adhesion of ETEC K88 to IPEC-J2 cells. Moreover the use of *L. pentosus* 199 14MT strain as starter in this fermentation improve the anti-adhesive properties of the brines isolated EPSs, probably by modulating the bacteria and yeast biofilms that live on olive surfaces. These new results will help to open new lines of research in the prevention of piglet diarrhoea.

7.4. References.

Aguilera, M., Cerdà-Cuéllar, M., Martínez, V., 2015. Antibiotic-induced dysbiosis alters host-bacterial interactions and leads to colonic sensory and motor changes in mice. Gut Microbes, 6, 10-23.

Al-Ghazzewi, F. H., Tester, R. F., 2014. Inhibition of the adhesion of *Escherichia coli* to human epithelial cells by carbohydrates. Bioactive Carbohydrates and Dietary Fibre, 4, 1-5.

Anguita, M., Gasa, J., Nofrarias, M., Martín-Orúe, S.M., Pérez, J.F., 2007. Effect of coarse ground corn, sugar beet pulp and wheat bran on the voluntary intake and physicochemical characteristics of digesta of growing pigs. Livest Sci, 107, 182–191.

Arroyo-López, F. N., Querol, A., Bautista-Gallego, J., Garrido-Fernández, A., 2008. Role of yeasts in table olive production. Int. J. Food Microbiol., 128, 189-196.

Arroyo-López, FN, Romero-Gil, V, Bautista-Gallego, J, RodríguezGómez, F, Jiménez-Díaz, R, García-García, P, Querol, A, GarridoFernández, A., 2012a. Yeasts in table olive processing: desirable or spoilage microorganisms? Int. J. Food Microbiol., 160:42–49.

Arroyo-López, F.N., Bautista-Gallego, J., Domínguez-Manzano, J., Romero-Gil, V., Rodriguez-Gómez, F., García-García, P., Garrido-Fernández, A., Jiménez-Díaz, R., 2012b. Formation of lactic acid bacteria-yeasts communities on the olive surface during Spanish-style Manzanilla fermentations. Food Microbiol., 32, 295–301.

Badel, S., Bernardi, T., Michaud, P., 2011. New perspectives for *Lactobacilli* exopolysaccharides. Biotechnol. Adv., 29, 54-66.

Barba-Vidal, E., Castillejos, L., López-Colom, P., Rivero Urgell, M., Moreno Muñoz, J. A., Martín-Orúe, S. M., 2017. Evaluation of the probiotic strain *Bifidobacterium longum subsp*. infantis CECT 7210 capacities to improve health status and fight digestive pathogens in a piglet model. Front Microbiol, 8, 533.

Becker, P. M., Van Wikselaar, P. G., Jansman, A. J. M., Van der Meulen, J., 2009. Pea dietary fiber for adhesion and excretion of enterotoxigenic *E. coli* K88 to prevent intestinal colonization. J. Anim. Sci., 87, 172-189.

Brunsgaard, G., 1998. Effects of cereal type and feed particle size on morphological characteristics, epithelial cell proliferation, and lectin binding patterns in the large intestine of pigs. J. Anim. Sci., 76, 2787-2798.

Chagnot, C., Listrat, A., Astruc, T., Desvaux, M., 2012. Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. Cell. Microbiol., 14, 1687-1696.

Chen, X. Y., Woodward, A., Zijlstra, R. T., Gänzle, M. G., 2014. Exopolysaccharides synthesised by Lactobacillus reuteri protect against enterotoxigenic *Escherichia coli* in piglets. Appl. Environ. Microb., AEM-01782.

Coppa, G. V., Zampini, L., Galeazzi, T., Facinelli, B., Ferrante, L., Capretti, R., Orazio, G., 2006. Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrhoeal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatr. Res., 59, 377.

Coppa, G.V., Facinelli, B., Magi, G., Marini, E., Zampini, L., Mantovani, V., Galeazzi, T., Padella, L., Marchesiello, R.L., Santoro, L., Coscia, A., Peila, C., Volpi, N., Gabrielli, O., 2016. Human milk glycosaminoglycans inhibit in vitro the adhesion of *Escherichia coli* and *Salmonella fyris* to human intestinal cells. Pediatr. Res., 79, 603–607.

Cozens, D., Read, R.C., 2012. Anti-adhesion methods as novel therapeutics for bacterial infections. Expert Rev Anti Infect Ther, 10, 1457–1468.

Day, C. J., Tran, E. N., Semchenko, E. A., Tram, G., Hartley-Tassell, L. E., Ng, P. S., King, R.M., Ulanovsky, R., McAtmney, S., Apicella, M.A., Tiralongo, J., Morona, R., Korolik, V., Tiralongo, J., 2015. Glycan: glycan interactions: high affinity biomolecular interactions that can mediate binding of pathogenic bacteria to host cells. PNAS, 112, E7266-E7275.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F.N., Garrido-Fernández, A., Jiménez-Díaz, R., 2012. Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. Int. J. Food Microbiol., 157, 230–238.

Fry, S.C., 1989. The structure and functions of xyloglucan. J. Exp. Bot., 40, 1–11.

González de Llano, D., Esteban-Fernández, A., Sánchez-Patán, F., Martínlvarez, P. J., Moreno-Arribas, M., Bartolomé, B., 2015. Anti-adhesive activity of cranberry phenolic

compounds and their microbial-derived metabolites against uropathogenic *Escherichia coli* in bladder epithelial cell cultures. Int J Mol Sci, 16, 12119-12130.

Gonzalez-Ortiz, G., Hermes, R. G., Jimenez-Diaz, R., Perez, J. F., Martin-Orue, S. M., 2013. Screening of extracts from natural feed ingredients for their ability to reduce enterotoxigenic *Escherichia coli* (ETEC) K88 adhesion to porcine intestinal epithelial cell-line IPEC-J2. Vet. Microbiol., 167, 494-499.

González-Ortiz, G., Pérez, J. F., Hermes, R. G., Molist, F., Jiménez-Díaz, R., Martín-Orúe, S. M., 2014. Screening the ability of natural feed ingredients to interfere with the adherence of enterotoxigenic *Escherichia coli* (ETEC) K88 to the porcine intestinal mucus. Br. J. Nutr., 111, 633-642.

Goldhar, J., Zilberberg, A., Ofek, I., 1986. Infant mouse model of adherence and colonization of intestinal tissues by enterotoxigenic strains of *Escherichia coli* isolated from humans. Infect. Immun., 52, 205-208.

Grange, P.A., Mouricout, M.A., Levery, S.B., Francis, D.H., Erickson, A.K., 2002. Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors Infect. Immun., 70, 2336–2343.

Hedemann, M.S., Mikkelsen, L.L., Naughton, P.J., Jensen, B.B., 2005. Effect of feed particle size and feed processing on morphological characteristics in the small and large intestine of pigs and on adhesion of *Salmonella enterica* serovar Typhimurium DT12 in the ileum in vitro. J. Anim. Sci., 83, 1554–1562.

Hermes, R. G., Manzanilla, E. G., Martín-Orúe, S. M., Pérez, J. F., Klasing, K. C., 2011. Influence of dietary ingredients on *in vitro* inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). Comp. Immunol. Microb., 34, 479-488.

Hotchkiss, A.T., Nuñez, A., Strahan, G.D., Chau, H.K., White, A.K., Marais, J.P.J., Hom, K., Vakkalanka, M.S., Di, R., Yam, K.L., Khoo, C., 2015. Cranberry Xyloglucan Structure and Inhibition of Escherichia coli Adhesion to Epithelial Cells. J. Agric. Food Chem., 63, 5622–5633.

International Olive Oil Council, IOOC, 2017, <http://www.internationaloliveoil.org>.

Jarrett, S., Ashworth, C. J., 2018. The role of dietary fibre in pig production, with a particular emphasis on reproduction. J Anim Sci Biotechnol, 9, 59.

Knudsen, K. E. B., Jensen, B. B., Hansen, I., 1993. Oat bran but not a β-glucan-enriched oat fraction enhances butyrate production in the large intestine of pigs. J. Nutr., 123, 1235-1247.

Knudsen, K. E. B., 2014. Fiber and nonstarch polysaccharide content and variation in common crops used in broiler diets. Poult. Sci., 93, 2380-2393.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., Bäckhed, F., 2016. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell, 165, 1332–1345.

Lavermicocca, P., Valerio, F., Lonigro, S. L., De Angelis, M., Morelli, L., Callegari, M. L., Rizzello, C.G., Visconti, A., 2005. Study of adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. Appl. Environ. Microb., 71, 4233–4240.

Laws, A.P., Chadha, M.J., Chacon-Romero, M., Marshall, V.M., Maqsood, M., 2008. Determination of the structure and molecular weights of the exopolysaccharide produced by *Lactobacillus acidophilus* 5e2 when grown on different carbon feeds. Carbohydr. Res., 343, 301–307.

Lee, J. H., Shim, J. S., Chung, M. S., Lim, S. T., Kim, K. H., 2009. In vitro anti-adhesive activity of green tea extract against pathogen adhesion. Phytother Res, 23, 460-466.

León-Romero, Á., Domínguez-Manzano, J., Garrido-Fernández, A., Arroyo-López, F. N., Jiménez-Díaz, R., 2016. Formation of in vitro mixed-species biofilms by Lactobacillus pentosus and yeasts isolated from Spanish-style green table olive fermentations. Appl. Environ. Microb., 82, 689-695.

Lucena-Padrós, H., Caballero-Guerrero, B., Maldonado-Barragán, A., Ruiz-Barba, J.L., 2014. Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques. Food Microbiol., 42, 154–165.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: Food sources and bioavailability. Am. J. Clin. Nutr., 79, 727–747.

Montagne, L., Pluske, J.R., Hampson, D.J., 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol., 108, 95–117.

Moonens, K., Van Den Broeck, I., De Kerpel, M., Deboeck, F., Raymaekers, H., Remaut, H., De Greve, H., 2015. Structural and functional insight into the carbohydrate receptor binding of F4 fimbriae-producing enterotoxigenic *Escherichia coli*. J. Biol. Chem., 290, 8409–8419.

Niehues, M., Euler, M., Georgi, G., Mank, M., Stahl, B., Hensel, A., 2010. Peptides from Pisum sativum L. enzymatic protein digest with anti-adhesive activity against Helicobacter pylori: Structure-activity and inhibitory activity against BabA, SabA, HpaA and a fibronectin-binding adhesin. Mol Nutr Food Res, 54, 1851–1861.

Patel, S., Goyal, A., 2011. Functional oligosaccharides: production, properties and applications. World J. Microb. Biot., 27, 1119-1128.

Payne, D., O'Reilly, M., Williamson, D., 1993. The K88 fimbrial adhesin of enterotoxigenic Escherichia coli binds to β1- linked galactosyl residues in glycosphingolipids. Infect. Immun., 61, 3673–3677.

Poli, A., Di Donato, P., Abbamondi, G. R., Nicolaus, B., 2011. Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. Archaea, 2011.

Rhoades, J. R., Gibson, G. R., Formentin, K., Beer, M., Greenberg, N., Rastall, R. a., 2005. Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. J. Dairy Sci., 88, 3455–3459.

Ritchie, J. M., 2015. Animal models of enterohemorrhagic *Escherichia coli* infection. In Enterohemorrhagic *Escherichia coli* and Other Shiga Toxin-Producing *E. coli*, 175-195. American Society of Microbiology.

Roberts, C.L., Keita, Å.V., Parsons, B.N., Prorok-Hamon, M., Knight, P., Winstanley, C., O'Kennedy, N., Söderholm, J.D., Rhodes, J.M., Campbell, B.J., 2013. Soluble plantain fibre blocks adhesion and M-cell translocation of intestinal pathogens. J. Nutr. Biochem., 24, 97–103.

Sánchez, J. I., Martínez, B., Guillén, R., Jiménez-Díaz, R., Rodríguez, A., 2006. Culture conditions determine the balance between two different exopolysaccharides produced by *Lactobacillus pentosus* LPS26. Appl. Environ. Microb., 72, 7495-7502.

Seviour, R. J., McNeil, B., Fazenda, M. L., Harvey, L. M., 2011. Operating bioreactors for microbial exopolysaccharide production. Crit. Rev. Biotechnol., 31, 170-185.

Stein, H. H., Lagos, L. V., Casas, G. A., 2016. Nutritional value of feed ingredients of plant origin fed to pigs. Anim. Feed. Sci. Tech, 218, 33-69.

Shahriar, F., Ngeleka, M., Gordon, J.R., Simko, E., 2006. Identification by mass spectroscopy of F4ac-fimbrial-binding proteins in porcine milk and characterization of lactadherin as an inhibitor of F4ac-positive *Escherichia coli* attachment to intestinal villi in vitro. Dev. Comp. Immunol., 30, 723–734.

Sharon, N., Lis, H., 2004. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology, 14, 53R-62R.

Sharon, N., 2006. Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta Gen Subj, 1760, 527–537.

Shi, C. X., Liu, Z. Y., Shi, M., Li, P., Zeng, Z. K., Liu, L., Huang, C.F., Zhu, Z.P., Li, D. F., 2015. Prediction of digestible and metabolizable energy content of rice bran fed to growing pigs. Asian-Austral. J. Anim., 28, 654.

Sun, X., Ganzle, M., Wu, J., 2017. Identification and characterization of glycopeptides from egg protein ovomucin with anti-agglutinating activity against porcine K88 enterotoxigenic *Escherichia coli* strains. J. Agric. Food Chem., 65, 777–783.

Toledo, C. C., Arvidsson, I., Karpman, D., 2011. Cross-Reactive Protection against Enterohemorrhagic *Escherichia coli* Infection by Enteropathogenic *Escherichia coli* in a Mouse Model. Infect. Immun., 79, 2224-2233.

Verhelst, R., Schroyen, M., Buys, N., Niewold, T., 2010. The effects of plant polyphenols on enterotoxigenic Escherichia coli adhesion and toxin binding. Livest Sci, 133, 101–103.

8. CONCLUSION

The results obtained in this Thesis allow concluding that under our in vitro experimental conditions:

1. Water soluble extracts of wheat, corn, oats, barley, rye, extruded full-fat soybean meal, soybean meal, as well as sweet whey powder, all demonstrate a clear affinity to attach ETEC K88. Extracts of rye, oats and also wheat, can also reduce ETEC K88 adhesion to IPEC-J2 cells ($P = 0.05$). Extracts from wheat bran, spelt bran, kamut bran, rye bran, oat bran and rice bran also can attach ETEC K88, being all able to reduce its adhesion to IPEC-J2 cells, except rice bran.

2. Microbial exopolysaccharides isolated from brines during the industrial fermentation of table green olives show a relatively constant chemical composition. Analysis of their monosaccharide content by GLC shows glucose and galactose followed by rhamnose and arabinose as their main components. The ^{1}H NMR spectrum demonstrate a very similar profile between industrial batches, and a more in-depth analysis reveals the presence of an α-pyranose in the form of α-D-Glc*p*-(1→) and two different α-furanoses, with chemicals shifts values, suggesting the presence of α-D-Glc*f* and α-D-Gal*f*.

3. Miniaturized *in vitro* tests demonstrate the ability of microbial EPS isolated from industrial olive brines to attach specifically to ETEC K88 and to effectively remove the pathogen once attached to IPEC-J2 cells.

4. By using different strains of *Lactobacillus pentosus* and *Candida boidinii,* as starters in the production of table green olives, is possible to modulate the fermentation process. Changes can be seen in fermenter pH and also in the number of yeast and LAB attaching to olive surfaces.

5. Miniaturized *in vitro* tests demonstrate that the use of different starters in the fermentation of table green olives can affect the anti-adhesive properties of the microbial EPS isolated from the brines. The mutant strain *L. pentosus* 119-14MT, high EPS variant, appears as the most effective starter to improve the antiadhesive properties of isolated EPS. This improvement is lost if this strain is combined with *C. boidinii* TOMC-Y13.