Universitat Autònoma de Barcelona

NATURAL SOURCES AGAINST VETERINARY PATHOGENS: EVALUATION OF THE ANTI-ADHESIVE AND ANTI-BIOFILM ACTIVITY OF WHEAT BRAN

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Que la memòria titulada "**Natural sources against veterinary pathogens: Evaluation of the anti-adhesive and anti-biofilm activity of wheat bran**", presentada per Gemma González Ortiz amb la finalitat d'optar al grau de Doctor en Veterinària amb menció europea, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzen la seva presentació per que sigui jutjada per la comissió corresponent.

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Dra. Susana M. Martín Orúe Dr. José F. Pérez Hernández

“Todo parece impossible hasta que se hace”

Nelson Mandela

“Gracias a todos por dejarme hacer”

Gemma González

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SUMMARY

Looking for natural sources may be a feasible alternative to prevent or treat bacterial infections, thus enhancing animal health and obtaining safer animal products. This Thesis was devised with the aim of screening among natural feed ingredients (FI) their ability to reduce bacterial adhesion, in particular against enterotoxigenic *Eschericha coli* (ETEC) K88, to prevent colibacillosis in young pigs. The hypothesis on the anti-adhesive role of wheat bran (WB) against enteropathogens derived from previous results from our group and was subsequently confirmed in the first two trials of the current Thesis. For this reason we considered that WB deserved further fractionation to elucidate the putative anti-adhesive molecules involved in the ETEC K88 recognition. Moreover, this Thesis professed into exploring the anti-biofilm activity of WB in an *in vitro* well-known biofilm model of *Staphylococcus aureus*, but also in its possible interference with bacterial quorum-sensing (QS) systems.

In order to achieve these main objectives a set of four trials (chapters 4 to 7) were designed.

Trial I was designed to screen the ability of different FI to bind ETEC K88 and to block its attachment to the porcine ileal mucus. The selected FI consisted of nine products of vegetable origin, one dairy and three microbial by-products, which could be considered as candidates to be included in a piglet's diet. Among the products screened, the soluble extracts from WB, locust bean (LB), locust gum (LG), guar gum (GG) and casein glycomacropeptide (CGMP) possess the highest anti-adhesive properties against ETEC K88. In contrast, soybean hulls (SO), sugar beep pulp (SBP), cranberry (CRA), fructo-oligosaccharides (FOS), Inulin (IN), exopolysaccharides (EPS), mannan-oligosaccharides (MOS) and the fermented extract from *Aspergillus oryzae* (AO) were not able to decrease the number of ETEC K88 to the porcine ileal mucus. For WB and LB, a fractionation based on their carbohydrate components was subsequently made, and each fraction was evaluated individually. None of the obtained fractions from WB reduced the adhesion of ETEC K88 to mucus as the original extract did, suggesting that a protein or glycoprotein could be involved in the recognition process. Regarding LB fractionation, the water extractable material reduced the adhesion of ETEC K88 to mucus similarly to that of the original extract, indicating, in this case, that galactomannans or phenolic compounds could be responsible.

The aim of **Trial II** was to screen, among different FI, their ability to reduce ETEC K88 attachment to the porcine intestinal epithelial cell-line (IPEC-J2). This trial was configured with mostly the same FI like in Trial I, which could complement the anti-adhesive response of the most promising FI, but in this case using another miniaturized *in vitro* assay, a monolayer of IPEC-J2, without the interference of the intestinal mucus. In this case, CGMP, LB, EPS and WB reduced the number of attached ETEC K88 to IPEC-J2 in a lineal dose-response way, but no anti-adhesive effect was found for SO, SBP, LG, FOS, IN, mushroom (MSH), MOS or AO. It was very interesting to see that EPS was not able to reduce ETEC K88 binding to the intestinal mucus in Trial I, but in this occasion it was the most efficient at reducing adhesion of the ETEC K88 strain to the IPEC-J2 cell-line among all FI tested, even better than LB, CGMP and WB.

Trial III was made trying to identify the mechanisms by which WB interferes in the attachment of ETEC K88 to IPEC-J2. One aliquot of the soluble extract of WB was used to fractionate it by molecular weight (MW) using a cut-off size filter of 300.000-Da. It was found that a proteinaceous compound in the >300-kDa fraction mediates the recognition of ETEC K88 to IPEC-J2. To further fractionate the >300-kDa fraction, size-exclusion chromatography (SEC) was performed. The fractionation of the >300-kDa fraction by SEC showed several proteins below 90-kDa, thus suggesting that the target protein involved in the recognition of ETEC K88 belongs to a high MW multicomponent protein complex. The identification of some relevant excised bands by mass spectrometry (MS) mostly revealed the presence of various protease inhibitors (PI): Serpin-Z2B, Class II chitinase, endogenous alpha-amylase/subtilisin inhibitor and alpha-amylase/trypsin inhibitor CM3. Furthermore, an incubation of the WB extract with ETEC K88 allowed for the identification of a 7S storage protein globulin of wheat, Globulin 3 of 66-kDa, which seems to be one of the most firmly attached WB proteins to ETEC K88.

Trial IV aimed to explore the *in vitro* potential of WB soluble extract to inhibit or destroy the biofilm formed by a cow mastitis strain of *S. aureus.* Also it was studied its possible interference with bacterial QS systems. The soluble extract of WB at 0.5% showed anti-biofilm activity, inhibiting the biofilm formation and also destroying the biofilm previously formed. Similarly to the WB extract did, the >300-kDa fraction had a significant anti-biofilm activity in both *in vitro* assays, inhibiting the biofilm

formation and also destroying it. The soluble extract of WB also showed a potential to interfere with the QS of bacteria as it was demonstrated to contain certain lactanase activity able to reduce acyl-homoserine lactones (AHL) concentration in the medium. This trial reveals two additional beneficial properties of WB extract never explored before, which may be related to the presence of defence compounds in the plant extract able to interfere with microbial biofilms and also QS systems.

Results exposed in this Thesis indicate that soluble extract of CGMP, EPS, LB and WB should be considered as possible natural sources for the development of new additives to be included in weaned piglet's diets and prevent post-weaning collibacillosis. The particular interest set on WB anti-adhesive properties led to the fractionation to gain understanding of the molecular recognition of the blocking process that takes place. The fractionation of WB by MW and SEC revealed that blocking activity is retained in the >300-kDa fraction. The identification of some relevant bands of low MW in this fraction by MS revealed the presence of various PI that could be implicated in the reported blocking effects. Finally, it was found that the soluble extract of WB and the >300-kDa fraction were able to inhibit the biofilm formation and also the destruction the biofilm previously formed. In the same trials was demonstrated that WB as well as the >300 kDa and the <300 kDa contain certain lactanase activity able to reduce AHL concentration in the medium. All the gathered information can eventually pave the way for the development of novel anti-adhesion, anti-biofilm and quorum quenching (QQ) therapeutic agents from wheat bran to prevent bacterial pathogenesis.

RESUMEN

La búsqueda de fuentes naturales podría ser una alternativa viable para prevenir o tratar infecciones bacterianas, así mejorando la salud de los animales y obteniendo productos de origen animal más seguros. Esta Tesis se elaboró con el objetivo de evaluar la capacidad de reducir la adhesión bacteriana, en concreto de *Escherichia coli* enterotoxigénica (ETEC) K88 por parte de diferentes ingredientes naturales (FI), para prevenir colibacilosis en lechones. La hipótesis sobre el papel anti-adhesivo del salvado de trigo (WB) en contra de enteropatógenos derivó de resultados previos de nuestro grupo y ha sido confirmado en los primeros dos ensayos de la presente Tesis. Por esta razón, consideramos que el WB merecía un fraccionamiento adicional para elucidar las moléculas anti-adhesivas involucradas en el reconocimiento de ETEC K88. Además, esta Tesis pretendió explorar la actividad anti-biofilm del WB en un modelo *in vitro* bien conocido de *Staphylococcus aureus*, pero también en su posible interferencia con los sistemas de comunicación bacteriana o, también conocidos como sistemas quorum sensing (QS).

Con el fin de alcanzar estos objetivos principales, una serie de cuatro ensayos (capítulos 4 a 7) fueron diseñados.

El **Ensayo I** se diseñó para evaluar la abilidad de diferentes FI para adherir y bloquear la adhesión de ETEC K88 al mucus ileal porcino. Los FI seleccionados consistieron en nueve productos de origen vegetal, un producto derivado de la leche y tres sub-productos microbianos, los cuales podrían ser candidatos para ser incluídos en la dieta de los lechones. De todos los productos evaluados, el extracto soluble de WB, la vaina conjuntamente con semillas de algarroba (LB), la goma de algarroba (LG) y el caseín glicomacropeptido (CGMP) possen la mayor propiedad anti-adhesiva en contra de ETEC K88. En contra, la cascarilla de soja (SO), la pulpa de remolacha (SBP), el arándano (CRA), los fructo-oligosacáridos (FOS), la inulina (IN), los exopolisacáridos (EPS), los mananoligosacáridos (MOS) y el extracto fermentado de *Aspergillus oryzae* (AO) no fueron capaces de reducir el número de ETEC K88 adheridos al mucus ileal porcino. Se realizó un fraccionamiento del WB y el LB en base a sus carbohidratos, y cada fracción se evaluó individualmente. Ninguna de las fracciones obtenidas del WB redujo la adhesión de ETEC K88 al mucus de la misma forma como lo hizo el extracto original, sugiriendo que una proteína o una glicoproteína podría estar implicada en

el proceso de reconocimiento. Respecto al fraccionamiento de LB, el material soluble extraído redujo la adhesión de ETEC K88 al mucus de la misma manera que lo hizo el extracto original, indicando, en este caso, que los galactomananos o los compuestos fenólicos podrían ser los responsables.

El objetivo del **Ensayo II** fue evaluar, entre diferentes FI, su abilidad para reducir la adhesión de ETEC K88 a las células epiteliales de intestino de lechón (IPEC-J2). Este ensayo se configuró con la mayoría de los mismos FI que en los usados en el Ensayo I, lo que podría complementar la respuesta anti-adhesiva de los FI más prometedores, pero en este caso usando otro test *in vitro* miniaturizado, una monocapa de IPEC-J2, sin la interferencia del mucus intestinal. En este caso, el CGMP, el LB, el EPS y el WB redujeron el número de ETEC K88 adheridas al cultivo celular de una forma lineal, pero no se encontró actividad anti-adhesiva en los extractos de SO, SBP, LG, FOS, IN, champiñones (MSH), MOS ni AO. Fue muy interesante observar que el EPS no fue capaz de reducir la adhesión de ETEC K88 al mucus intestinal en el Ensayo I, pero en esta ocasión fue el ingrediente más eficaz interfiriendo en la adhesion de ETEC K88 a IPEC-J2 entre todos los FI evaluados, incluso mejor que el LB, el CGMP y el WB.

El **Ensayo III** se realizó para tratar de identificar los mecanismos por los cuales WB interfiere en la adhesión de ETEC a IPEC-J2. Una alícuota del extracto soluble del WB se fraccionó por peso molecular (MW) usando unos filtros de tamaño de corte de 300,000 Da. Se encontró que un compuesto proteíco en la fracción >300 kDa es el responsable del reconocimiento de ETEC K88 a IPEC-J2. Posteriormente se realizó un fraccionamiento adicional de la fracción >300 kDa mediante cromatrografía de exclusión molecular (SEC). El fraccionamiento por SEC mostró varias proteínas por debajo de 90-kDa, así sugiriendo que la proteína responsable involucrada en el reconocimiento de ETEC K88 pertenece a un complejo proteico de alto MW compuesta por varios componenetes. La identificación por espectrometría de masas (MS) de las bandas más relevantes recortadas principalmente reveló la presencia de varios inhibidores de proteasas (PI): Serpina-Z2B, Chitinasa de classe II, inhibidor endógeno de alfa-amilasa/subtilisina e inhibidor CM3 alfa-amilasa/tripsina. Además, la incubación del extracto de WB con ETEC K88 permitió la identificación de una proteína globulina de almacenamiento 7S del trigo, la Globulina 3 de 66 kDa, la cual parece ser una de las proteínas del WB más firmemente unidas a ETEC K88.

El objetivo del **Ensayo IV** fue explorar el potencial in vitro del extracto soluble del WB para inhibir o destruir el biofilm formado por una cepa de *S. aureus* procedente de un episodio de mastitis de vaca. También se estudió su posible interferencia en sistemas de QS. El extracto soluble de WB al 0.5% mostró actividad anti-biofilm, inhibiendo la formación de biofilm y también destruyendo el biofilm previamente formado. De forma similar a como el extracto de WB lo hizo, la fracción >300 kDa tuvo una actividad anti-biofilm significativa en ambos tests *in vitro*, inhibiendo la formación de biofilm y también destruyéndolo. El extracto soluble de WB también mostró potencial de interferencia con el QS de las bacterias ya que demostró contener cierta actividad lactanasa capaz de reducir la concentración de acil-homoserina lactonas (AHL) en el medio. Este Ensayo revela dos propiedades beneficiosas adicionales al extracto de WB nunca explorados anteriormente, los cuales podrían estar relacionados con la presencia de compuestos de defensa en el extracto vegetal capaces de interferir con los biofilms microbianos y, también, con los sistemas de QS.

Los resultados expuestos en esta Tesis indican que los extractos solubles de CGMP, EPS, LB y WB podrían considerarse como posibles fuentes naturales para el desarrollo de nuevos aditivos para ser incorporados en las dietas de los lechones al destete y prevenir la colibacilosis post-destete. El interés particular fijado en las propiedades anti-adhesivas del WB llevó al fraccionamiento para comprender mejor el reconocimiento molecular del proceso de bloqueo que tiene lugar. El fraccionamiento del WB por MW y SEC reveló que la actividad de bloqueo se mantiene en la fracción >300 kDa. La identificación por MS de algunas de las bandas más relevantes de bajo MW en esta fracción, reveló la presencia de varios PI que podrían estar implicados en lo efectos de bloqueo presentados. Finalmente, se encontró que el extracto soluble de WB y la fracción >300 kDa fueron capaces de inhibir la formación de biofilm y también de destruir el biofilm previamente formado. En ese mismo ensayo se demostró que el WB así como la fracciones >300 kDa y <300 kDa contienen cierta actividad lactanasa capaz de reducir la concentración de AHL en el medio. Toda la información recogida puede considerarse para el desarrollo de nuevos agentes anti-adhesion, anti-biofilm y quorum quenching (QQ) a partir del salvado de trigo para prevenir infecciones bacterianas.

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ABBREVIATIONS

Aap:	Acumulation-associated protein	**IBD:**	Intestinal inflammatory diseases
ACN:	Acetonitrile	**IC50:**	Half maximal inhibitory concentration
AE1D:	First alkali treatment of the cellulosic residue of WB	**IN:**	Inulin
AE2D:	Second alkali treatment of the cellulosic residue of WB	**IPEC-J2:**	porcine intestinal epithelial cell line J2
AGP:	Antibiotic growth promoters	**LB:**	Locust bean
AHL:	Acyl-homoserine lactone	**LBM:**	Luria broth medium
AI-1:	Autoinducer -1	**LG:**	Locust gum
AI-2:	Autoinducer -2	**MALDI-TOF:**	Matrix-assisted laser desorption/ionization time-of-flight
AIEC:	Adherent-invasive *Escherichia coli*	**MAN:**	D-Mannose
AIP:	Autoinducing peptides	**MIC:**	Minimal inhibitory concentration
ANOVA:	One-way analysis of variance	**MOS:**	Mannanoligosaccharides
AO:	*Aspergillus oryzae* fermented extract	**MS:**	Mass spectrometry
AT:	Adhesion test	**MSCRAMMs:**	Microbial surface components recognizing adhesive matrix molecules
Bap:	Biofilm-associated protein	**MSH:**	Mushroom
BSA:	Bovine serum albumine	**MW:**	Molecular weight
BT:	Blocking test	**NDS:**	AHL non-degrader control strain
CD:	Crohn disease	**NF:**	Non-fimbriated
CFU:	Colony forming units	**NSP:**	Non-starch polysaccharide
CGMP:	Casein glycomacropeptide	**OD:**	Optical density
CP:	Crude protein	**ON:**	Overnight
CRA:	Cranberry	**PBS:**	Phosphate buffered saline
DEMI:	Demineralized	**PDI:**	Protein disulphide isomerase
DMEM:	Dulbecco's modified eagle medium	**PI:**	Protease inhibitor
DS:	AHL degrader control strain	**PIA:**	Polysaccharide intercellular adhesin
ECM:	Extracellular matrix	**PSM:**	Phenol-soluble modulins
eDNA:	Extracellular DNA	**QQ:**	Quorum quenching
Embp:	Extracellular matrix binding protein	**QS:**	Quorum sensing
EPEC:	Enteropathogenic *Escherichia coli*	**RFU:**	Relative fluorescence units
EPS:	Exopolysaccharides	**RT:**	Room temperature
ETEC:	Enterotoxigenic *Escherichia coli*	**SBP:**	Sugar beep pulp
FI:	Feed ingredient	**SDS-PAGE:**	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
FnPB:	Fibronectin binding proteins	**SEC:**	Size-exclusion chromatrography
FOS:	Fructooligosaccharides	**SO:**	Soybean hulls
GalNAc:	N-acetyl-D-galactosamine	**TFA:**	Trifluoroacetic acid
GG:	Guar gum	**TSB:**	Tryptone soy broth
GIT:	Gastrointestinal tract	**UPEC:**	Uropathogenic *Escherichia coli*
GlcNAc:	N-acetyl-D-glucosamine	**WB:**	Wheat bran
GLMM:	Generalized linear mixed model	**WEM:**	Water-extractable material

1. GENERAL INTRODUCTION

For the last fifty years, the common practice in animal production was to include antibiotics in feedstuffs at sub-therapeutic levels to act as growth promoters (Antibiotic Growth Promoters; AGP). However, concerns about a promotion on the antimicrobial resistance and a likely transference of antibiotic resistance genes from animal to human microbiota led to the ban on the use of AGP in the European Union since January 1, 2006. As a consequence, a high pressure on livestock has emerged because the incidence of certain common illnesses, which remained under control. Therefore, there is the need to look for viable alternatives that do not compromise performance, lowering pathogens presence in the livestock chain and also reducing antibiotics administration for therapeutic purposes.

A great list of alternatives have been proposed to be included in animal feed that could be possible solutions such as prebiotics, probiotics, symbiotics, plant extracts, organic acids, essential oils, enzymes or microminerals (Gaggia et al., 2010; Arguello et al., 2013; Thacker. 2013). The general effects of these feed additives are their ability to improve the intestinal resistance to pathogenic bacteria colonization and the enhanced immunity response. Both are known to change the intestinal environment due mainly to the proliferation of beneficial bacteria and the modification of the fermentative pattern. However, there are other novel strategies to combat pathogens (Lynch and Wiener-Kronish, 2008) by targeting in the specific bacterial virulence systems. The recognition of host receptors by bacteria's adhesion proteins (fimbriae) is considered as the first requirement in many pathogenic diseases (Ofek et al., 2003a). Because of that, blocking this initial attachment step provides a strategy to prevent bacterial colonization of the epithelial surface. Moreover, certain part of the typical bacterial lifecycle involves attachment to the desired surface and biofilm formation, deteriorating the health status of animals. Biofilms are broadly defined as communities of bacterial cells encased in a protective extracellular matrix. These bacterial reservoirs are difficult to combat, leading to subsequent infections. To prevent biofilm formation or other virulent factors by interfering in the communication systems (quorum sensing [QS]), would be another feasible option once the infection has unfortunately been established (Bergamo-Estrela and Abraham, 2010).

In this sense, a promising approach to combat bacterial pathogenesis in the veterinary practice is to develop useful and cheap therapeutics that target specific aspects of the bacterial activity. It can be predicted that soluble glycans or host glycan coming from food, could be used to block the initial attachment of microbes to cell surfaces. Thus, preventing or suppressing infection (Gornik et al., 2006) may provide a safer alternative than antibiotics. Naturally occurring derivatives have exhibited effective

fimbriae-blocking activity in a variety of assays (Sharon. 2006), and represent a viable option for novel anti-adhesive therapies (Lane et al., 2010). The large list of food components, mainly oligosaccharides, able to interfere with bacterial adhesion to epithelial cells offers a consistent background to encourage this field of research. The greatest benefit in using anti-adhesion agents may be that these compounds do not act by killing the bacteria or arresting their growth, thereby reducing the threat of resistance. In addition, because most of the anti-adhesive candidates are found naturally in food, these products are unlikely to be toxic or immunogenic.

Nevertheless, there are some feed ingredients (as fibrous ones) that have demonstrated anti-adhesive ability but due to their complex composition, it is difficult to conclude the responsible molecule of such activity. So, the assignation of a responsible structure to block bacterial adhesion is sometimes partially empiric. Moreover, extra difficulty and feasibility of performing *in vivo* trials is found when is examined the effect of a particular molecule inside a normal alive ecosystem which makes hard the outcomes interpretation. For that reason, *in vitro* methods allow the study of isolated compounds in a much more controlled environment.

On this basis it seems opportune to screen among different feed ingredients (FI) their ability to interfere with bacterial adhesion to the intestine using *in vitro* based models. In particular this Thesis will study the potential of different natural FI to reduce the adhesion of one of the most relevant pathogens in the pig industry during the post-weaning period: the enterotoxigenic *Escherichia coli* (ETEC) K88. The Thesis will also try to go deeper into the molecular mechanisms involved in the effects observed for the wheat bran, also evaluating its ability to interfere in biofilm formation and complementarily in QS mechanisms.

2. LITERATURE REVIEW

2.1. BACTERIAL ADHESION

2.1.1. INTRODUCTION

Adherence of pathogens to host cell surfaces is considered an essential step to start infection (Ofek et al., 2003a; Lloyd et al., 2007). By means of specific adhesion molecules, the microorganism is able to recognize specific receptors located on the host cell surface and then attaches itself. Thus, microbes gain access to host tissues by mechanisms of adherence, upset the integrity of the cells of the organ surface and cause tissue infection. One of the immediate consequences after the attachment to mucosal surfaces is the production and release of pro-inflammatory cytokines which can triggers both local and systemic infection (Yuehuei et al., 2000).

Carbohydrate- or oligosaccharide-bearing moieties are mainly, the most common host cell receptors recognized by pathogenic bacteria. The identification and characterization of these specific bacteria-host interactions have revealed important insight into the molecular mechanisms of the action of pathogens, but also provides a basis for the development of anti-adhesion therapies (Shoaf-Sweeney and Hutkins, 2009).

2.1.2. MOLECULAR BASIS FOR PATHOGEN-HOST INTERACTIONS

Animals and humans are susceptible to develop infectious diseases by the attachment of bacteria to the host surface. Bacterial adhesion involves the interaction of complementary molecules on the surface of the bacteria and host epithelium. Therefore, it requires a strict series of steps leading to stable interactions between pathogens and host. In particular, three types of adhesion-receptor interactions have been described, depending on the molecules' nature that is taking part in the process: hydrophobin-protein, lectin-carboydrate or protein-protein (Ofek et al., 2003a).

2.1.2.1. HYDROPHOBIN-PROTEIN INTERACTIONS

The repulsive forces generated by the negative charges naturally found on the host tissues and the pathogen surface keep bacteria far away from an attachment episode (Shoaf-Sweeney and Hutkins, 2009). However, some bacteria are able to overcome such repulsive forces, and van der Waals' attractions, Columbic forces, hydrophobic interactions, and eventually, complementary interactions are established allowing the bacterial binding in two-step kinetic model (Hasty et al., 1992). Firstly, bacteria becomes loosely and reversible bound by hydrophobins that interact with hydrophobic moieties (e.g., fatty acids) on the host cell surface in a none-specific way (Hui and Dykes,

2012). Subsequently, specific surface structures of bacteria adhere to the substratum resulting in an irreversible attachment (Goulter et al., 2009) by one of the other two types of adhesion-receptor interaction (lectin-carbohydrate or protein-protein interactions).

2.1.2.2. LECTIN-CARBOHYDRATE INTERACTIONS

The lectin-carbohydrate interactions are the most well studied bacteria-host interactions (Ofek et al., 2003a). These interactions are those involving bacterial lectins and complementary oligosaccharides receptors, such as glycolipids, glycoproteins, and/or proteoglycans, found on the mucosal glycocalyx layer on the top of the host epithelial surface (Shoaf-Sweeney and Hutkins, 2009). Lectin-carbohydrate types of recognition can occur when the lectin is on the bacterial surface or on the host surface. The type in which the lectin is on the bacterial surface predominates and undoubtedly contains the largest group of adhesins so far described among bacterial pathogens. However, there are examples of other lectin-carbohydrate adhesive interactions in which the lectin is located on the host cell surface (e.g. macrophages binding to capsular polysaccharides and lipopolysaccharides of the outer membrane of the Gram-negative bacteria) (Targosz et al., 2006). In Gram-negative bacteria, lectins are usually presented as fimbriae or pili that bind host oligosaccharides receptors (Klemm and Schembri, 2000). In Gram-positive bacteria, lectins are positioned inside the peptidoglycan layer, so by traversing this wide layer are extended beyond the cell wall (Vengadesan and Narayana, 2011). The lectin adhesins are usually classified by sugar specificity. This specificity can be determined by inhibiting the adhesion with a battery of different carbohydrates, either presented alone or inside complexes, which compete with the binding adhesion to host cells (Pieters. 2007).

2.1.2.3. PROTEIN-PROTEIN INTERACTIONS

Protein-protein interactions occur between a protein on the bacterium and a complementary protein on the mucosal surface. Protein-protein interactions usually involve the extracellular matrix components (ECM) of the host cell when this one has been compromised (Schennings et al., 2012). There are some bacterial proteins able to bind one or more of the ECM such as fibronectin, laminin, collagen and elastin (Zulfakar et al., 2012). The best protein-protein interaction example is those that involve fibronectin-binding proteins and bacterial proteins. In this sense, several bacteria such as *Staphylococcus aureus* and diverse species of *Streptococcus* spp. have developed the expression of FBP to ensure their infectious success (Yamaguchi et al., 2012; Geoghegan et al., 2013).

2.1.3. MOLECULAR SUPPORT BY THE SPECIFICITY AND AFFINITY IN BACTERIAL ADHESION

2.1.3.1. THE SPECIFICITY IN BACTERIAL ADHESION

Considering the above molecular basis for pathogen adhesion, it is accepted that bacterial attachment is not a random process. Bacterial lectins can recognize complementary oligosaccharides on the host cell surface by means of small globular carbohydrate recognition domains that can distinguish subtle chemical diversities among bacterial pathogens (Lloyd et al., 2007). This binding specificity explains the attraction a pathogen has for a particular host tissue. However, specificity is not altogether a function of the presence or not of a particular oligosaccharide. For instance, *E. coli* that possess mannose-specific adhesins do not colonize all mannose-containing tissues (Shoaf-Sweeney and Hutkins, 2009).

2.1.3.2. THE AFFINITY IN BACTERIAL ADHESION

The affinity is also another significant feature to consider in the bacterial adhesion process (Weis and Drickamer, 1996). A single oligosaccharide molecule would have very low affinity to its corresponding protein, but the use of multiple interactions by multivalent carbohydrates, thus increasing the valency of the protein-oligosaccharide interaction, significantly enhances the affinity of the protein for its target (Lloyd et al., 2007; Pieters. 2009). Bacteria strategically create these multivalent oligosaccharide-binding proteins by assembling individual protein subunits that contains numerous individual oligosaccharide-binding sites into a filamentous structure. This complex allows the bacteria to bind to numerous individual oligosaccharides receptors on the host cell like a “Velcro” which ensure an efficient attachment process (**Figure 2.1**) (Mulvey et al., 2001; Pieters. 2007).

2.1.4. BACTERIAL ADHESINS

Colonization depends on the ability of the bacteria to compete successfully with the host’s normal microbiota for essential nutrients. Specialized structures that allow bacteria to compete for surface attachment sites also are necessary for colonization (Prescott et al., 2005). Bacterial pathogens adhere with a high degree of specificity to particular tissues. These interactions are mainly mediated by proteinaceous structures expressed on the bacterial surface commonly called bacterial adhesins (Kline et al., 2009). Adhesins are specialized molecules generally found on the tips of bacterial

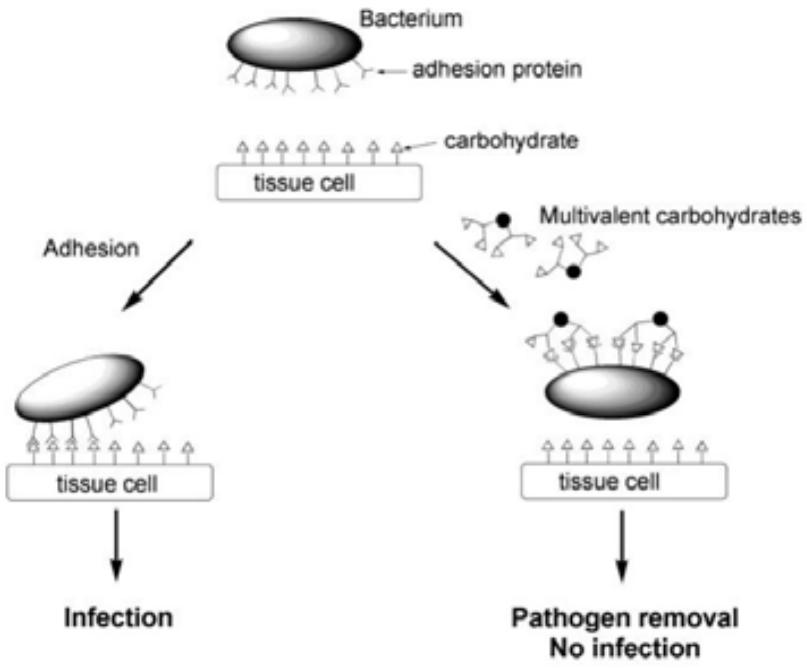

Figure 2.1. The scenario of bacterial adhesion to bind to numerous individual oligosaccharides receptors on the host cell like a "Velcro" followed by infection (left) is contrasted to a scenario in which multivalent carbohydrates are present that bind the pathogen (right). The latter scenario leads to removal of the bacterium by regular secretion mechanisms and no infection will result (From Pieters 2007).

appendages (fimbrial adhesins) that bind to complementary receptor sites, frequently carbohydrates, on the host cell surface, but may also be anchored in the bacterial outer membrane (afimbrial adhesins) (**Figure 2.2**). At first, adhesins were related to provide a means for bacteria to colonize a site and a way to avoid clearance by mucosal secretion and peristalsis. In a second term, it is currently known that, after their ligand interactions mediate, a series of signalling events occurs. These may affect bacterial uptake or invasion and/or promote pro- or anti-inflammatory events by affecting innate immune receptors response (Frick et al., 2006; Friberg et al., 2008). Thus, adhesins may become in very efficient virulence factors. Even though bacterial adhesins share the same infectious objective (attaching specifically on target molecules present on the host cell surface) they can be differentiated depending on the mechanism of assembly. This is a complex process that requires the participation of a great number of auxiliary proteins (Salyers and Whitt, 2002; Kline et al., 2009). It has been defined four main mechanisms of assembly: Chaperon-Usher Pathway, Type IV secretion pathway, Sortase Assembled pathways and Curly (**Table 2.1**). Between them, the chaperone-usher pathway is the most studied being reported as the responsible for the assembly for over 100 pilus and nonpilus surfaces structures in a lot of bacterial pathogens involved in a great number of diseases (Ofek et al., 2003a).

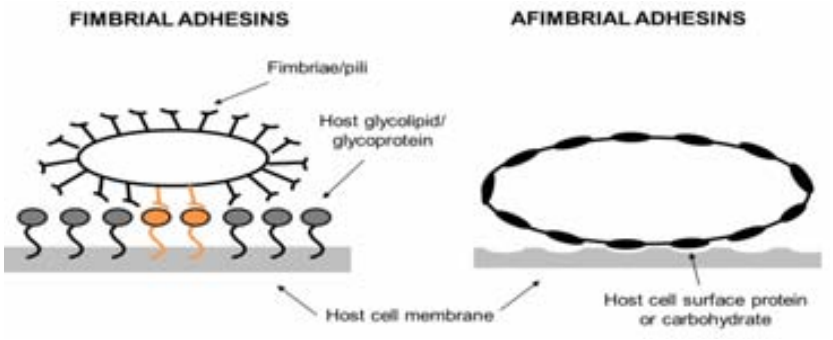

Figure 2.2. Two types of bacterial adherence mechanisms (Adapted from Salyers and Whitt, 2002).

2.1.4.1. FIMBRIAL ADHESINS

The fimbrial adhesins encompass various types of multisubunit protein polymers structured in rod-shaped structures allocated on the cell-surface. They have a pivotal role in the colonization of specific host tissues by Gram-positive and Gram-negative bacteria (Jonson et al., 2005). These structures are shown evenly distributed over the surface of the bacteria, but in some cases are stacked preferentially on one part of them. Besides their assembly procedures (Kline et al., 2009), they can also be differentiated according to their morphology, mainly flagella, fimbriae and/or pilin structures (Juge. 2012).

2.1.4.1.1. FLAGELLA

Flagella are the best studied of all prokaryotic motility structures. The flagellum consists of three parts: the basal body, the hook, and the filament (Prescott et al., 2005). It is composed of several thousands of copies of flagellin subunits, the major structural protein of the flagellar filaments. It comprises N- and C-terminal conserved regions and the central hypervariable region. The hypervariable region provides antigenic differences in diverse flagellins and contributes to the unique adhesive properties of flagella in distinct serotypes (Stocker and Newton, 1994). The investigations from the last decade have demonstrated that the flagella and their flagellin monomers from a wide range of pathogens like *Clostridium difficile*, *Salmonella enterica* serovar Typhimurium and *E. coli* possess adhesive properties (Juge. 2012). This property was suggested to be required for colonization of the gastrointestinal tract (GIT), including adhesion and subsequent invasion (Allen-Vercoe and Woodward, 1999; La Ragione et al., 2000). In this sense, recent investigations have demonstrated that flagella are required for efficient F18ab *E. coli* adhesion, invasion, biofilm formation

Table 2.1. Main mechanisms of pilus/fimbriae assembly emphasizing some typical examples inside each classification group

Chaperon-Usher Pathway

This pathway involves a periplasmic chaperone and an outer membrane usher protein. The pilus subunits are secreted via the Sec translocation machinery from the cytoplasm to the periplasmic side. Firstly, the distal (tip) end is assembled first, and additional subunits are then incorporated sequentially into the base of the fiber in a defined order.

Pathogen	General adhesin type	Gene cluster	Adhesin	Ligand Receptor	Disease
Uropathogenic *E. coli* (UPEC)	P fimbriae	*pap*	PapG	Globobiose	Pyelonephritis
UPEC; Enterotoxigenic *E. coli* (ETEC); Enteropathogenic *E. coli* (EPEC); *S.* Typhimurium	Type 1 pilus	*fim*	FimH	Mannose residues	Diarrhea, Dysentery, Cystitis
ETEC	CFA fimbriae	*cfa*	CfaE	Glycoproteins?	Diarrhea

Type IV secretion pathway

The whole pilus fiber is assembled in the periplasm and finally an outer-membrane secretin pore translocates the pilus to the cell surface. As the chaperone-usher pathway, the distal end of the pilus is formed first.

Pathogen	General adhesin type	Gene cluster	Adhesin	Ligand Receptor	Disease
Neisseria gonorrhoeae	Type IV pili	*pil*	PilC	CD46, integrin, proteinaceous ligand	Gonhorrea
Vibrio cholerae	TCP	*tcp*	TcpA	Human receptor?	Cholera
EPEC	Type IV pili	*bfp*	Bundle-forming pili	Nonintimate binding	Infant diarrhea, Traveler's diarrhea

Sortase Assembled pathways

On Gram-positive bacteria, after the Sec system mediates secretion of the pilins, the enzyme called sortase link the pilins subunits to each other and eventually transfer the pilus to the peptidoglycan cell wall.

Pathogen	General adhesin type	Gene cluster	Adhesin	Ligand Receptor	Disease
Streptococcus pneumoniae	Rrg pili	*rlrA*	RrgA	Not defined	Pneumonia
Staphylococcus aureus	Fibronectin-binding protein	*fnbA y fnbB*	FnBP	Fibronectin (Components of extracellular matrix)	Epithelium Endothelium
Enterococcus faecalis	Ebp pili	*ebp*	EbpA	Not defined	Endocarditis, Biofilms Urinary tract infections

Curli

Pilus subunits are secreted as soluble proteins and precipitated onto thin fibers on the surface of the bacteria. These structures are very sticky without exhibiting a clear ligand-binding specificity.

Pathogen	General adhesin type	Gene cluster	Adhesin	Ligand Receptor	Disease
E. coli *S. enterica*	Curli fibers	CsgA	Amyloid structure	Contact phase system	Gastroenteritis

Adapted from Kline et al. (2009)

and IL-8 production under *in vitro* conditions (Duan et al., 2012; Duan et al., 2013).

2.1.4.1.2. FIMBRIA AND/OR PILI

Fimbriae and/or pili are, by far, the most common adhesive structures expressed by Gram-negative bacteria (Ofek et al., 2003b). The fimbriae are composed of major protein subunits (pilins) organized into a tube-like structure, having diameters ranging from 2 to 8 nm and usually extend 1-2 µm from the bacterial surface (Pratt and Kolter, 1998). In contrast, pili are similar appendages to fimbriae, but present on the bacterial surface in a less extent than fimbriae, about 1 to 10 pili per cell and often are larger than fimbriae (around 9 to 10 nm in diameter) but shorter. Even though they present morphological differences, the literature frequently interchanges the words (Juge. 2012). These fibers are used for adherence by both Gram-negative and Gram-positive bacteria, but are substantially different depending on the linkages between pilins structures. Pilins from Gram-negative are not covalently linked, whereas the pili of Gram-positive bacteria are composed of multiple pilin subunits covalently coupled to each other by the transpeptidase activity of the pilin-specific sortase (Schneewind et al., 1993).

There are numerous types of fimbriae, and they differ from each other according to the assembling procedure, the morphology and their receptor binding specificities (**Fig. 2.3**). The fibers of Gram-negative bacteria have been extensively studied; particularly, those of the *Enterobacteriaceae* including *E. coli* and *Salmonella* spp. (Salyers and Whitt, 2002). In this sense, the most thoroughly characterized fimbriae are the type 1 and P fimbriae of *E. coli* (Ofek et al., 2003a). Type 1 fimbriae are expressed on virtually all enterobacteria, encodes the subunit FimH which determines the target receptor specificity, being in this case, mannose rich residues (Chen et al., 2009) whereas the subunit PapG in the P fimbriae binds to globoseries of the glycolipids, presents mainly in the urinary tract (Striker et al., 1995).

2.1.4.1.3. THE PARTICULAR CASE OF THE FIMBRIAL ADHESINS OF E. COLI K88

The *E. coli* K88, besides to produce enterotoxins, is also characterized by the production of adhesins that mediate bacterial adherence to the intestine of piglets causing diarrhea (Fairbrother et al., 2005). In particular, the K88 (F4) fimbria is the most common adhesin type associated with diarrhea in nursing pigs as well as in weaned pigs. K88 are flexible fimbriae that occur as K88ab, K88ac, or K88ad variants, but the K88ac antigenic variant is by far the most common type (Blanco et

al., 1997). The “a” antigenic region is conserved and the second antigenic variant is variable and designated as “b”, “c” and/or “d”. Each of the three variant of K88 fimbrial adhesin (K88ab, ac and ad) has a different carbohydrate binding specificity as evidenced by the existence of multiple phenotypes of pigs whose intestinal brush borders bind different combinations of K88 adhesin variants (Billey et al., 1998). In addition, there are differences in binding specificity by pigs as some of them are susceptible to all three types, some are susceptible to two types, some are only susceptible to a single type, but there are some resistant piglets to all the antigenic varieties (Francis et al., 1999).

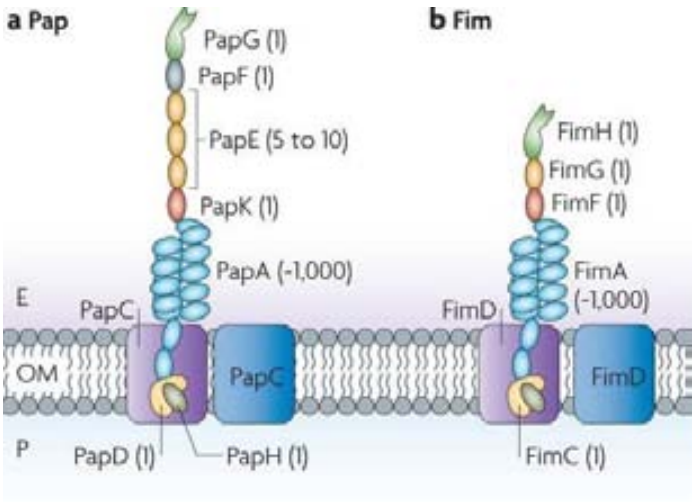

Figure 2.3. Schema of P (Pap) and Type 1 (Fim) fimbriae remarking their main structural differences. From Allen-Vercoe and Woodward, (1999).

2.1.4.2. AFIMBRIAL ADHESINS

Alternatively, afimbrial adhesins are bacterial surface proteins that are not organized in a rod-like structure and mediate tight binding between bacteria and host cell. These adhesins, commonly referred to as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), have been shown to bind ECM components with the objective to initiate infection (Patti and Hook, 1994). MSCRAMM are secreted through the cytoplasmic membrane and their morphological appearance is “fuzzed” because are short filaments (Ofek et al., 2003b). Many are then attached covalently to the peptidoglycan layer by the sortase enzyme. Several pathogenic bacteria bind throughout these structures to ECM components including fibronectin, collagen, laminin, and elastin, as the four main fibrous proteins (Chagnot et al., 2012). Two of the most widely studied systems of bacteria-ECM interaction are *S. aureus* and *Streptococcus pyogenes* binding to fibrinogen (Boland et

al., 2000). However, neither the MSCRAMM proteins nor the sortase enzyme are exclusive to these pathogens; they are present in a great number of Gram-positive bacteria. Fibronectin is one of the major plasma glycoproteins incorporated into the fibrin matrix during blood clot formation. In *S. aureus*, adherence has been found to involve at least two MSCRAMM fibronectin-binding proteins (FnBPs), known as FnbpA and FnbpB. In general, FnBPs are surface proteins anchored in the cell wall that contain an amino acid sequence motif, [Leu-Pro-X-Thr-Gly (LPXTG, where X can be any aminoacid)], and have a short positively charged C-terminal tail where fibrinogen recognition occurs within sequences repeats of 35-40 amino acids residues (Patti and Hook, 1994). Regarding *S. pyogenes,* multiple MSCRAMMs have been identified, which could possibly explain how it is able to colonize different host tissue and confer various tissue tropisms (Shoaf-Sweeney and Hutkins, 2009).

2.1.4.2.1. ADHESINS OF *STAPHYLOCOCCUS AUREUS* CAUSING MASTITIS

Staphylococcus aureus is one of the most frequently isolated contagious pathogens causing clinical or subclinical mammary gland infection (Melchior et al., 2006b). Multiple strains can be involved, not only in different herds from a similar or a different geographical environment, but also within a single herd (Joo et al., 2001). The adhesion of *S. aureus* is not only dependent in one single type of virulence factor but in the combined action of more than forty different toxins, enzymes and cell surfaces structures, that makes this bacteria extremely difficult to combat (Zecconi et al., 2006). The expression of virulence factors of *S. aureus* is dependent on the bacterial growth phase (Projan and Novick, 1997). Most adhesins are expressed from the early exponential to the mid-exponential phase of growth, when cell density is low (Lowy. 1998). As the growth cycle progresses and cell density increases in the late exponential phase, most adhesins are down-regulated. At the same time, the expression of many of the secreted virulence factors, such as cytotoxins, superantigens, and proteases, is upregulated. The organization of these pattern of expression across the *S. aureus* growth cycle is regulated by active regulatory systems, such as quorum sensing molecules (Arvidson and Tegmark, 2001; Cheung and Zhang, 2002). Therefore *S. aureus* express several MSCRAMMs and all of them could play a strategic role in the different steps of pathogenesis (**Table 2.2**), however the FnBPs are essential for adhesion and invasion of bovine mammary epithelial cells (Lammers et al., 1999; Fluit. 2012).

Table 2.2. Most important microbial surface components of *S. aureus* recognizing adhesive matrix molecules (MSCRAMM).

Surface protein (Abreviation)	Gene	Ligand specificity/function
Fibronectin-binding protein A (FnBPA)	*fnbA*	Fibronectin, fibrinogen, elastin; intercellular adhesion
Fibronectin-binding protein B FnBPB	*fnbB*	Fibronectin, fibrinogen, elastin; intercellular adhesion
Clumping factor A (ClfA)	*clfA*	Fibrinogen; platelet aggregation
Clumping factor B (ClfB)	*clfB*	Fibrinogen, cytokeratin 10, desquamated nasal epithelial cells; platelet aggregation
Collagen-binding protein (Cbp)	*cna*	Collagen; intercellular adhesion
Fibrinogen-binding protein (Fbp)	*fib*	Fibrinogen; intercellular adhesion
Laminin-binding protein (Lbp)	*eno*	Laminin; adhesion, ECM destruction, favouring dissemination
Elastin-binding protein (Ebps)	*ebps*	Soluble Elastin, tropoelastin; adhesion to ECM, regulates cells growth

From Heilmann (2011)

2.1.5. PRESENCE OF HOST RECEPTORS IN TARGET TISSUES

The biology of tissues and host cells is critical in identifying mechanisms of bacterial adherence. Although animals and humans are organized by different specialized tissues, the epithelial cells exhibit all of the general components of cell membranes (Ofek et al., 2003a). The formed epithelium possesses three basic characteristics: (i) the cell layer that has a free apical surface, keratinized (e.g., the skin) or not keratinized, but coated with a layer of mucus (e.g., intestine); (ii) the contiguous cells that are joined by junctional complexes; and (iii) the cell layer that is attached basally to an extracellular matrix specialization termed the basal lamina, which itself sits on other ECM components called lamina propia. The lamina propia is composed by connective tissues (e.g. collagen, fibrinectin, and elastin), various immunity cell types, as well as neural and vascular ramifications. Besides the differences among tissues and their higher or lower complexity degree, two distinct types of receptors could be categorized: cell surfaces structures and ECM components.

2.1.5.1. RECEPTORS ON CELL SURFACE STRUCTURES

The receptors on cells surface are, in general, submerged inside the sticky gel mucus layer. Mucus is a biochemically complex medium of variable thickness, which contains a number of specific components as mucins, exfoliated cells and other trapped materials (Gouyer et al., 2011; Ambort et al., 2012; Juge. 2012). The mucins constitute the major structural components of the mucus layers, which are synthetized by goblet cells. Mucins possess a core with repeated sequences of proline,

threonine and serine residues. To these aminoacidic structures, the 80% of mucins are *O*-glycosilated and, the *N*-glycosylations (mainly high-mannose-, hybrid/complex-type glycans), are quantitatively minors. The glycosylation process is initiated in the Golgi apparatus by the addition of an N-acetyl-D-galactosamine (GalNAc) to the Ser and Thr of the core structures (Tran and Ten Hagen, 2013). After further elongation to variable lengths and ramifications, the chains are terminated by L-fucose, D-galactose, D-mannose, GalNAc or sialic acid (Miner-Williams et al., 2009). The *O*-glycosylated extracellular domain protrudes at least 800 nm above the cell surface being a strategic point for cell adhesion, whereas the domain anchored in the cytoplasmic is involved in signal transduction (Marth and Grewal, 2008; Jonckheere and Van Seuningen, 2010). Depending on the structure of mucin glycosilations, they can act as a releasable decoy ligand for bacterial adhesins, thereby limiting attachment of pathogens (Linden et al., 2009), or can also act as receptor molecules for bacterial lectins.

There have been reported innumerable receptors able to recognize different microbial lectins. Just to mention some examples, the presence of α1-2 fucosylated receptors present in the colonic mucins is related to bind *S. enterica* Typhimurium which encodes the *std* operon (Chessa et al., 2009). Sialic acids and Gal are responsible for the attachment of enterotoxigenic *E. coli* K99 fimbrial adhesins (Vazquez et al., 1996) whereas F17 has affinity for N-acetyl-β-D-glucosamine-presenting receptors (Buts et al., 2003). The P fimbriae of UPEC bind to galabiose-containing glycolipid receptors (Sung et al., 2001). The glycan linkages of mammalian host are an abundant and attractive target to establish an infection through bacterial lectin binding (Marth and Grewal, 2008), although the following paragraph will only briefly emphasize the receptors for *E. coli* K88.

2.1.5.1.1. RECEPTORS FOR E. COLI K88

Several putative K88 adhesin receptors have been identified in the brush borders of epithelial cells, from intestinal membranes, and from mucus of piglets (Jin and Zhao, 2000). These putative receptors have been reported as being either glycoproteins or glycolipids allocated on the jejunum and the ileum (Fairbrother et al., 2005). By using binding kinetic studies, it was detected the presence of high- and low- affinity receptors for K88 adhesin (Sellwood. 1984), depending on the adhesin's antigenic variants. K88ad adhesin appears to preferentially bind to glycolipids, while K88ab and K88ac adhesins preferentially bind to glycoproteins (Grange et al., 1999). Receptors for K88ac variant were characterized as *O*-linked mucin-type sialoglyconjugates (Erickson et al., 1994) also called intestinal

mucin-type glycoprotein, but digestion techniques revealed that the terminal sialic acid did not contribute to the binding of K88ac adhesin to the receptors (Grange et al., 1998). Regarding K88ab receptors, it was demonstrated that adhesion was mediated by the monosialylated and monofucosylated glycan chain of the *N*-acetyllactosamine type present in the intestinal transferrin GP74 receptor (Grange et al., 1997). Lastly, the intestinal neutral receptors for K88ad appear to be neolactotetraosylceramide (nLc4Cer, Galβ1-aGlcNAcβ1-3Galβ1-4Glcβ1-1-Cer). Nevertheless, what is clearly demonstrated is the ability of all K88 variants to recognize carbohydrate structures as *β*-galactose, *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetylglymannoseamine and D-galactosamine (Jin and Zhao, 2000).

2.1.5.2. EXTRACELLULAR MATRIX COMPONENTS

The components of the ECM are important bacterial receptors when the integrity of the apical enterocytes surface is compromised, or when the pathogen is able to invade cells from the basal side (Schennings et al., 2012). In a recent review, it has been described in detail the composition, structure and organization of ECM (**Figure 2.4**), and the most important protein components involved into binding bacteria (Chagnot et al., 2012). Bacteria that possess the collagen binding domains promote strong binding to collagen such as some strains of *S. aureus* and *Enterococcus faeceium* (Nallapareddy et al., 2003; Zong et al., 2005). The fibronectin and fibrinogen binding domains have clearly attracted the most interest in bacterial adhesion and have been reported in a lot of bacteria (Heilmann. 2011; Chagnot et al., 2012). Other binding domains have not received so great deal of attention like those specific binding laminin or elastin. Nevertheless laminin has been described to serve as an attach point for pathogens like enterohemorragic *E. coli* (Wells et al., 2009) and *Leptospira* spp. (Pinne et al., 2010). Because cell surface structures are the most well studied binding-appendages with respect to ECM, much remains to be learned on specific binding to other ECM proteins via single or supramolecular protein structures (Chagnot et al., 2012).

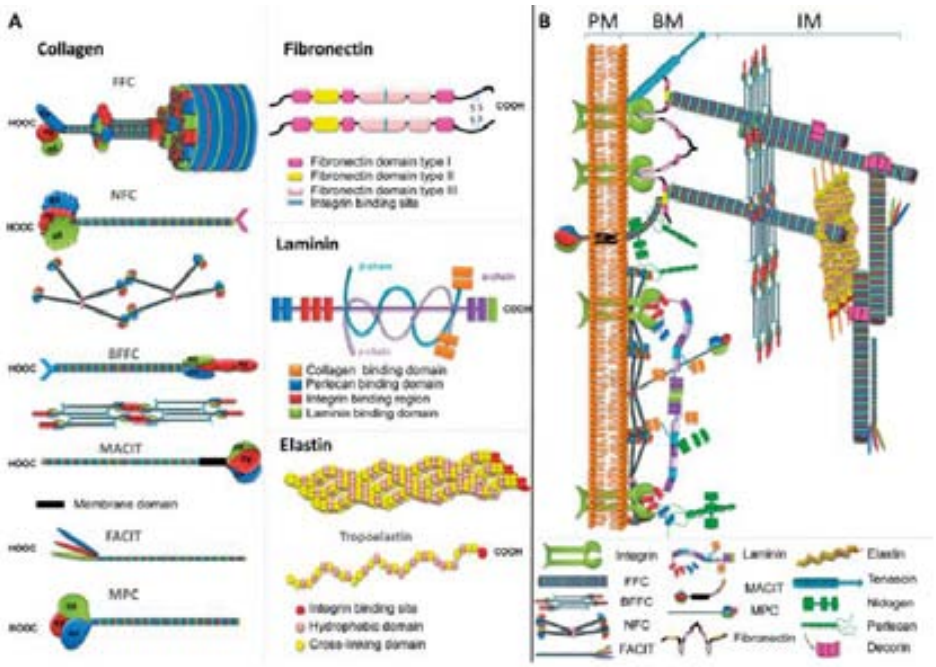

Figure 2.4. Schematic representation of the (A) molecular structure and (B) supramolecular organization in tissue of the main ECM components. FFC, fibril-forming collagen; BFFC, beaded filaments-forming collagen; FACIT, fibril-associated collagen with interrupted triple helices; MACIT, membrane-associated collagen with interrupted triple helices; NFC, network-forming collagen; MPC, multiplexin collagen; PM, plasma membrane; BM, basement membrane; IM, interstitial matrix (Chagnot et al., 2012).

2.2. MICROBIAL BIOFILMS

2.2.1. INTRODUCTION

After bacterial adhesion to biotic and also abiotic surfaces, several microorganisms have the ability to form a structured community of bacterial cells enclosed in a self-produced polymeric matrix commonly called biofilm (Costerton et al., 1999). In the living organism, biofilms can display different roles or effects with variable health impact: (i) play a protective or probiotic role, (ii) be associated with commensal communities and non-invasive colonization, or (iii) be pathogenic in nature, resulting in chronic diseases (**Table 2.3**) (Ehrlich et al., 2004). For instance, the presence of biofilms of mixed bacterial species attached to the epithelial cells in the intestine, forms an important barrier against invading foodborne pathogens (Probert and Gibson, 2002), nevertheless dysbiosis in this particular microbial community could be behind some gastrointestinal conditions such as inflammatory bowel diseases (Macfarlane et al., 2011). Biofilms are therefore also involved in a significant amount of chronic infections, moreover taken into account that microorganisms in biofilms are less susceptible to antimicrobials than their freely suspended bacteria counterparts (Goldstone et al., 2012). In veterinary

medicine, maybe mastitis in dairy cows is on the top among diseases related with biofilm production. *S. aureus* is the prime etiological agent causing mastitis in bovines (Bradley et al., 2007) and besides the production of many MSCRAMMs that mediate epithelial adhesion (Clarke and Foster, 2006), the formation of biofilms, is also recognized as an important virulence factor of this pathogen (Otto. 2013). In this sense, many staphylococci growing in biofilms in infected tissues, like in the udder of dairy cows, develop an innate resistance to almost all therapeutic agents (Melchior et al., 2006a). The natural structure of biofilms makes the treatment with biocides and antibiotics frequently ineffective to overcome bacterial infections (Costerton et al., 1999). By this reason, it is important to avoid the unfavourable biofilm formation by inhibiting bacterial attachment, but also providing methods for biofilm control, as preventing its formation or destroying it (Blackledge et al., 2013).

Table 2.3. Partial list of infections involving biofilms related to host tissues

Infection or disease	Common biofilm bacterial species
Dental caries	Acidogenic Gram-positive cocci (e.g. Streptococcus)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	*P. aeruginosa*, *E. coli*, *S. aureus*, pneumococci and *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g. *Staphylococci*)
Necrotizing fascitis	Group A *Streptococci*
Biliary tract infection	Enteric bacteria (eg. *Escherichia coli*)
Osteomyelitis	Various bacterial (*S. aureus*) and fungal species – often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Group *Streptococci*
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Meliodiosis	*Pseudomonas pseudomallei*
Mastitis	Group *Staphylococci*

2.2.2. DEVELOPMENT AND STRUCTURE OF BIOFILMS

All bacteria form biofilms in essentially the same manner regardless of which environment they inhabit (Costerton et al., 1999). Surfaces are normally conditioned with water, lipids, albumin, extracellular matrix or other nutrients from the surrounding environment (Dunne. 2002). Despite of differences among the numerous candidate surfaces and bacteria able to perform biofilms, a general model for biofilm development has been proposed (Lemon et al., 2008). Biofilm development includes initial attachment, maturation, and final detachment (Otto. 2013). Attachment may occur directly to a surface (such as the polymeric surface of an indwelling medical device) or to a “conditioning film” formed by host matrix molecules. Then, biofilm maturation proceeds via the agglomeration of cells,

which is dependent on adhesive molecules. Formation of the characteristic channel-containing biofilm structure is dependent on disruptive factors, which also ultimately facilitate the last phase of biofilm development, detachment.

2.2.2.1. INITIAL ATTACHMENT

Planktonic bacteria, those floating, drifting or motile in the liquid medium, initiate the contact with a surface either randomly or in a directed way (Jiang and Pace, 2006). During the initial interaction between bacterial cells and the surface, different physical, chemical and biological processes take place. On abiotic surfaces, bacterial attachment is generally mediated by non-specific interactions such as electrostatic, hydrophobic, or van der Waals forces; whereas adhesion to biotic surface is through specific molecular (lectin or adhesin) docking mechanisms (Dunne. 2002). Even so, the motility of bacteria is another point to consider in planktonic bacteria to make initial contacts with surfaces. For instance, the flagella-mediated motility can bring the cell within close proximity to the surface overcoming repulsive forces between bacterium and the surface where bacterium will be attached, so forming the first monolayer (Shirtliff et al., 2002; Lemon et al., 2007). In a similar way, type IV pili are responsible for a form of surface-associated movement known as twitching motility, and have been shown to play an important role in bacterial adhesion to eukaryotic cell surfaces and subsequent microcolony formation (O'Toole and Kolter, 1998).

After binding, bacterial cells start the process of irreversible adhesion, proliferation, and accumulation as multilayered cell clusters (Jiang and Pace, 2006).

2.2.2.2. MATURATION

The development of biofilms requires multicellular behaviour, which can involve more than one bacterial species. The observation that biofilms of many different bacterial species develop through similar stages, suggests that biofilm formation is a genetically regulated process (McDougald et al., 2011). Once bacterial cells have been irreversibly attached, bacteria start to form microcolonies either by aggregation of already attached cells, cell division or cell recruitment of planktonic cells from the bulk liquid (Jiang and Pace, 2006). The efforts within the colony are focused into downregulate non-interesting components for the bacterial community, such as flagellum or other motility components, but prioritizing exopolysaccharides (EPS) synthesis (Davies and Geesey, 1995). The microcolonies develop into a mature biofilm that is associated with the production of different EPS, a wide range of

proteins, extracellular DNA (eDNA) and other polymers which form a more complex structure (**Figure 2.5**) (Joo and Otto, 2012). They are considered to be essential in cementing bacterial cells together in the biofilm structure, in helping to trap and retain nutrients for biofilm growth, and in protecting cells from dehydration and antimicrobial agents.

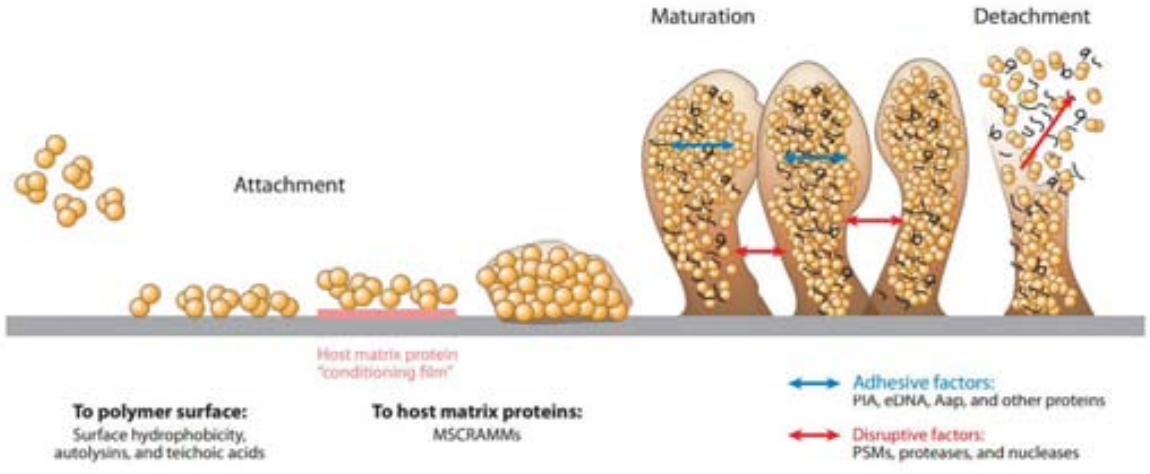

Figure 2.5. Phases of biofilm development. Molecular determinants shown or suspected to be involved in the respective biofilm development phases in staphylococci are noted on the bottom. Abbreviations: Aap, accumulation-associated protein; eDNA, extracellular DNA; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PIA, polysaccharide intercellular adhesin; PSMs, phenol-soluble modulins. Aadapted from Otto. (2013).

In the centre of mature biofilm structures, only a subpopulation of cells will undergo lysis. These killed cells provide nutrients for the bacteria that will become the dispersal cells (McDougald et al., 2011). Only for some well-studied biofilm systems, the genetic regulators, the effectors and their modes of action for killing a subpopulation of cells are known.

The structural development is a dynamic multifactorial entity, which is constantly fluctuating both in time and space (Hall-Stoodley and Stoodley, 2002). Bacteria growing in these matrix-enclosed microcolonies, possess open water channels which facilitate nutrient uptake by allowing the flow to permeate into the structure (Stoodley et al., 1994). Environmental signals as osmolarity, pH, iron availability, oxygen tension and temperature, as well as the surrounding hydrodynamics affect the architecture of biofilms. In this sense, biofilms can present a rich repertoire of structures, that can range anywhere from patchy monolayers, to thin or thick flat biomasses, to more organized mushrooms, ripples and filamentous streamers (Stoodley et al., 1999). For instance, biofilms growing in fast-moving water tend to form filamentous streamers (Reysenbach and Cady, 2001). In the

contrary, the biofilms formed in quiescent water are like mushroom or mound structures (Goller and Romeo, 2008). However, the underlying mechanisms that promote biofilm architectural development are yet to be completely characterized.

The senses of bacteria to grow enclosed in a biofilm matrix provide several ecological advantages. Speculations about the benefits of living in community have been regarding different factors like the protection from the environment (Vu et al., 2009), the nutrient availability and metabolic cooperativity (Costerton et al., 1995) and, finally the acquisition of new genetic traits (Roder et al., 2013).

The polysaccharide intercellular adhesin (PIA, also called poly-N-acetyl glucosamine [PNAG]) and the teichoic acids are the most important components of the extracellular matrix in staphylococci (Otto. 2013). The PIA is associated with biofilm infection in animal infection models, but is also found in other biofilm-forming bacteria, including many staphylococci and even Gram-negative bacteria (Kaplan et al., 2004; Wang et al., 2004). Teichoic acids interact with other surface polymers and function as a scaffold for protein adhesion (Heilmann. 2011).

In addition, proteins may have a role in formation of the biofilm matrix (Rohde et al., 2007). The accumulation-associated protein (Aap) contributes to the establishment of intercellular connection by forming fibrils on the cell surface in *S. epidermidis* (Rohde et al., 2005). In *S. aureus* and *S. epidermidis*, other surface proteins such as protein A, the *S. aureus* surface proteins SasC and SasG, extracellular matrix binding protein (Embp), biofilm-associated protein (Bap), and the fibronectin-binding proteins FnbpA and FnbpB were implicated in biofilm formation (Corrigan et al., 2007; Gruszka et al., 2012; Geoghegan et al., 2013; Otto. 2013).

Finally, the eDNA which is released from dying cells, is a component of the extracellular biofilm matrix (Gloag et al., 2013). DNA is a polyanionic molecule that is believed to interact with other matrix polymers of opposite charge, thereby contributing to the matrix network. It has been suggested to act as a resistance mechanism against prejudicial conditions for the biofilm (Rajendran et al., 2013).

2.2.2.3. FINAL DETACHMENT

The detachment process is an important feature of the biofilm life cycle which plays a fundamental role in dissemination, contamination and ultimately long-term survival, and therefore becoming in persistent infections (Otto. 2013). The formation of detached cells and their eventual dispersion is initiated through diverse and remarkably sophisticated mechanisms, suggesting that

there are evolutionary pressures for dispersal from an otherwise largely sessile biofilm (McDougald et al., 2011). The released dispersed cells as well as the passive dispersed cells resulting from biofilm erosion have the same opportunity to initiate new biofilm structure elsewhere.

Focusing in the specialized dispersed cells, these scape by coordinated evacuation from break points (Purevdorj-Gage et al., 2005), leading to the characteristic hollowing of biofilm microcolonies that is observed during the dispersal stage for many biofilms. To break biofilm bonds, bacteria have developed a number of enzymes, surfactants and bacteriophages that will degrade the biofilm matrix, eDNA, polysaccharides and proteins, leading to dispersal (Karatan and Watnick, 2009). The decision of bacteria enclosed in a biofilm to disperse is thought to depend from a range of environmental cues, including alteration in the availability of nutrients (such as carbon sources), oxygen depletion, low levels of nitric oxide, changes in temperature and high or low levels of iron (McDougald et al., 2011). In addition, quorum sensing (QS) derived signals (for more details see 2.2.3 section) are also involved in to induce dispersal, including acyl-homoserine lactones (AHL), cell-cell autoinducing peptides, diffusible fatty acids and D-amino acids (Jiang and Pace, 2006). In this sense, the review of Joo and Otto (2012), pointed the important insight into the QS controlled factors that structure biofilms and cause detachment. In both *P. aeruginosa* and staphylococci, surfactants function via disruption of non-covalent interactions between biofilm cells and matrix molecules.

2.2.3. QUORUM SENSING IN MICROBIAL BIOFILMS

2.2.3.1. THE CONCEPT OF QUORUM SENSING

Quorum sensing (QS) is an important regulatory mechanism of biofilm lifestyle in a variety of bacterial species (McDougald et al., 2011). The QS involves the accumulation of signalling molecules in the surrounding environment which enables a single cell to sense the density of the number of bacteria and the signalling molecules, and therefore the population as a whole, can make a coordinated response (Goldstone et al., 2012). These cell-cell communication systems regulate various functions as diverse as motility, virulence, sporulation, antibiotic production, DNA exchange, and development of more complex multicellular structures such as biofilm (Jiang and Pace, 2006).

2.2.3.2. BACTERIAL QUORUM SENSING SIGNALS

Several putative bacterial signals have been discovered, but in general QS systems have been divided into at least three classes: acyl-homoserine lactones (AHL) known as autoinducer-1 (AI-1)

signals in Gram-negative bacteria (Galloway et al., 2011), amino acids and short cyclic peptide signals in Gram-positive bacteria known as autoinducing peptides (AIP) (Kleerebezem et al., 1997), and a furanosyl borate diester known as autoinducer-2 (AI-2) signals of both groups (Chen et al., 2002).

The synthesis of AHL by Gram-negative bacteria is dependent on the LuxI/LuxR-type QS. The LuxI-like enzymes produce a specific AHL which freely diffuses across the cell membrane and increases in concentration in proportion to cell density. The transcriptional regulator, LuxR-like receptor protein binds to the diffusing AHL, thus activating transcription of target genes (Miller and Bassler, 2001). There are hundreds of Gram-negative bacteria identified to use LuxI/LusR-type QS to control a wide range of cellular processes (Nasuno et al., 2012). Each species produces a unique AHL or a unique combination of AHL, but only the members of the same species recognize and respond to its own signal molecule (Purohit et al., 2013).

In contrast, a number of Gram-positive bacteria have been shown to employ small, modified oligopeptides as extracellular signalling molecules known as autoinducing peptide (AIP) (Sturme et al., 2002). These peptides are recognized by two-component signal transduction system, which consists of a membrane-associated, histidine kinase protein sensing the AIP, and a cytoplasmic response regulator protein enabling the cell to respond to the peptide via regulation of gene expression.

The third class of QS has been described in both Gram-negative and Gram-positive bacteria which are called autoinducer 2 (AI-2) (Pereira et al., 2013). The widespread occurrence of AI-2 among bacteria is often considered the evidence for a universal language for inter-species communication (Rezzonico et al., 2012). The AI-2 molecule is a furanosyl borate diester produced by the enzyme LuxS (Chen et al., 2002). Different homologues of LuxS have been found in many species of bacteria, suggesting having important roles in microbial ecology (Pereira et al., 2013).

2.2.3.3. QUORUM SENSING TO FORM BIOFILMS

These QS signals have been detected in clinically relevant biofilms, but the precise role of QS in regulating biofilm formation and maturation is still not clear but great efforts in research are being performed (Li and Tian, 2012). **Table 2.4** is trying to show a general overview of the use of QS in the regulation of biofilm formation by some examples of Gram-negative and Gram-positive bacteria. Whereas the importance of AHL-dependent QS involved in biofilm formation has been relatively well studied, there is also an increasing body of research concerning the involvement of the third type of QS derived from the enzyme LuxS, the AI-2 (Miller et al., 2004). What it seems certain is that QS does

not appear to affect the initial attachment of cells to a surface (except for *Listeria* spp.), but rather the maturation of biofilm structure and dispersal (Goldstone et al., 2012; Joo and Otto, 2012).

2.2.4. MICROBIAL BIOFILMS IN THE GASTROINTESTINAL TRACT

The gastrointestinal tract (GIT) contains a rich and diverse microbiota along its length (Zhu et al., 2010). Bacteria in the large intestine can occur independently as individual cells, but also may exist in microcolonies growing in biofilms. Biofilms associated with different regions of the GIT are usually multispecies consortia whose development is determined by environmental and nutritional factors, as well as by the chemical composition of the substratum and host defensive mechanisms associated with the innate and adaptive immune systems (Macfarlane and Dillon, 2007). Nevertheless, it is the case that bacteria growing in a biofilm in the mucus layer of the gut are likely to play a pivotal role in gut health and disease (Probert and Gibson, 2002).

2.2.4.1. INFLUENCE OF THE GASTROINTESTINAL AREA ON THE FORMATION OF BIOFILMS

While much is known about the composition, structure and metabolism of oral biofilms, their study has been focused with less attention in the GIT. The anatomy of the large intestine, the physiology of host digestive processes and the mechanics of movement leads in two different biofilm-forming sites (**Table 2.5**): luminal biofilm and mucosal biofilm communities (Macfarlane and Dillon, 2007).

2.2.4.1.1. LUMINAL BIOFILM COMMUNITIES

Not only can bacteria exist as biofilms on the colonic epithelium and within the mucus layer covering it, but they can also form associations with food particles in the lumen (Macfarlane et al., 1997). Bacteria that are able to rapidly colonize food residues in the ceacum serve as inocula for new food residues entering the colon, and they have the occasion to form biofilms. The diversity of bacterial biofilms associated with food particles in the rumen and the large intestine of animals and humans facilitate hydrolysis and fermentation of the ingested material (Flint and Bayer, 2008; Rosewarne et al., 2011).

Table 2.4. Overview of the use of QS (type, system and genes involved) in the regulation of biofilm formation by Gram-negative and Gram-positive bacteria

Species	Colonization surfaces	Type of QS signaling	QS system	Genes	Function	Reference
Gram-negative						
Pseudomonas aeruginosa	Soil, water, skin, pulmonary tract, urinary tract, burns, wounds	AHL	rhl	*rhlI, rhlR*	Production of biosurfactant rhamnolipid	Brint and Ohman, 1995; Ochsner and Reiser, 1995
Serratia liquefaciens	Water, soil, digestive tract rodents, plants, insects, fish, humans	AHL	SwrRI	*swrA*	Synthesis of a lipopeptide biosurfactant	Lindum et al., 1998
				bsmA, bsmB	Development of highly differentiated filamentous biofilms	Labbate et al., 2004
Vibrio cholerae	Water, GIT humans&animals	AHL	HapR	*hapR*	Repression of matrix exopolysaccharide production	Karatan and Watnick, 2009
		AI-2		*luxS*	Repression of matrix exopolysaccharide production Synthesis of proteases	Ali and Benitez, 2009
Salmonella enterica	Foodborne pathogen	AI-2	LuxS/Autoinducer-2	*luxS*	Positive effect on biofilm formation	Choi et al., 2007
Escherichia coli	Ubiquitarious, GIT, reproductive tract	AI-2	-	wza	Production of biofilm matrix component colanic acid	Dong et al., 2007
Gram-positive						
Staphylococcus aureus	Skin, respiratory tract, wounds, indwelling medical devices,	Peptides	agr	*hld, hlaA*	Production of proteases that induce biofilm detachment	Boles and Horswill, 2008
Listeria monocytogenes	GIT, meninges, foodborne pathogen	Peptides	agr	*prfA*	Promotes attachment and biofilm formation	Luo et al., 2013
Enterococcus faecalis	GIT	Peptides	fsr	*fsrD*	Production of proteases which modulates the biofilm growth	Desouky et al., 2013
Bacillus subtilis	GIT	Peptides	SinR	*sinI*	Formation of biofilm matrix components by causing potassium leakage in the cell	López et al., 2009
Streptococcus mutans	Oral cavity	AI-2	com	*luxS*	Promotes biofilm formation	Li et al., 2002
Staphylococcus epidermidis	Skin	AI-2	agr	*luxS*	Production of PIA polysaccharide	Xu et al., 2006

Bacteria growing in association with food particles possess a different metabolic profile to their planktonic counterparts. For example, these bacteria are more efficient in digesting polysaccharides, while non-adhering communities break down oligosaccharides more rapidly. The formation of biofilm leads to significant production of acetate (Van Wey et al., 2011), even when biofilms constitute only 5% of bacterial population in faecal samples (Macfarlane and Macfarlane, 2006). In contrast, high levels of butyrate seem to be produced by non-adherent populations.

Table 2.5. Comparison between lumenal and mucosal bacterial populations in the human colon (from Probert and Gibson, 2002)

Lumenal/planktonic bacteria	Mucosally-associated/biofilm bacteria
Bacteroides	*Bacteroides*
Eubacterium	*Fusobacterium*
Bifidobacterium	Spirochaetes
Lactobacillus	*E. coli*
Gram positive cocci	Helicobacter
Clostridium	*Bifidobacterium*
	Gram positive cocci

2.2.4.1.2. MUCOSAL BIOFILM COMMUNITIES

Bacterial communities inhabiting the epithelial surface are also able to form biofilms. In this sense, the mucosal biofilms differs significantly in composition to bacterial communities in faecal material (Zoetendal et al., 2002). The formation of the biofilm community structure is determined by a variety of host factors, including immune system, the synthesis and composition of mucus, epithelial turnover, diet, availability of adhesion sites, lysozyme production, pancreatic substances, colonization resistance mediated by the normal commensal microbiota and gut motility. The study of colonic bacteria in humans showed that mucosal biofilms often occurred in microcolonies, which seems to be a characteristic of mucosal communities throughout the GIT (Macfarlane et al., 2004). In terms of fermentation, this microbiota is better adapted to utilise long chain endogenous substrates, such as mucin, when compared to planktonic bacteria.

In particular, fusobacteria associated with mucosal tissue have been found to have a “bridging” function within biofilms, forming coaggregation/coadhesion with other bacterial strains such as *H. pylory*, *E. coli* and spirochaetes (Probert and Gibson, 2002). This may explain why only very low numbers of fusobacteria are isolated from faeces since their role is predominantly mucosally-associated.

2.2.4.2. BIOFILMS IN GASTROINTESTINAL DISEASES

A state of mutualism exists between bacteria residing in the mucus layer and the host. Mucosal biofilm seems to confer a form of colonisation resistance against potential pathogens, rendering more difficult for opportunistic organisms to penetrate the intestinal mucosa and elicit and infection (Isolauri et al., 1999). Alterations in this host-bacteria interaction or cross-talk have been postulated as one possible pathogenic mechanism for intestinal inflammatory diseases (IBD). The incidence and prevalence of IBD are increasing with time in different regions around the world, indicating its emergence as a modern global disease (Molodecky et al., 2012). The increased consumption of polysaccharides in western diets may promote dysbiosis of gut microbes and contributes to diseases susceptibility (Nickerson and McDonald, 2012). The structure, organization and the composition of microbiota for IBD patients have demonstrated to greatly differ from healthy subjects (Swidsinski et al., 2008). Ileal lesions of patients with Crohn disease (CD) are colonized by adherent-invasive *E. coli* (AIEC) that is able to adhere and invade intestinal epithelial cells, to replicate within macrophages, and to form biofilm (Chassaing and Darfeuille-Michaud, 2013). The study of the specific biofilm formation between AIEC and non-AIEC strains, demonstrated that AIEC were more efficient biofilm producers (Martinez-Medina et al., 2009). Even though IBD are new disorders with unknown aetiology, but probably multifactorial reasons; there have been a number of recent studies that have shown the link between biofilms and disease in the GIT of humans (von Rosenvinge et al., 2013). Together to other factors responsible of IBD, the possible role of an altered biofilm structure may be also an important issue to further investigate in the prevention of these pathologies.

2.2.4.3. DIETARY INFLUENCE ON GASTROINTESTINAL BIOFILMS

Whether gut microbial biofilms have not been extensively explored, very little is known about the significance of diet on these gut biofilms. However, a lot of studies have shown that the microbial composition of mucosal communities can be manipulated through the use of prebiòtics, probiotics and synbiotics (Gaggia et al., 2010; Guerra et al., 2013). In the particular case of biofilms, Langlands et al. (2004) reported an increase in bifidobacterial and eubacterial numbers in mucosae of the proximal and distal colons in patients fed a prebiotic mixture. Similarly, the inclusion of inulin-type fructans in the diet modulated the gut microbiota by stimulation of biofilm mucosa-associated bifidobacteria as well as by partial reduction of *S.* Typhimrium in rats (Kleessen and Blaut, 2005). Several investigations have

demonstrated that even small dietary changes have profound effects on the mucosal microbiota, thus opening up new therapeutic strategies for tackling bacteria-associated gut diseases.

2.3. WAYS TO PREVENT BACTERIAL ADHESION AND BIOFILM FORMATION

2.3.1. INTRODUCTION

The management of bacterial infections is becoming increasingly difficult due to the emergence and increasing prevalence of bacterial pathogens that are resistant to antibiotics (Klemm et al., 2010). Due to this problematic in veterinary practice, as well as in human medicine, alternatives to antibiotic therapies are increasingly required. Targeting bacterial virulence functions directly is an attractive alternative. The present review will focus in two target ways: anti-adhesion and anti-biofilm therapies.

2.3.2. ANTI-ADHESION THERAPY

Considering that adherence is a prerequisite to initiate colonization and infection, preventing bacterial adhesion would appear to be an ideal strategy for reducing infectious disease (Shoaf-Sweeney and Hutkins, 2009). Several approaches have been evaluated, with each showing different levels of success and feasibility (**Figure 2.6**).

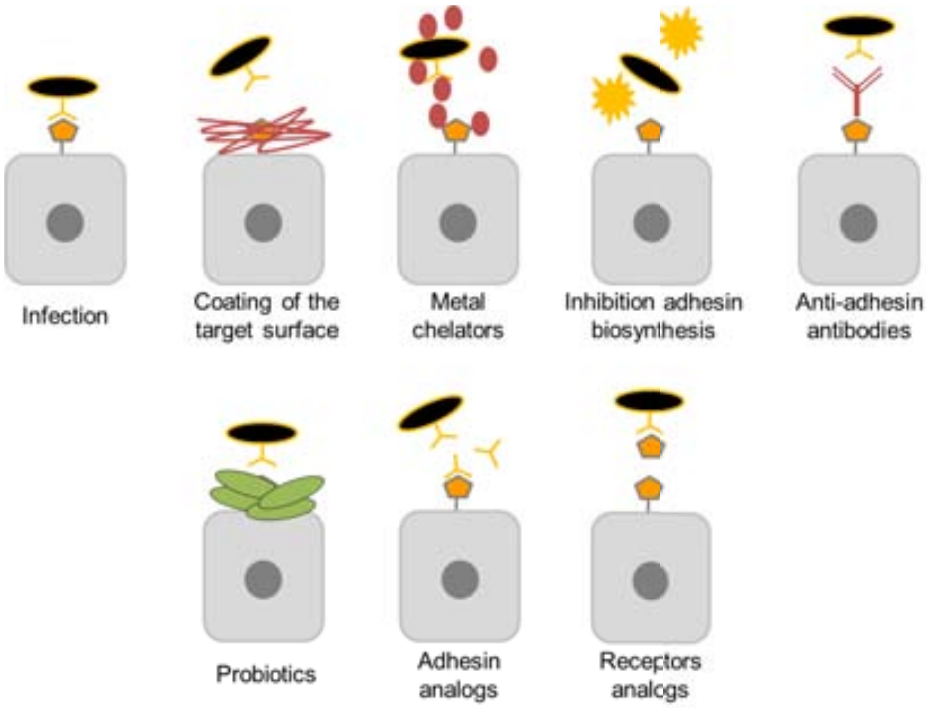

Figure 2.6. Pictures showing different strategies to inhibit bacterial adhesion.

These methods have involved both industrial and biomedical applications and are based on the following interactions: (i) changing surface interactions by coating inert surfaces with inhibitory macromolecules like α-tropomyosin (Vejborg et al., 2008); (ii) metal chelators such as citrate that interfere with bacterial growth, attachment and biofilm formation (Banin et al., 2006; Bosma et al., 2010); (iii) prevention by interfering in the adhesin biosynthesis (e.g. the bycyclic 2-pyridones interferes in the synthesis of fimbriae and curli) (Pinkner et al., 2006; Cegelski et al., 2009); (iv) anti-adhesive vaccines (Scarpa et al., 2010); (v) probiotics as anti-adhesives (Reid and Burton, 2002; Das et al., 2013); (vi) adhesin analogs (Leach et al., 2005; Sharon. 2006); and (vii) inhibition with receptor analogs (Ofek et al., 2003a). The latter three will be covered in more detail.

2.3.2.1. PROBIOTICS AS ANTI-ADHESIVES

Probiotics are live cultures that, when administered to humans or animals in adequate amounts, equilibrate the intestinal microbiota and benefit the host (Fuller. 1989). The most widely used probiotics are lactobacilli and bifidobacteria, but other microorganisms, including *E. coli*, enterococci, bacilli, and yeasts have also been used. The modification of the intestinal environment is the result of several mechanisms such as the synthesis of inhibitor compounds, increase of mucins secretion, adherence-site competition against pathogens and for nutritional sources, bacterial toxins inhibition and improvement of nutrients absorption (Strompfova et al., 2004). All these mechanisms, and other, are important and potentially play a role in probiotic functionality. However, the competitive binding of probiotics to host tissues at the expense of pathogens is clearly an anti-adhesive effect (Shoaf-Sweeney and Hutkins, 2009). Several probiotic organisms have been shown to inhibit pathogen adhesion to different surfaces, not only in the GIT, but also in the oral cavity, the mammary gland, the reproductive tract among others (**Table 2.6**).

The new genetic and molecular tools have allowed investigating the surface-layer proteins from some probiotic strains responsible to act as anti-adhesives against pathogens. An extracellular serine/threonine-rich protein from *Lactobacillus plantarum* NCIMB 8826 have recently been identified as an aggregation promoting factor with affinity to mucin which may be the responsible to limit attachment of pathogens to the gut mucosa (Hevia et al., 2013). Similarly, a 72-kDa surface layer collagen binding protein from *L. plantarum* 91 reduced 60% adhesion of *E. coli* O157:H7 on immobilized collagen (Yadav et al., 2013). S-layer proteins in lactobacilli seem to play an important role to inhibit *E. coli* K88 and *S. enteritidis* 50335 adhesion to Caco-2 (Zhang et al., 2013). In addition,

some investigations have revealed that the isolated extracellular proteins from probiotics are even able to prevent adhesion (Sanchez and Urdaci, 2012). Even though *in vitro* studies are promising, there have been no carefully controlled clinical human and animal studies to test the effect of probiotics as anti-adhesives.

2.3.2.2. ADHESIN ANALOGS

The adhesin analog strategy is based on the assumption that the isolated adhesion molecule or its synthetic or recombinant fragment binds to the receptor and thereby competitively blocks adhesion of bacteria (Shoaf-Sweeney and Hutkins, 2009). This type of anti-adhesive activity has been limited to use in practical applications because most of the time are macromolecules, that must be used in high concentrations, and due to their innate nature may be toxic and/or immunogenic (Ofek et al., 2003a). Nevertheless, modern proteomics and recombinant biotechnology have allowed the development of some potential adhesion analogs.

Both proteinaceous and non-proteinaceous analogs have been studied. For example, a synthetic peptide comprising the Bar sequence (the streptococcal SspB polypeptide) potently inhibited *Porphyromonas gingivalis* adherence to *Streptococcus gordonii* and prevents the development of *P. gingivalis* biofilms (Daep et al., 2006). Similarly, a synthetic peptide of 20 amino acids copied from the sequence of an *S. mutans* cell surface adhesin inhibited the binding of streptococci to the immobilized salivary receptor (Kelly et al., 1999). However, these results should be interpreted with caution because the adhesion of *S. mutans* is able to utilize other adhesins that bind to other cell surfaces. Thus the application of multiple analogs may be required for this approach to be effective (Ofek et al., 2003a).

Among the non-proteinaceous adhesins, lipoteichoic acid mediates the adhesion of groups A and B streptococci and their use under experimental *in vivo* conditions showed to reduce colonization of bacteria (Cox. 1982). Hyaluronic acid has also demonstrated their anti-adhesive properties against streptococci colonization (Cywes et al., 2000). However, more attention has been placed on receptor analogs to inhibit bacterial adherence.

Table 2.6. Examples of probiotic bacteria inhibiting the adhesion of pathogens

Probiotic	Pathogen	Substrata for adhesion assay	Justified probiotic inhibitor	Reference
L. salivarius ZJ616 *L. reuteri* (ZJ616, ZJ617, ZJ621, ZJ623)	*E. coli* K88 *S. enteritidis*	Caco-2 cell line	S-layer protein (34-130 kDa and 42 kDa)	(Zhang et al., 2013)
L. plantarum 91	*E. coli* O157:H7	Immobilized collagen type-1	72 kDa protein	(Yadav et al., 2013)
L. plantarum KSBT 56	*S. enteritidis*	HCT-116 cell line	Some virulence factor located in the Salmonella Pathogenicity island 1	(Das et al., 2013)
L. plantarum CS24.2	*E. coli* O26:H11	HT-29 cell line	NC	(Dhanani and Bagchi, 2013)
L. casei CIRM-BIA 667	*S. aureus* Newbould305 *S. aureus* RF122	Bovine mammary epithelial cells	NC	(Bouchard et al., 2013)
L. plantarum *L. salivarius*	*S. aureus*	Caco-2	Some cell surface properties	(Ren et al., 2012)
L. rhamnosus GG	*Campylobacter* spp.	Chicken intestinal mucus	NC	(Ganan et al., 2013)
L. reuteri	*E. coli*	HT-29 cell line	Mechanistically way: inhibition by competitive exclusion	(Bujnakova and Kmet, 2012)
L. paracasei DSMZ16671	*S. mutans*	Caries model in rats	NC	(Tanzer et al., 2010)
L. jensenii	*Neisseria gonorrhoeae*	Hec-1-B cell line	Protein in the inhibitory	(Spurbeck and Arvidson, 2010)
L. helveticus R0052	*C. jejuni*	Human colon T84 epithelial cells Embryonic intestine 407 epithelial cells	Mechanistically way: inhibition by competitive exclusion	(Wine et al., 2009)
B. lactis Bb12	*Salmonella* spp *Clostridium* spp. *E. coli*	Pig intestinal mucus	NC	(Collado et al., 2007)
B. longum *B. catenulatum*	*S.* Typhimurium *C. difficile*	Human intestinal mucus	NC	(Collado et al., 2006)
E. coli Nissle 1917	*S.* Typhimurium	IPEC-J2 cell line	Secretory components	(Schierack et al., 2011)

NC: No comment regarding the probiotic inhibitor in the article refereed.

2.3.2.3. INHIBITION WITH RECEPTOR ANALOGS

This strategy is based on the observation that bacterial adherence is often mediated by interactions between bacterial surface proteins and complimentary oligosaccharide receptors located at the surface of host cells. Therefore, soluble oligosaccharides may competitively inhibite the adherence process because resemble or mimic the host glycoprotein or glycolipid receptors. In a more illustrative approach (**Figure 2.6**), pathogens bind to the soluble oligosaccharide decoy and are displaced from the environment preventing infection initiation (Ofek et al., 2003a). Although many of the receptor analogs that have been studied are derived synthetically, there are numerous reports describing anti-adherence activities from natural sources, such as milk, plant compounds, and other foods. Moreover, there is now considerable evidence demonstrating that soluble oligosaccharides with specificity for an adhesin can competitively inhibit binding to target cells not only in the GIT, but also in a variety of other tissues (Shoaf-Sweeney and Hutkins, 2009).

2.3.2.3.1. SYNTHETICALLY DERIVED ANALOGS

In some cases where the interactions between the bacterial lectins to their complementary oligosaccharide ligands have been well studied, the information has been used to synthetically design and develop receptor mimetics or decoys. Because the molar concentrations of the carbohydrate required for effective inhibition of adhesion are usually high (in the millimolar range), the affinity of such saccharides to recognize the bacterial lectins is, in general low. Multivalency phenomena are used to achieve strong adhesion to the cell surfaces (Pieters. 2011). Therefore, several efforts have been focused into increase the valency of such oligosaccharides to get better inhibition results (**Figure 2.1**) (Salminen et al., 2007; Pieters. 2009).

One of the most studied interactions are the urinary tract infections caused by *E. coli*. A trisaccharide analog, the globotriose (Galα1,4Galβ1,4Glc) reduced the P-fimbriated *E. coli* attachment to the glycolipids of the globo series (Leach et al., 2005). On the other hand, a multimeric heptyl mannoside resulted in a strong inhibitor of type 1 fimbriated *E. coli* adhesion in a murine cystitis model (Gouin et al., 2009).

Other carbohydrates sequences that serve as receptors for enteric pathogens have been identified (Shoaf-Sweeney and Hutkins, 2009). The receptors for EPEC, located on the surface of host epithelial cell are often comprised of galactose, *N*-acetyl-galactosamine, lactosyl glycans, and fucosylated and sialylated oligosaccharides (Vanmaele et al., 1999). Similarly, the inhibitory potential

of *N*-acetyllactosamine to EPEC was also demonstrated (Hyland et al., 2006). About the particular case of ETEC K88, data from monosaccharide-blocking studies indicated the involvement of *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), N-acetylglymannosamine (Anderson et al., 1980), and D-galactosamine (Sellwood. 1980) in the binding. In this sense, results from glycoprotein-blocking studies confirmed the role of terminal GlcNAc, GalNAc, and galactose in the interaction of the fimbriae adhesion with the brush border receptors from piglets (Anderson et al., 1980). Besides oligosaccharides, glycolipids also play a role in the adhesion of K88 to pig intestinal borders (Grange et al., 1999). More recently, Coddens et al. (2011) characterized different glycoesphingolipids that were recognized by K88 fimbriae, with the ultimate goal to create a platform for synthesis of anti-adhesive substances.

2.3.2.3.2. NATURALLY OCCURRING ANALOGS

The possibility that pathogens can be inhibited by naturally occurring compounds is especially attractive and has captured significant attention (Shoaf-Sweeney and Hutkins, 2009). These assumptions comes from empirical observations which suggested that certain natural compounds may have beneficial effects preventing bacterial infections (Ofek et al., 2003a). Milk source, plant-derived compounds, and microbial by-products have been the most important candidates to be considered (Lane et al., 2010).

Milk contains a huge amount of constituents such as lactoferrin, casein peptides, and oligosaccharides which have been long investigated into their ability to bind pathogenic bacteria (Saadi et al., 1999; Schwertmann et al., 1999; Rhoades et al., 2005). Data collected over the years, strongly suggest that milk oligosaccharides are exceptional anti-adhesives. Gangliosides and sialic acids inhibit the adhesion of a wide range of enteropathogenic bacteria (ETEC, EPEC, *L. monocytogenes*, *S.* Typhimurium, *Shigella sonnei*, *C. jejuni* and *H. pylori* to Caco-2 (Salcedo et al., 2013). Milk oligosaccharides reduced the cellular invasion of *C. jejuni* (Ruiz-Palacios et al., 2003) and protected infants from diarrheal diseases (Morrow et al., 2005). Casin glycomacropeptide (CGMP), a glycoprotein originating from the C-terminal portion of κ-casein during cheese manufacture, inhibited *E. coli* attachment to the intestinal mucosa of piglets (Hermes et al., 2013). The reason of such biological activity maybe the high presence of sialylated and fucosylated oligosaccharides, which have a structural homology to the glucan moieties of intestinal mucosal cell surface (Newburg et al., 2005).

While milk has a restricted availability, plant material are generally abundant or can be engineered to become available. For this reason, vegetable products with anti-adhesin activities are attractive candidates as therapeutic agents. Theoretically, these lectins could interact with host cell receptors to block adhesion by competition; but they also could interact with bacterial adhesins to enhance the clearance of bacteria by exclusion (Slifkin and Doyle, 1990). The *in vitro* assessment of the interactions between vegetable products and pathogenic bacteria has been demonstrated with a miniaturized adhesion test in several works (Becker et al., 2007; Becker and Galletti, 2008; Molist et al., 2011). Simultaneously, Molist et al. (2010) also found less *E. coli* attached to the ileal mucosa after an ETEC K88 challenge in piglets when received wheat bran.

Among the large number of plant extracts studied (**Table 2.7**), cranberry is probably the most thoroughly studied (Wang et al., 2012). Summary of the *in vitro* data support that may be the proanthocyanidins (a flavonoid, also referred to as condensed tannin) which inhibit adherence (Shmuely et al., 2012). However, the target of this anti-adhesive activity seems to be the bacterial adhesin, not the animal cell receptor (Howell. 2007).

Other vegetable products rich in mannose, such as locust bean and locust seed, have demonstrated the anti-adhesive abilities against different enteropathogens. Badia et al. (2012c) showed a reduction in the adhesion of ETEC K88 on cell surface of porcine intestinal IPI-2I cells by a β-galactomannan product isolated from the locust bean gum. In a similar study, this product also demonstrated its ability to inhibit the association of *S.* Typhimurium with IPI-2I cell line (Badia et al., 2012a,b). And more recently, it was reported reductions in the number of ETEC K88 adhered to IPEC-J2 (Hermes et al., 2011) when incubated with locust bean extract. These products with more than 50% of mannan may act as binding sites for type-1 fimbriae bacteria, attracted by mannose receptors (Becker and Galletti, 2008).

Finally, by-products obtained from the microbial fermentation are a very interesting source because contain different kind of oligosaccharides, such as glucans and mannan. These structures resemble that of the surface glycoprotein containing mannose, as for example in the mucosal surface of the intestine, thus avoiding the binding of mannose-specific type-1 fimbriae such as *Salmonella* spp. and *E. coli*, resulting in their excretion from the intestine (Borowsky et al., 2009; Tiralongo and Moran, 2010). Some examples with demonstrated anti-adhesive capacity are mannooligosaccharides

(Becker and Galletti, 2008; Castillo et al., 2008), the *Aspergillus oryzae* fermented extract (Hermes et al., 2011) and microbial exopolysaccharides (Alp et al., 2010).

2.3.3. ANTI-BIOFILM THERAPY

Bacterial biofilms cause chronic infections that can persist despite the use of antibiotic treatments. It seems that bacteria in biofilms are significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species (Bryers. 2012). Moreover, recent studies have shown that sublethal doses of antibiotics can enhance biofilm formation suggesting a natural defence mechanism of bacteria to avoid the lethal effects of antibiotics (Bagge et al., 2004; Hoffman et al., 2005). Once bacteria are irreversibly attached to a surface, strategies for interfering structure development and differentiation of biofilms are being developed by many research groups (Yang et al., 2012). In this sense, several biological strategies for combating bacterial biofilms have been successfully discussed in a recent review (Blackledge et al., 2013). Some of these alternatives are: antibody therapy (Martinez and Casadevall, 2005), synthetic peptide vaccine and antibody therapy (Cachia and Hodges, 2003), disruption of biofilm EPS matrix (Longhi et al., 2008), negating biofilm formation by disrupting iron metabolism (Kaneko et al., 2007) and inhibiting or negating cell-cell signalling (Otto. 2004). Only the last three strategies are listed below.

2.3.3.1. ENZYMATIC DEGRADATION OF MATRIX COMPONENTS

This approach is based into destroying the integrity of the biofilm matrix, typically by enzymatic degradation of components of the EPS, leading to subsequent detachment of cells from the biofilm. This mechanism of biofilm disruption is an innate phenomenon employed by several diverse bacterial species (Kaplan. 2010). Alginate lyase has been shown effective to degrade alginate of *P. Aureuginosa* in *in vivo* and *in vitro* models (Bayer et al., 1992; Alkawash et al., 2006). Biofilms formed in the presence of DNase exhibit reduced biomass resulting from a reduced number and size of microcolonies within the biofilm, and decreased antibiotic tolerance (Tetz and Tetz, 2010). Other enzymatic anti-biofilm approaches include the use of proteases to modulate biofilms by degradation of the protein component of the biofilm matrix. Some endogenous proteases like Esp from *S. epidermidis*, have demonstrated activity against established biofilms of *S. aureus* (Iwase et al., 2010).

Table 2.7. Examples of plant-derived compounds that inhibit bacterial adhesion

Plant source	Chemical putative compound	Targeted bacteria	Tested tissue	Reference
Cranberry *Vaccinium macrocarpon*	Flavonols, anthocyanins, proanthocyanidins	*S. pneumoniae* UPEC	Human bronchial cells (Calu-3) Uroepithelial cell line	Huttunen et al., 2011 Ermel et al., 2012
Okra fruit *Abelmoschus esculentus*	Polysaccharides rich in glucuronic acid	*H. pylori* *C. jejuni*	Human gastric mucosa Colonic mucosa from chicken	Wittschier et al., 2007
Plantain bananas *Musa* spp.	Non-starch polysaccharides	*S.*Typhimurium *Shigella somnei* ETEC *C. difficile*	Caco-2	Roberts et al., 2012
Turmeric *Curcuma longa*	Essential oil components	*H. pylori*	Human gastric mucosa sections	O'Mahony et al., 2005
Blakcurrant seed *Carissa spinarum*	High molecular weight galactans	*H. pylori*	Human gastric mucosa sections	Langsfeld et al., 2004
Gingseng *Panax gingseng*	Acidic polysaccharide	*H. pylori*	Caco-2	Lee et al., 2004
Dandelion root *Taraxacum officinale*	Polysaccharides	*C. jejuni*	HT-29 cells	Bensch et al., 2011
Licorice *Glycyrrhiza glabra*	Acidic fraction from purified polysaccharides, glucuronic acid	*C. jejuni*	HT-29 cells	Bensch et al., 2011
Cayenne *Capsicum annum*	Alkaloids (capsaicin and dihydrocapsaicin), carotinoids and acyclic diterpene glycosides	*C. jejuni*	HT-29 cells	Bensch et al., 2011
Ginger *Zingiber officinale*	Gingerols (polyphenoplic compounds)	*C. jejuni*	HT-29 cells	Bensch et al., 2011
Valencia orange *Citrus sinensis*	Pectin oligosaccharides	*C. jejuni*	Caco-2	Ganan et al., 2010
Green tea *Camellia sinensis*	Acidic polysaccharide	*H. pylori* *P. acnes* *S. aureus*	AGS gastric cells NIH 3T3 fibroblast cells NIH 3T3 fibroblast cells	Lee et al., 2006 Lee et al., 2006 Lee et al., 2006
Edible mushroom *Lentinus edodes*	Solvent extract fractions (< 5 kDa)	*S. mutans*	Hydroxyapatite	Signoretto et al., 2012
Wheat bran *Triticum aestivum*	Arabinoxylooligosaccharides	*S.* Enteritidis ETEC K88 *E. coli*	Fecal samples from chickens IPEC-J2 *In vivo* experiment piglets	Eeckhaut et al., 2008 Hermes et al., 2011; Molist et al., 2011
Locust bean *Ceratonia siliqua*	Galactomannans	*S.* Typhimurium ETEC K88 ETEC K88 ETEC K88 *P. aureuginosa*	IPI-2I cell line IPI-2I cell line IPEC-J2 Fecal shedding from piglets Hemagglutination test	Badia et al., 2012b Badia et al., 2012c Hermes et al., 2011; Guerra et al., (2013) Zinger-Yosovich and Gilboa-Garber, 2009

2.3.3.2. IRON METABOLISM INTERFERENCE

Iron is critical for bacterial growth and the function of key metabolic enzymes. Thus, sequestration of iron is an early evolutionary strategy of host defence (Banin et al., 2006). On the one hand, iron-binding compounds demonstrated to reduce biofilm formation of *P. aeruginosa* (O'May et al., 2009). Gallium has many features similar to Fe^{3+}, including a nearly identical ionic radius, and biological systems are often unable to distinguish Ga from Fe^{3+}. Thus, placing Ga, rather than Fe, in such enzymes renders them non-functional. Ga readily binds to the siderophores (bacterially derived iron chelators) of *Pseudomonas* spp., *S. aureus*, *S. epidermidis*, *E. coli*, *E. faecalis* and *S.* Typhimurium. Ga efficiently interfered iron uptake and biofilm formation by *P. aeruginosa* (Kaneko et al., 2007).

2.3.3.3. DISRUPTION OF CELL TO CELL COMMUNICATION

It is generally accepted that in a number of clinically relevant bacterial pathogens, most notably in *S. aureus* and *P. aureuginosa*, QS signalling plays an important role in the control of virulence factor expression as well as biofilm formation (Boyen et al., 2009). Therefore, targeting for QS signalling systems might offer a new strategy to combat bacterial infections (**Figure 2.7**). Approaches that interfere with proper microbial QS signalling are called “quorum quenching (QQ)” (Dong et al., 2007). Currently, there are three main paradigms being pursued for the development of QQ agents as potential prophylactics or therapeutics (Zhu and Kaufmann GF., 2013). Firstly, inhibition of the synthesis of QS signal molecules, for example, LuxS in the case of AI-2 QS or LuxI-type proteins for AHL-based QS systems; second, neutralization of the QS signal molecules by using, for example, degrading enzymes; and third, ligand mimicry at the receptor level by analogs of the QS signal molecules.

2.3.3.3.1. NATURAL SOURCES WITH QUORUM QUENCHING ACTIVITY

In recent years, the screening and evaluation of foods with QQ activity have gained increasing popularity (**Table 2.8**) (Nazzaro et al., 2013). For example, the furanones produced by the red algae *Delisea pulchra* can interfere with AHL QS in *P. aeruginosa*, which affects maturation of the biofilm architecture, enhancing dispersal and causing the formation of substantially thinner biofilms (Hentzer et al., 2002). These compounds appear to function by displacing the AHL from the receptor protein, or by increasing the turnover rate of the receptor (Manefield et al., 1999; Manefield et al., 2002).

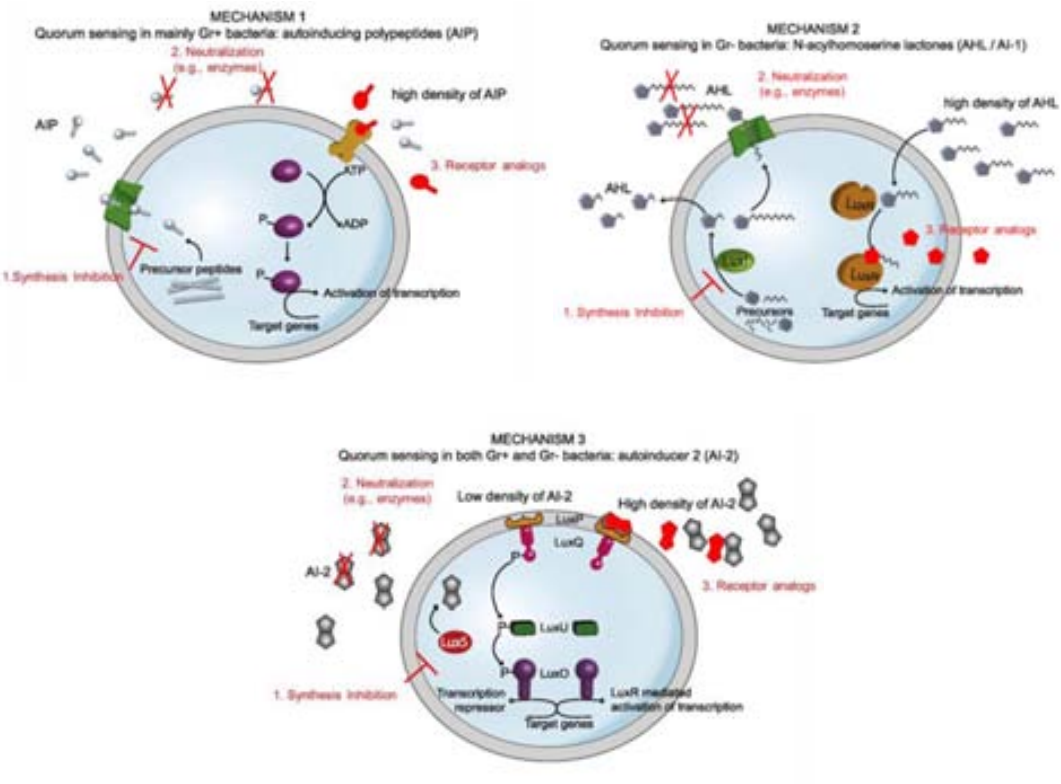

Figure 2.7. Simplified models of the described QS mechanisms and the most important ways to use QQ agents. Adapted from Boyen et al. (2009).

Moreover, such furanone compounds have also been shown to affect biofilm formation in *S.* Typhimurium (Janssens et al., 2008) and *E. coli* (Ren et al., 2004), bacteria which are not thought to utilize AHL-based QS. In these species, the furanone compounds are thought to be interfering with the LuxS/AI-2 signalling system and in this way affecting biofilm formation. In both cases, the furanone may supress QS by displacing the signal ligand from its cognate receptor (Manefield et al., 1999; Ren et al., 2004). Garlic extract is a further example of naturally occurring QS inhibitor detected following the screening of natural and synthetic compound libraries. Garlic has been shown to have anti-fungal, anti-cancer and anti-microbial properties (Ankri and Mirelman, 1999), and has been shown to specifically inhibit QS-regulated gene expression in *P. aeruginosa* (Harjai et al., 2010). Two compounds isolated from broccoli, named sulforaphane and erucin, also quenched QS signalling of the *las* system an overall virulence in *P. aeruginosa* (Ganin et al., 2013). In this sense, salicylic acid has interesting properties. Salicylic acid inhibited adherence (55%), growth, and biofilm production of *S. epidermidis* (Farber and Wolff, 1992). In addition, salicylic acid decreased the biofilm formation of *Candida* spp (Stepanovic et al., 2004). A dramatic reduction in biofilm formation by *P. aeruginosa*

grown with salicylic acid was attributed by the inhibition of the *las* QS system (Bryers et al., 2006; Bryers. 2012). Also, enzyme-based QQ agents have been documented, such as the AHL-degrading lactonases from soil bacteria like the *Bacillus* genera (Augustine et al., 2010).

It is not strange that natural sources would provide a lot of QQ agents since they might have co-evolved to prevent bacterial encroachment and infection or to outcompete microbial counterparts in the environment (Zhu and Kaufmann GF., 2013). Despite the fact that a plethora of QQ agents have been discovered and characterized *in vitro* and *in vivo*, no candidate has reached clinical stage development yet. So, the application of these natural sources as QQ agents in humans and animals, may provide prophylactic protection against bacterial infections, but need further studies.

Table 2.8. Phytochemicals with proved anti-quorum sensing activity and anti-biofilm activity

Molecule	Natural source	Pathogens	Reference
Gallic acid	Tea leaves, oak bark, grape seeds	*P. aeruginosa* *S. aureus* *L. monocytogenes* *E. coli*	Borges et al., 2012
Ferulic acid	Oat flours	*P. aeruginosa* *S. aureus* *L. monocytogenes* *E. coli*	Borges et al., 2012
Proanthocyanidins	Cranberry	*Staphylococcus* spp.	LaPlante et al., 2012
Flavonones	Orange	*Y. enterocolitica*	Truchado et al., 2012
Curcumin	Turmeric	*P. aeruginosa*	Rudrappa and Bais, 2008
Cinnamaldehyde	Cinnamon	*Vibrio* spp.	Brackman et al., 2008
Furo-coumarins	Grapefruitf	*E. coli* O157:H7 *S.* Typhimurium *P. aeruginosa*	Girennavar et al., 2008
Rosmarinic acid	Sweet basil	*E. coli*	Vattem et al., 2007
Salicylic acid	Bark from willow tree	*P. aeruginosa*	Bryers, 2012
Sulforaphane and erucin	Broccoli	*E. coli* O157:H7	Lee et al., 2011
		P. aeruginosa	Ganin et al., 2013
Furanones	Algae (*Delisea pulchra)*	*P. aeruginosa*	Hentzer et al., 2002

The present review has pretended to illustrate how bacterial adhesion is an essential step that triggers in an infection process, not only in the GIT but also in other environments. In some cases, biofilm production is the later consequence of bacterial adhesion. The multi-drug resistant behaviour of these bacterial communities is a grave threat, and is truly untreatable with the current list of antibiotics. Moreover, the ban of antibiotic inclusion in feedstuffs for animal production originated the need to look for viable alternatives. The possibility that pathogens can be inhibited by naturally compounds through as in the interference of biofilm production is especially attractive. Taking this in consideration the research of new alternative therapies based on natural products aimed to interfere with virulence factors as microbial adhesion, biofilms and mechanisms of QS, would be of particular interest for the veterinary practice.

3. OBJECTIVES AND EXPERIMENTAL DESIGN

Increasing antimicrobial resistance in pathogenic bacteria has created the need for the development of novel preventive and therapeutic agents in the animal industry. Our group [Animal Nutrition, Management and Welfare research group (SGR2009SGR1160)] has been involved in the recent years on the evaluation of new feed strategies aimed to fight intestinal pathogenic bacteria and particularly the search of new functional ingredients (AGL2005-07428-C02-01/GAN; AGL2007-60851/GAN; AGL2009-07328/GAN; AGL2012-31924). This Thesis has been specifically funded by the projects: AGL2009-07328/GAN and AGL2012-31924 to focus on the research of new natural ingredients with anti-adhesive and or anti-biofilm properties against pathogenic bacteria of veterinary interest. This work also was possible with the cooperation of other research centres: the Department of Medicinal Chemistry & Chemical Biology, Utrecht University (The Netherlands) and the Department of Biosciences, General Microbiology, University of Helsinki (Finland).

Therefore, the current thesis has been developed, fixing the following statements as its main objectives:

1. To screen, among different natural compounds, their ability to reduce enterotoxigenic *E. coli* K88 (ETEC) attachment to the intestinal mucus and to the intestinal porcine epithelium.
2. To identify the putative anti-adhesive molecules in the wheat bran soluble extract able to interfere in the ETEC K88 adhesion to the porcine intestine.
3. To evaluate the anti-biofilm ability of the soluble extract of wheat bran and its possible implication in quorum sensing inhibition.

To asses these three objectives, four different trials were performed. Results will be included in chapters 4 to 7.

Trial 1 (Gonzalez-Ortiz et al., 2013a). The soluble extracts of natural feed ingredients were used to screen their ability to bind ETEC K88 and to block its attachment to natural porcine ileal mucus by using a miniaturized *in vitro* test with 96-well high-binding polystyrene microtitration plates. A dose-response assay was performed with the most promising feed ingredients. Also, a digestion procedure with the most active vegetable products was included trying to identify the active fractions.

Trial 2 (Gonzalez-Ortiz et al., 2013b). This time the soluble extracts were tested in their ability to block the binding of ETEC K88 to intestinal porcine epithelial cell-line (IPEC-J2). It was performed a

dose response assay to check the minimum concentration needed to achieve successful anti-adhesive results.

Trial 3. Focused on the wheat bran, we aimed to identify the responsible molecule of the soluble extract able to specifically recognize ETEC K88. Different methodological strategies were used. Fractionations obtained by different digestion processes, molecular weight and size-exclusion chromatography were tested using the miniaturized *in vitro* adhesion tests and blocking tests to IPEC-J2 and by Dot Blot assays. The identification of the possible candidate active compounds was performed by proteomic approaches.

Trial 4. To evaluate the possible anti-biofilm activities of wheat bran we designed a fourth trial to test the possible activity against a well characterized biofilm, as is that produced by *Staphylococcus aureus*. For that, we used two *in vitro* tests, one to evaluate the ability to inhibit biofilm formation and the other to study the ability to destroy the biofilm previously formed. Also the ability of wheat bran extract to interfere with quorum sensing signalling molecules was studied.

4. SCREENING THE ABILITY OF NATURAL FEED INGREDIENTS TO INTERFERE WITH THE ADHERENCE OF ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) K88 AT THE PORCINE MUCOSAL INTESTINE

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4.1. ABSTRACT

Inhibiting the attachment of bacteria to the intestine by receptor analogs could be a novel approach to prevent enterotoxigenic *Escherichia coli* (ETEC) K88-induced diarrhoea in piglets. The objective of the present study was to screen the ability of different feed ingredients (FI) to bind ETEC K88 (adhesion test: AT) and to block its attachment to the porcine intestinal mucus (blocking test: BT), using *in vitro* microtitration-based models. In the AT, wheat bran (WB), casein glycomacropeptide (CGMP) and exopolysaccharide showed the highest adhesion to ETEC K88 ($P < 0.001$). In the BT, WB, CGMP and locust bean (LB) reduced the number of attached ETEC K88 to the intestinal mucus ($P < 0.001$). For WB and LB, a fractionation based on their carbohydrate components was subsequently made, and each fraction was evaluated individually. None of the obtained fractions from WB reduced the adhesion of ETEC K88 to mucus as the original extract did, suggesting that a protein or glycoprotein could be involved in the recognition process. Regarding LB fractionation, the water extractable material reduced the adhesion of ETEC K88 ($P < 0.001$) to mucus similarly to that of the original extract ($P < 0.001$), indicating, in this case, that galactomannans or phenolic compounds could be responsible. It is concluded that among the FI screened, the soluble extracts from WB, LB and CGMP possess the highest anti-adhesive properties against ETEC K88 on BT. These results suggest that they may be good candidates to be included in weaned piglet's diets to prevent ETEC K88-induced diarrhoea.

4.2. INTRODUCTION

Colibacillosis caused by strains of enterotoxigenic *Escherichia coli* (ETEC) is a common diarrheic disease in neonatal and early weaned pigs (Fairbrother et al., 2005), and is the most important diseases worldwide in the swine industry (Zhang et al., 2007). Specifically, ETEC K88 serotype which expresses fimbrial adhesin F4, is the most prevalent serotype responsible for this disease. Susceptibility of piglets to ETEC K88 is inherited as an autosomal dominant trait and is determined by the expression of receptors on the enterocyte brush borders (Jensen et al., 2006). So, the recognition of the intestinal receptors by the bacterial fimbriae and attachment of *E. coli* to the brush border of epithelial cells is considered as the first requirement in the pathogenesis of colibacillosis. After attachment, ETEC K88 can also produce enterotoxins that induce water and electrolyte secretion into the intestinal lumen, resulting in dehydration and metabolic acidosis (Erume et al., 2008).

Receptors for ETEC K88 fimbriae appear to be glycoconjugates which are sparsely located on the mucosal surface of the small intestine of piglets, preferentially in the ileum (Hermes et al., 2013). Different antigenic variants, referred to as K88ab, K88ac and K88ad fimbriae, bind to their own set of receptors. Whereas K88ab and K88ac adhesins preferentially bind to glycoproteins, the K88ad adhesin appears to bind to glycolipids (Jin and Zhao, 2000). In both cases, carbohydrates seem to have a key role in the receptors to bind ETEC K88 fimbrial adhesins (Erickson et al., 1994). In this sense, the importance of *N*-acetylgalactosamine, fucosylated tetra- and pentasaccharides, GalNAc(β1-4)Gal containing sequences, *β*-Galactose, N-acetyllactosamine (LacNAc) and phosphatidylethanolamine was pointed out (Shoaf-Sweeney and Hutkins, 2009). Taking this into account, on the basis of their glycoside composition, the incorporation of some receptor analogs in the diet would be a practical strategy to reduce the number of some intestinal pathogens (Lane et al., 2010), including ETEC K88 (Ofek et al., 2003a). Until now, numerous reports about the ability of natural feed ingredients (FI) to bind to or block the attachment of ETEC K88 to the intestinal mucosa based on their complex composition in carbohydrates have been published (Roubos-van den Hil et al., 2009; Zinger-Yosovich and Gilboa-Garber, 2009; Roubos-van den Hil et al., 2010; Hermes et al., 2011). Some vegetables have been proposed as attractive alternatives to antibiotics for swine production (Windisch et al., 2008). Other natural products, such as the casein glycomacropeptide (CGMP) (Lane et al., 2010; Hermes et al., 2013) and microbial by-products, like exopolysaccharides

(EPS) and mannooligosaccharides (MOS), have been also suggested to act as enteropathogen anti-adhesives (Ruas-Madiedo et al., 2006a; Ruas-Madiedo et al., 2006b; Baurhoo et al., 2007a; Baurhoo et al., 2007b). However, no evidence about the specific interaction between these ingredients and ETEC K88 has been reported regarding their ability to block the adhesion to the intestinal mucus under *in vitro* conditions, evaluating solely their interactions.

The objectives of the present study were to perform an *in vitro* screening comparison of the ability of different compounds to bind ETEC K88 and to block or reduce its attachment to the intestinal mucus using microtitration-based adhesion tests.

4.3. MATERIAL AND METHODS

4.3.1. ANIMALS AND MUCUS ISOLATION

The experiment received previous approval from the Animal Protocol Review Committee of the Universitat Autònoma de Barcelona (Nº 689). The treatment, management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines (The Council of the European Communities, registered under Nº 11GCE007-R).

Five weaned piglets (28 days of age) from a commercial farm were selected on the basis of their genotype for ETEC K88 susceptibility by a DNA marker-based test (Jensen et al., 2006), allowing for the genomic characterization for presence of F4 receptors in the intestinal epithelium. Piglets were placed to the experimental facilities of the Universitat Autònoma de Barcelona. The animals were fed with a commercial feed treated with colistin for five consecutive days (Coliplus® Solution, Divasa Farmavic SA; Barcelona, Spain, 102,500 UI/kg of body weight) and complementarily by i.m. administration (Trimixin, S.P. Veterinaria SA; Barcelona, Spain) in order to reduce the microbial load in the GIT of animals (Hermes et al., 2013). Piglets were euthanized with an intravenous sodium phentobarbital overdose (200 mg/kg body weight) and the abdomen was immediately opened. The ileum was extracted and immersed into the binding buffer (3.84 mM NaH_2PO_4, 6.16 mM Na_2HPO_4, 0.15 M NaCl, pH 7.2). After that, sections were split along the mesenteric border and washed with sterile PBS. The intestinal mucus was recovered following Fang et al. (2000) procedures. Briefly, the mucus was collected by gentle scraping with a glass slide and then was transferred into 20 mL of the binding buffer. All processes were performed in an ice-cold bath. The scrapes of all of the animals were pooled, mixed, and centrifuged at 10,000 x ***g*** at 4°C for 15 min to remove particulate material.

Aliquots of supernatants containing the ileal mucus were stored at -80ºC until their use. Before their utilization in the *in vitro* tests, the ileal mucus was analysed for protein concentration and for the absence of any indigenous microbial contamination by monitoring the optical density (OD) of its culture in Luria broth mèdium (LBM) (650 nm, every 10 min for 12h at 37ºC). Before each blocking test (BT), mucus was thawed and diluted 1:2 in sterile phosphate buffered saline (PBS) to prepare the coating suspension well.

Table 4.1. Feed ingredients used in the adhesion and blocking tests, which also specify the feed ingredient abbreviation, the provider company and the country of origin.

	Product	Abbreviation	Provider company	Country of origin
Controls				
1	Phosphate buffer saline	PBS	Sigma	Spain
2	D-Mannose	MAN	Sigma	Spain
Products of vegetable origin				
3	Wheat bran	WB	Local Mill	Spain
4	Soybean hulls	SO	Local Mill	Spain
5	Sugar beet pulp	SBP	Local Mill	Spain
6	Locust bean*	LB	Armengol, S.A.	Spain
7	Locust gum	LG	Polygal, S.A.	Switzerland
8	Guar gum	GG	Polygal, S.A.	Switzerland
9	Cranberry extract	CRA	Cran Max, S.A.	United States
10	Fructoligosaccharides	FOS	Beghin Meiji, S.A.	France
11	Inulin	INU	Orafti, S.A.	Belgium
Dairy products				
12	Casein glycomacropeptide	CGMP	Arla Foods, S.A.	Denmark
Microbial products				
13	Exopolysaccharides†	EPS	IG-CSIC	Spain
14	Mannanoligosaccharides	MOS	Alltech, S.A.	Spain
15	*Aspergillus oryzae* fermented extract	AO	Molimen, S.L.	Spain

*Product obtained from the carob tree (*Ceratonia siliqua*) including a meal mixture of carob pods and carob seeds.

†Exopolysaccharides obtained from olive fermentation brines.

‡IG-CSIC: Instituto de la Grasa – Consejo Superior de Investigaciones Científicas (Sevilla, Spain).

4.3.2. FEED INGREDIENTS EXTRACTION

The FI evaluated in the tests are described in **Table 4.1**, including ingredients of vegetable origin, a dairy protein and three microbial by-products.

All ingredients were prepared following the protocol described by Becker et al. (2007). Briefly, coarse ingredients, like wheat bran (WB), soybean hulls (SO) and sugar beet pulp (SBP) were finely ground in an analytical grinder. All products were suspended in PBS to a solid-to-liquid ratio of 1:100 (w/v). Subsequently, the suspensions were sonicated three times for 30s each (Unheated Ultrasonic Bath, JP Selecta; Spain) and then centrifuged at 460 x *g* for 5 min (Mikro 220R, Hettich Zentrifugen; Germany). The supernatants were stored at -20ºC until used in the *in vitro* tests.

4.3.3. WHEAT BRAN AND LOCUST BEAN FRACTIONATION

Wheat bran and locust bean, which are ingredients rich in fibre and non-starch polysaccharides (NSP), were also fractionated according to Maes and Delcour (2002) procedures, which characterizes different water extractable and water un-extractable fibrous fractions from WB. Of each FI, three different fractions were obtained. The water extractable material (WEM) was the first fraction obtained after enzymatic digestion [α-amylase (90ºC - 30 min), protease (55ºC – 4h) and amyloglucosidase (60ºC – overnight; ON)], which in the case of WB, according to authors, it is mainly composed by more or less 50% of the total of non-cellulosic sugars (glucose, xylose and arabinose) and high protein content (33%), but little arabinoxylan content (10%) (Maes and Delcour, 2002). A first alkali treatment of the cellulosic residue led to a fraction, designated as AED1, and a second alkali treatment to the fraction AED2. During the dialysis step, more than 90% of the ash content and 30% of proteins were removed. Authors reported that these two fractions (AED1 and AE2D) have similar monosaccharide composition, and in both extracts above 90% are arabinoxylans when referring to WB samples (Maes and Delcour, 2002). Although Maes and Delcour (2002) apply this fractionation only to wheat bran, in this work we also apply this procedure to locust bean looking for a similar physicochemical fractionation of NSP. The three fractions obtained from WB and LB were included in the *in vitro* adhesion and blocking tests.

4.3.4. *ESCHERICHIA COLI* STRAINS

Two different *Escherichia coli* strains were used in this experiment. The first one was an enterotoxigenic *E. coli* (ETEC) K88 strain isolated from a colibacillosis outbreak in Spain (Blanco et al., 1997), serotype (O149:K91:H10 [K-88]/LT-I/STb) that was generously provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The other one was a non-fimbriated *E. coli* (F4 -, F6 -, F18 -, LT1 -, ST1 -, ST2 +, Stx2e -) isolated from the faeces of a post-weaning piglet and kindly donated by the Departament de Sanitat i d'Anatomia Animals of the Universitat Autònoma de Barcelona. ETEC K88 was cultured in unshaken LBM at 37ºC (Snellings et al., 1997), while the non-fimbriated *E. coli* was cultured in shaking media. Bacteria were serially cultured every 48h, at least three times.

Bacterial cells were collected by centrifugation of 15 ml of an ON culture (1,700 x *g*, 5 min**;** Hettich Zentrifugen Mikro 220R; United Kingdom). Supernatants were removed and PBS buffer were added to the cell pellet to achieve an OD of the bacterial suspension of one (650 nm) that was used in the AT (approximately log 9-8.5 CFU [colony forming units]/ml). For the BT, bacterial suspensions were serially diluted to 6.5-7 log CFU/ml.

4.3.5. ADHESION TEST

The ability of the different feedstuffs to adhere to ETEC K88 was determined using an adaptation of the *in vitro* AT described by Becker et al. (2007). The procedure involves the use of 96-well high-binding polystyrene microtitration plates (Microlon F plate 655 092; Greiner Bio-One BV; Alphen a/d Rijn, The Netherlands). Briefly, after an ON incubation at 4ºC with 300 µl of the FI extracts, the plates were washed with sterile PBS to remove non-binding material. Afterwards, the non-specific adhesion sites were blocked by incubating the plates with 1% bovine serum albumin (BSA) + 0.5% sodium azide in PBS (w/v) at 4ºC for 1h. Thereafter, plates were washed twice with sterile PBS and then 300 µl of the bacteria suspensions (ETEC K88 or non-fimbriated *E. coli*) were added. The plates were incubated for 30 min at room temperature and washed three times with sterile PBS in order to remove the non-attached bacteria. Finally, 300 µl of sterile Luria broth were added and the sigmoideal growth of bacteria was measured in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation; Sunnyvale, California, USA) at 37ºC, following the protocol described by Becker et al.

(2007). Bacterial growth was monitored as OD at a wavelength of 650 nm at intervals of 10 min for 12h. All readings were performed in two independent assays and in triplicate per trial.

4.3.6. BLOCKING TEST

The BT was performed by adapting the methodology described above. Briefly, 300 µl per well of the mucus suspensions were pipetted into the flat-bottom 96 wells of high-binding polystyrene microtitration plates and incubated overnight at 4ºC. Plates were washed with 300 µl PBS to remove non-binding material. In order to avoid non-specific adhesion, wells were treated with 350 µl of a mixture containing 1% BSA in PBS and 0.5% sodium azide at 4ºC for 1h. Two washing steps with 300 µl of PBS were subsequently performed. Separately, 500 µl of each bacteria suspension were added to 500 µl of each tested FI extract (1%) and incubated at 37ºC for 30 min. After that, 300 µl of the co-incubated mixtures were added, in triplicate, into the microtitration plate wells and were allowed to adhere on the mucus at room temperature for 30 min. Afterwards, an equal number of washing steps, medium addition and bacterial growth monitoring procedures were managed as in the *in vitro* AT protocol described above.

Additionally, the absence of any microbial growth in the FI extracts likely interfering with the test was evaluated by including controls of each extract co-incubated with PBS instead of with bacteria. To discard the presence of antimicrobial compounds or nutrients in the FI extracts that could modify the number of viable bacteria after the co-incubation, microbial counts in Luria agar plates were also performed after the co-incubation step (30 min at 37ºC and 30 min at room temperature). No differences related to PBS were detected. Supernatant of the FI that showed the highest specific blocking properties (WB, CGMP and LB) were also tested at 0.2, 0.4, 0.6 and 0.8% (w/v) in different dosage-response BT.

4.3.7. BACTERIAL COUNTS AND $t_{OD=0.05}$ CORRELATIONS

In trying to translate the OD values ($t_{OD=0.05}$**)** to CFU per well initially attached to the FI extracts (in the AT) or mucus (in the BT), an assay was performed with ETEC K88 and the non-fimbriated *E. coli* strains. Both bacteria were serially diluted in LBM. The CFU were determined after serial dilutions in PBS, plating on Luria agar and incubation at 37ºC for 48h. At the same time, three replicates of each bacteria and dilution were incubated, adding 300 µl of each dilution per well in the microtitration

plate. The growth characteristics were determined at 37ºC for 18h in the microplate reader (SPECTRAmax 384 Plus, Molecular Devices Corporation; Sunnyvale, California, USA) as described above in the AT and BT protocols. The $t_{OD=0.05}$ (h) and CFU values obtained for each dilution were used to fit the following linear models: y = -1.6371 x + 13.543 (R^2 = 0.994) for ETEC K88 and y = -1.2875 x + 11.999 (R^2 = 0.996) for the non-fimbriated *E. coli*, where "*y"* corresponded to the $t_{OD=0.05}$ and "*x*" to the log of CFU per well.

4.3.8. STATISTICAL ANALYSIS

All statistical analyses were performed using SAS 9.2 (SAS Inc.; Cary, NC, USA). The OD data from the AT and the BT were processed by non-linear regression analysis using the non-linear P-NLIN (Gauss-Newton method) procedure (SAS 9.2, SAS Inc., Cary, NC, USA) following the equations previously described (Becker et al., 2007). From the time at which the bacterial growth reached an OD of 0.05 ($t_{OD=0.05}$, h), the log CFU were calculated for each FI using the previously described linear models. Significant differences on the log CFU among FI and between bacterial strains were determined by one-way analysis of variance (ANOVA). Linear, quadratic and cubic contrasts were performed to analyse the dose response of each FI. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons.

4.4. RESULTS

4.4.1. *IN VITRO* ADHESION TEST

Table 4.2 shows the ability of different FI to adhere to ETEC K88 and non-fimbriated *E. coli* as the number of bacteria attached to each well (log CFU per well). For most of the FI extracts, a higher attachment was observed for ETEC K88 than for the non-fimbriated *E. coli,* showing that fimbriae play an important role in bacterial adherence. The results showed three different levels of adhesive ability to ETEC K88. A first group, including WB, CGMP and EPS, showed the highest adhesion, with more than 7 log ETEC K88 CFU per well. Soybean hull (SO), locust gum (LG), guar gum (GG) and mannanoligossaccharides (MOS) showed values between 7 and 6 log ETEC K88 CFU per well. Finally, the remaining FI, locust bean (LB), fructoligosaccharides (FOS), D-Mannose (MAN), inulin (INU), *Aspergillus oryzae* fermentation extract (AO), sugar beet pulp (SBP) and cranberry (CRAN) showed similar results as those of the negative control (PBS).

Table 4.2. Number of bacteria (log CFU per well) attached to wells coated with different feed ingredient extracts in the adhesion test (AT)†.

Feed ingredient extracts*	Incubated bacteria‡			
	ETEC K88	Non-fimbriated *E. coli*	SEM	*P*-Value *E. coli* type
Phosphate buffer saline	5.42^{de}	6.26^{ab}	0.207	0.008
D-Mannose	5.29^{d-f}	5.43^{c-e}	0.114	0.203
Wheat bran	7.01^{a}	5.69^{bc}	0.332	0.008
Soybean hulls	6.11^{bc}	5.58^{cd}	0.140	0.010
Sugar beet pulp	5.05^{f}	5.28^{c-f}	0.152	0.141
Locust bean	5.61^{d}	5.26^{c-f}	0.107	0.016
Locust gum	6.35^{b}	6.48^{a}	0.122	0.257
Guar gum	6.00^{c}	4.52^{g}	0.172	0.010
Cranberry	5.00^{f}	4.69^{fg}	0.091	0.015
Fructooligosaccharides	5.34^{d-f}	4.84^{fg}	0.085	0.002
Inulin	5.00^{f}	4.90^{e-g}	0.103	0.291
Casein glycomacropeptide	7.15^{a}	5.03^{d-g}	0.107	<0.001
Exopolysaccharides	7.18^{a}	5.78^{bc}	0.181	0.001
Mannanoligosaccharides	6.22^{bc}	4.53^{g}	0.138	<0.001
***Aspergillus oryzae* fermented extract**	5.15^{ef}	4.80^{fg}	0.153	0.050
SEM	0.113	0.193		
***P*-Value feed ingredients‡**	<0.001	<0.001		

Feed ingredients tested: PBS: phosphate buffer saline; MAN: D-Mannose; WB: wheat bran; SO: soybean hulls; SBP: sugar beet pulp; LB: locust bean; LG: locust gum; GG: guar gum; CRA: cranberry; FOS: fructooligosaccharides; INU: inulin; CGMP: casein glycomacropeptide; EPS: exopolysaccharide obtained from olive fermentation brines; MOS: mannanoligosaccharide; AO: fermented extract from *Aspergillus oryzae*.

***After extracted, all feed ingredient extracts were tested at 1% (w/v).**

†Each value (log CFU per well) results from the average of three replicates in, at least, two independent *in vitro* assays according to the fitted equations.

‡Different superscripts within a column indicate significant differences among feed ingredients

4.4.2. *IN VITRO* BLOCKING TEST

Table 4.3 shows the number of bacteria attached to the intestinal mucus after co-incubation with the different ingredient extracts (log CFU per well). In this case, a lower number of bacterial cells are associated with a higher blocking activity of the compounds. The results showed that five ingredients, WB, LB, LG, GG and CGMP, significantly ($P < 0.05$) decreased the attachment of ETEC K88 to mucus, as compared to PBS. In contrast, none of these ingredients were able to significantly decrease the number of the non-fimbriated *E. coli* attached to mucus, thus demonstrating their specificity to ETEC K88. Among the ingredients, WB, LB and CGMP showed the highest effects with reductions of bacteria by more than one log unit.

The dose response assay with WB, CGMP and LB extracts revealed a significant linear response ($P < 0.001$) among the dosages evaluated for the three ingredients (**Figure 4.1**). Nonetheless, the quadratic and cubic contrast were also significant for LB ($P < 0.001$) and CGMP ($P < 0.05$), respectively.

4.4.3. FRACTIONATION OF CARBOHYDRATES COMPONENTS OF WB AND LB

Carbohydrate fractions obtained from the two vegetable ingredients that showed the highest blocking activity (WB and LB) were evaluated in relation to their adhesive and anti-adhesive properties. All WB fractions (WEM and AED1 and AED2) showed lower adhesive properties than did the sonicated WB, while fraction AED2 was not significantly different from PBS (**Figure 4.2**). Regarding LB, neither sonicated LB extract nor any of the LB fractions showed any relevant ability to adhere to ETEC K88.

The ability of these WB and LB fractions blocking ETEC K88 attachment to the intestinal mucus is presented in **Figure 4.3**. Fractions from WB (WEM, AED1 and AED2) did not have the same ability to block ETEC K88 as did sonicated WB at 1%. Regarding LB, the sonicated extract, together with the LB WEM fraction, demonstrated a similar ability to block ETEC K88 attachment. However, the AED1 and AED2 fractions did not show anti-adhesive properties.

Table 4.3. Number of bacteria (log CFU per well) that attached to the natural mucus after being co-incubated with different feed ingredient extracts tested in the blocking test (BT) †.

Feed ingredient extracts*	Incubated bacteria‡			
	ETEC K88	Non-fimbriated *E. coli*	SEM	*P*-Value *E. coli* type
Posphate buffer saline	5.13^{b-d}	5.03^{a-c}	0.194	0.558
D-Mannose	5.60^{a}	4.93^{bc}	0.050	<0.001
Wheat bran	3.87^{fg}	5.19^{ab}	0.002	<0.001
Soybean hulls	5.10^{cd}	5.46^{a}	0.157	0.048
Sugar beet pulp	5.35^{a-c}	4.94^{bc}	0.175	0.045
Locust bean	3.90^{fg}	5.24^{ab}	0.152	<0.001
Locust gum	4.66^{e}	4.78^{b-e}	0.063	0.075
Guar gum	4.12^{f}	4.77^{b-e}	0.134	0.004
Cranberry	4.88^{de}	5.03^{a-c}	0.032	0.364
Fructooligosaccharides	5.06^{b-e}	4.61^{c-e}	0.050	<0.001
Inulin	5.03^{c-e}	4.41^{de}	0.171	0.012
Casein glycomacropeptide	3.70^{g}	4.63^{c-e}	0.046	<0.001
Exopolysaccharides	4.77^{de}	4.38^{c-e}	0.309	0.194
Mannanoligosaccharides	5.48^{ab}	4.90^{b-d}	0.099	0.002
***Aspergillus oryzae* fermented extract**	5.51^{ab}	5.04^{a-c}	0.204	0.049
SEM	0.136	0.169		
***P*-Value feed ingredients**	<0.001	<0.001		

Feed ingredients tested: PBS: phosphate buffer saline; MAN: D-Mannose; WB: wheat bran; SO: soybean hulls; SBP: sugar beet pulp; LB: locust bean; LG: locust gum; GG: guar gum; CRA: cranberry; FOS: fructooligosaccharides; INU: inulin; CGMP: casein glycomacropeptide; EPS: exopolysaccharide obtained from olive fermentation brines; MOS: mannanoligosaccharide; AO: fermented extract from *Aspergillus oryzae*.

***After extracted, all feed ingredient extracts were tested at 1% (w/v).**

†Each value (log CFU per well) results from the average of three replicates in, at least, two independent *in vitro* assays according to the fitted equations.

‡Different superscripts within a column indicate significant differences among feed ingredients.

4.5. DISCUSION

Using two simple *in vitro* methods, the present study is able to show differences among FI regarding their ability to bind ETEC K88 and to interfere in its attachment to the intestinal mucus of piglets. These adhesive and blocking activities, when they occurred, were specific for the ETEC K88 strain but not for the non-fimbriated *E. coli,* thus confirming the role of fimbria F4 in the adhesion of the bacteria to different substrates. Among the FI, WB, CGMP and EPS extracts showed the highest binding capacity to ETEC K88. Wheat bran and CGMP, together with LB, also showed the highest blocking activity in the *in vitro* blocking assay.

4.5.1. CASEIN GLYCOMACROPEPTIDE

The ability of CGMP to block the adhesion of ETEC K88 to the ileal mucus showed a positive both linear and cubic dose-response in the range of 0.2% to 1%. Brody (2000) reviewed the evidence that CGMP from bovine milk binds *E. coli* toxins, inhibits bacterial and viral adhesion, promotes bifidobacteria growth and modulates the immune system response of the animals. Moreover, Nakajima et al. (2005) and Malkoski et al. (2001) reported anti-adhesive properties of CGMP against *E. coli*, arguing for the presence of glycoprotein structures. Casein glycomacropeptide contains three glycosilation sites with a heterogeneous array of glycans, based on a core of Galβ(1→3)GalNAc- and NeuAc(2→5)Gal-, which may act as potential receptor analogs (Grange et al., 2002; Rhoades et al., 2005). In this sense, searching for new theranostic systems, the specific recognition of CGMP to ETEC K88, instead of to the non-fimbriated *E. coli,* was seen by using chronoamperometric measurements (Espinoza-Castañeda et al., 2013). In piglets, CGMP was able to reduce *in vitro* the attachment of ETEC K88 to porcine intestinal epithelial cells (IPEC-J2) (Hermes et al., 2011) and also to reduce *in vivo* diarrheic incidence and *E. coli* attachment to intestinal villi (Hermes et al., 2013).

4.5.2. WHEAT BRAN

Wheat bran is the by-product of the wheat milling industry. It is the outermost covering of wheat grain, which is rich in carbohydrates (40% of NSP, 34% of starch) and protein (12%) (Palmarola-Adrados et al., 2005). In human medicine, WB has been proposed as a cholesterol reducer and for the prevention of certain gastrointestinal cancers (Mohsin-Javed et al., 2012). To our knowledge, the pathogenic anti-adhesive properties of WB have only been suggested in swine nutrition (Hermes et

al., 2009; Molist et al., 2009; Hermes et al., 2011; Molist et al., 2011), which can be confirmed by the results obtained in our research group.

Wheat bran extract showed positive results in both adhesion and blocking assays. In addition, a significant linear dose-response was demonstrated. Other *in vivo* experiments carried out with piglets orally challenged with ETEC K88 also showed that dietary inclusion of WB reduced diarrhoea incidence and the attachment of *E. coli* to the ileal mucosa (Molist et al., 2009). These results also agree with other findings (Hermes et al., 2009a; Molist et al., 2011), reporting that WB in the diet reduce the ubiquitous *E. coli* in the intestinal tract. Moreover the ability of WB soluble extract to block ETEC K88 adhesion to IPEC-J2 has also been demonstrated (Hermes et al., 2011).

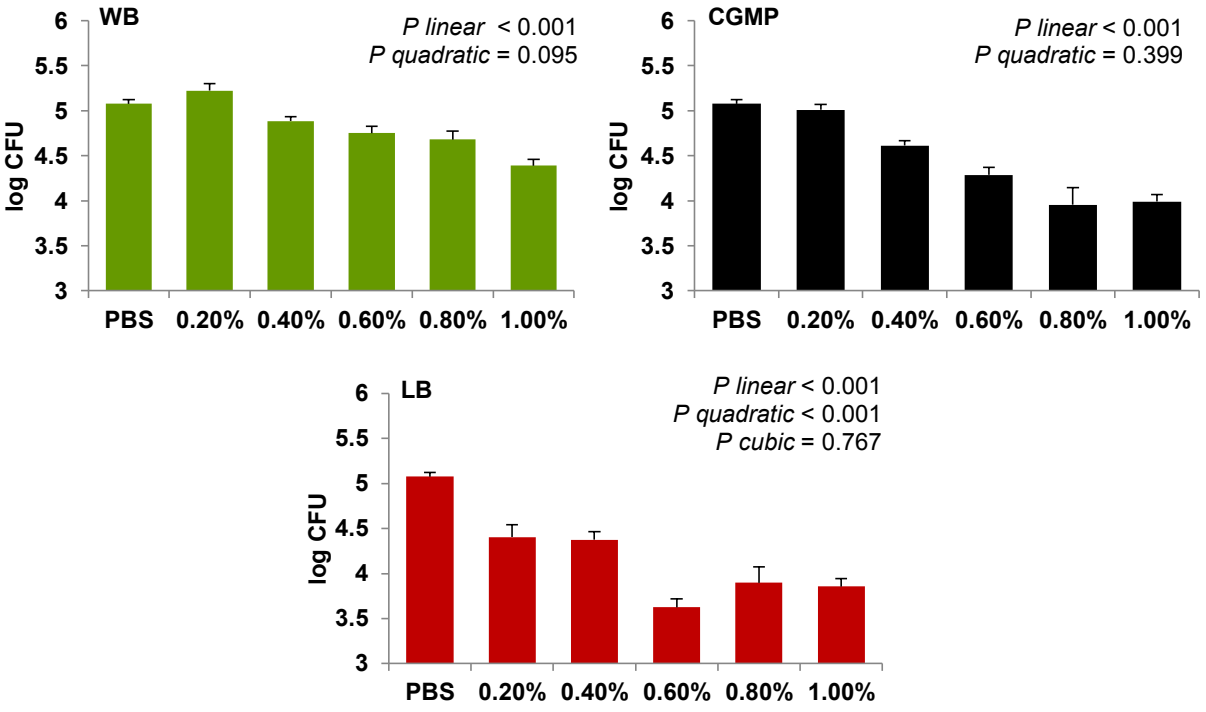

Figure 4.1. Dose response relationships of the ability of wheat bran (WB), casein glycomacropeptide (CGMP) and locust bean (LB) extracts to block the attachment of ETEC K88 in the natural ileal mucus. Log CFU: number of bacteria attached to the natural ileal mucus that were not blocked by the feed ingredient in the blocking test (BT). The lower the log CFU counts, the higher the blocking adhesion ability.

Linear, quadratic and cubic contrasts were performed to analyse the dose response of each feed ingredient. Data result from the experiments performed in triplicate in two independent assays. Error bars represent the standard error of the mean.

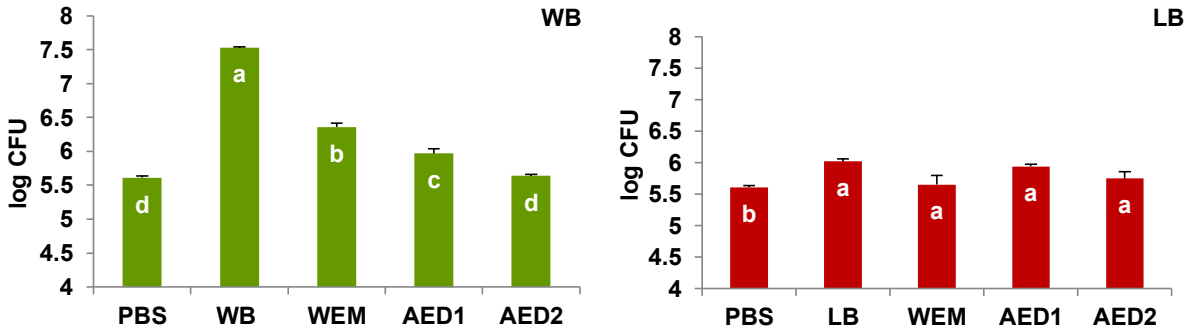

Figure 4.2. Number of ETEC K88 attached to wells (log CFU per well) coated with the different fractions obtained after the digestion process of wheat bran (WB) and locust bean (LB) in the adhesion test (AT). WEM: water extractable material after enzymatic digestion (α-amylase, protease and amyloglucosidase) and dialysis; AED1: fraction obtained after the first alkali treatment of the cellulosic residue; AED2: fraction obtained after the second alkali treatment. The higher the log CFU counts, the higher the adhesion ability.

[a-d]Different letters mean significant differences ($P < 0.05$) between fractions. Data result from the experiments performed in triplicate in two independent assays. Error bars represent the standard error of the mean.

The water extractable material (WEM) did not show the same ability as did the sonicated WB extract to adhere to ETEC K88. The alkaline extracts, AED1 and AED2, did not show a higher adhesion capacity compared to the whole WB extract either. A similar response was observed in the blocking test, in which none of the fractions presented the same blocking ability as did the sonicated WB extract. Both fractions AED1 and AED2 are reported to contain xylose (40%) and arabinose (33%) as the main monosaccharides (Maes and Delcour, 2002). The failure of these fractions to block ETEC K88 adhesion could be due to the digestion process used with α-amylase, protease and amyloglucosidase enzymes that probably may have broken the functional structure of a putative glycoprotein or protein responsible for ETEC K88 fimbriae recognition. In this sense, the involvement of a glycoprotein or a complex of proteins from WB could be the reason for the specific binding phenomenon. That possibility is no wonder, because some reports have suggested the ability of protein fractions from plant-derived compounds to act as anti-adhesive substrates (Lengsfeld et al., 2004; Wittschier et al., 2007). It also could suggest that the ability of WB to adhere ETEC K88 can be reduced along the GIT through digesta. However, this hypothesis merits further *in vivo* studies.

4.5.3. LOCUST BEAN

The LB used in this study comprised the milled mixture of carob's pods and part of their seeds. This FI is highly rich in galactomannans, a neutral polysaccharide consisting of a β-(1→4)-mannan backbone to which single D-galactopyranosyl residues are attached via α-(1→6) linkages. Apart from the wide range of technological applications as feed additive (Rinaudo. 2008), galactomannans are also interesting because it has been reported that mannan residues are able to inhibit fimbrial adhesins of enteropathogens such as *E. coli* and *Salmonella* (Swanson et al., 2002).

Locust bean extract reduced the adhesion of ETEC K88 to mucus in a similar way as did WB; however, LB did not show any ability to adhere to the pathogen. The linear and quadratic contrasts significantly resulted in the dose-response assay. Previous studies using plant products rich in galactomannans have demonstrated anti-adhesive properties to many pathogens. A strong inhibition against *Pseudomonas aeruginosa* by LB, guar galactomannans and acacia gum was observed (Zinger-Yosovich and Gilboa-Garber, 2009). In the same line, Hermes et al. (2011) showed the anti-adhesive response against ETEC K88 using LB in porcine intestinal epithelial cells, whereas, more recently, Badia et al. (2012b) reported the anti-adhesive effect to *Salmonella* by a highly rich β-galactomannan purified from the carob bean gum. The chemical organization and ramification of sugars of these leguminous plants seem to be essential in disrupting pathogen-receptor recognition (Badia et al., 2012c).

It is intriguing why the positive effects observed were associated with LB, but not with LG nor in purified D-Mannose sugar. The LB used in this study comes from the mixture of seeds and pods where the percentage of endosperm per one pod is around 6% (Albanell et al., 1996), while LG is processed from the seed milling, where 52% of the weight of the seed corresponds to the endosperm (where galactomannans are). Therefore, the content of galactomannans is much lower in LB than in LG. On this basis, LB composition is more heterogeneous. The locust pod is especially rich in insoluble dietary fibre and diverse polyphenolic compounds (83 mg/kg) (Papagiannopoulos et al., 2004). Phenolic compounds have been reported to be involved in the blockage of bacterial adhesion (Huttunen et al., 2011; Kurek et al., 2011; Riihinen et al., 2011) and specifically in the recognition of ETEC K88 (Wittschier et al., 2007). Thus, it could be hypothesised that the anti-adhesive response given by LB could have been mediated by the phenolic compounds interfering in F4 recognition rather than were galactomannans. In this way, Liu et al. (2008) demonstrated the effect of proanthocyanidins

from cranberry juice to compress the *P* fimbriae of uropathogenic *E. coli* and interfere in bacterial adhesion.

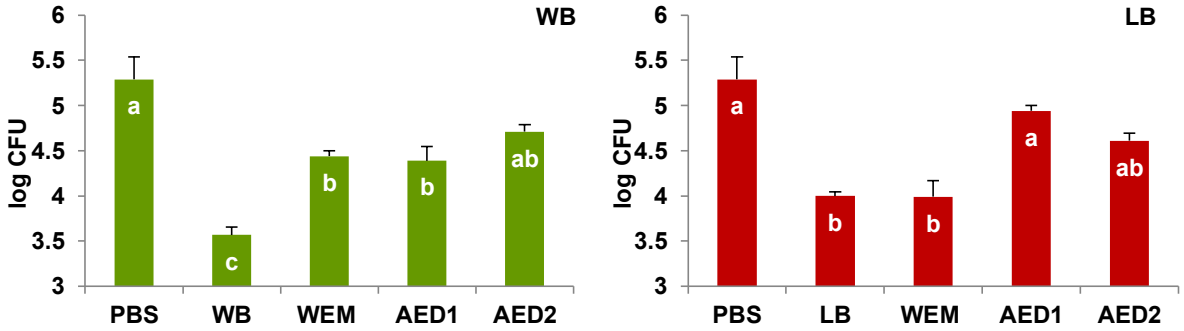

Figure 4.3. Number of ETEC K88 attached to wells (log CFU per well) coated with the natural ileal mucus after pre-incubation with the different fractions obtained after the digestion process of wheat bran (WB) and locust bean (LB) in the blocking test (BT). WEM: water extractable material after enzymatic digestion (α-amylase, protease and amyloglucosidase) and dialysis; AED1: fraction obtained after the first alkali treatment of the cellulosic residue; AED2: fraction obtained after the second alkali treatment. The lower the log CFU counts, the higher the blocking adhesion ability.

[a-b]Different letters mean significant differences ($P < 0.05$) between fractions. Data result from the experiments performed in triplicate in two independent assays. Error bars represent the standard error of the mean.

Regarding the fractions obtained from LB, the first fraction after enzymatic digestion, WEM, showed the same anti-adhesive capacity as did the sonicated extract, whereas the dialysed products did not. This result could suggest that the digestion process did not modify the responsible moiety. From this point of view soluble galactomannans and/or phenolic compounds, could be the responsible for the recognition of the Type 1 fimbriated *E.coli* (Swanson et al., 2002; Zinger-Yosovich and Gilboa-Garber, 2009) as none of the three enzymes included in the digestion process were expected to break down their linkages.

4.5.4. EXOPOLYSACCHARIDES

Exopolysaccharides are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives, mainly glucose, galactose and rhamnose, in different ratios (Welman and Maddox, 2003). They are exocellular molecules excreted during bacterial growth. Exopolysaccharides from *Lactobacillus* and *Bifidobacteria* have been shown to play an important role in the formation of biofilms (Lebeer et al., 2007). The product tested in this work came from the natural fermentation process of green olives in which *L. pentosus* and yeasts have had a relevant role (Garrido-Fernández

et al., 1997; Arroyo-López et al., 2008). Results demonstrated the ability of ETEC K88 to adhere to this substrate, but it was not able to promote significant reductions of ETEC K88 adhesion to the intestinal mucus. It seems as if adhesion of fimbria F4 to EPS would not be enough to interfere in the blocking process. We can hypothesize that bacterial EPS, as complex chemical structures, could offer a variable number of complementary molecules to bacterial and mucus lectins. If EPS could attach not only to fimbria F4 but also to other molecular structures in mucus, EPS could even favour the attachment of the bacteria. In this regard, the potential of different EPS adhering probiotics or enteropathogens to mucus have been previously described by other authors (Ruas-Madiedo et al., 2006a; Ruas-Madiedo et al., 2006b). The lack of ability we found, to reduce the attachment to mucus, do not fully discard the potential of EPS to interfere in bacterial adhesion to the epithelial cells. Adhesion to epithelial cells is a requisite for infection, nevertheless, adhesion to mucus can be considered as a way to maintain bacteria far from the epithelium border line (Variyam. 2007). In vitro studies with epithelial cells could confirm this hypothesis. In this regard it can be found different works describing how EPS can reduce the *E. coli* adhesion to Caco-2 (Alp et al., 2010) and to porcine erythrocytes (Wang et al., 2010).

4.5.5. OTHER INGREDIENTS

All ingredients used in this study were chosen based on previously reported properties interfering in bacterial adhesion and/or by their chemical composition, particularly regarding a carbohydrate profile. However, several of them showed limited or non-significant effects in the present study.

Locust gum and GG seemed to be of interest for testing because of their reported high galactomannnan content (Mathur. 2012) and by the bacterial reduction observed in the digesta of piglets (Van Nevel et al., 2005). However our results did not show any effect on adhesion or blocking activity. Soybean meal oligosaccharides, mainly composed of fructose, galactose and glucose, are reported to promote competitive exclusion of potential pathogens (Qiang et al., 2009). Studies by van der Meulen et al. (2010) demonstrated the effects of different legume seeds and their hull fractions as preventive of ETEC K88 colonization. Nonetheless, the present *in vitro* test was not able to evaluate competitive exclusion effects, rather the processes only evaluated pathogen-mucus interaction.

Sugar beet pulp (SBP) is an important by-product from the sugar extraction industry rich in heterogenous pectins such as rhamnogalacturonan, among others (Guillon and Thibault, 1983). The inclusion of SBP in swine diets modified the intestinal microbiota and the fermentative pattern by a bifidogenic effect (Hermes et al., 2009b) related to the presence of long-chain arabino-oligosaccharides (Holck et al., 2011). However, SBP did not show adhesive and/or anti-adhesive effects using these *in vitro* microtitration-based models.

Regarding CRA, MOS, AO, FOS and IN, no significant effect either in the adhesion or the blocking tests was found, despite the previous evidence found in the literature. Cranberry extract was known to prevent human urinary tract infections by disrupting bacterial ligand-uroepithelial receptors and by changing the physicochemical surface properties of *E. coli* (Liu et al., 2008). The MOS is an extract from yeast cell walls that has already been shown to have beneficial effects in piglets by reducing the jejunal number of enterobacteria (Castillo et al., 2008) and by reducing faecal coliform after an ETEC K88 challenge (White et al., 2002). Mannanoligosaccharides had been described by many authors as being able to specifically interfere with adhesion of pathogens, such as *E. coli* (Baurhoo et al., 2007a; Baurhoo et al., 2007b) or *Salmonella* (Fernández et al., 2000) to the intestine. Similar properties have been described for AO (Becker and Galleti, 2008). The *Aspergillus oryzae* fermented extract also presents a very unique composition based upon a primary fermentation containing high amounts of mannoproteins. Even though the anti-adhesive properties of those oligosaccharides such as FOS and IN against bacterial pathogens were proposed (Gibson et al., 2005), no interference was found against ETEC K88 in the present *in vitro* study.

4.6. CONCLUSION

In summary, the use of these two microtitration-based *in vitro* models have allowed for the identification of certain FI to specifically adhere to and to inhibit the adhesion on mucus of ETEC K88. However, it should be stated that the absence of effects observed in this study for some of the tested ingredients does not completely discard the possibility that they could have a potential role against intestinal colibacillosis through other mechanisms not evaluated in the present *in vitro* models, such as blocking the adhesion to the epithelial cells. Among all of the FI screened in the present study, wheat bran, casein glycomacropeptide and locust bean showed anti-adhesive properties against ETEC K88, representing the most promising FI. Results from the fractionation of wheat bran suggest that the

responsible molecule may be a glycoprotein or complex of different proteins which specifically recognise ETEC K88. In contrast, galactomannans or phenolic compounds could be the responsible anti-adhesive in locust bean. Despite the fact that molecules responsible for the anti-adhesive response have not been elucidated, the positive effects demonstrated in this work make them suitable candidates to be included in the piglet's diet with supported functional activity. Even though the purpose of the present work was to evaluate the anti-adhesive properties of "intact" natural products, it should be consider that some of these FI could be digested in the small intestine of piglets (Gdala et al., 1997; Kiarie et al., 2007), and then activities may be modified. In this sense, further studies are needed in order to check their anti-adhesive activity throughout the intestinal tract as well as their efficacy to prevent pathogen attachment under field conditions.

5. 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

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5.1. ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) K88 is the most prevalent enteropathogen in weaned piglets, with the ability to express fimbria F4 and specifically attach to intestinal receptors in the young piglet. The prevention of ETEC K88 adhesion to the epithelium by interfering in this fimbria-receptor recognition provides an alternative approach to prevent the initial stage of disease. The aim of this study is to screen, among different feed ingredients (FI), their ability to reduce ETEC K88 attachment to the porcine intestinal epithelial cell-line (IPEC-J2). The selected FI consisted of products of a vegetable or dairy origin, and microbial by-products, which could be suitable to be included in piglet's diet. Incubation of a mixture of each FI extract with the bacteria on IPEC-J2 monolayer was allowed. After washing with PBS to remove the non-adhered bacteria, the culture medium was added to grow the adhered bacteria and, simultaneously, to keep the cells alive. Then, the bacterial growth was monitored in a spectrophotometer reader for 12h. Casein glycomacropeptide (CGMP), locust bean (LB), exopolysaccharide (EPS) and wheat bran (WB) reduced the number of attached ETEC K88 to IPEC-J2, but no anti-adhesive effect was found for soybean hulls (SO), sugar-beet pulp (SBP), locust gum (LG), fructooligosaccharides (FOS), inulin (IN), mushroom (MSH), mannanoligosaccharides (MOS) or the fermented product from *Aspergillus oryzae* (AO). The lineal analysis of dose responses demonstrated lineal activity ($P < 0.0001$) for CGMP, LB, EPS and WB. These *in vitro* results suggest CGMP, LB, EPS and WB as good candidates to be included in piglet's diet with supported functional activity against colibacillosis.

5.2. INTRODUCTION

The ability of pathogens to survive and initiate infection depends on their ability to adhere to host cell-tissues. Adherence involves the interaction of complementary molecules on the surface of bacteria and the surface of the host epithelium. The overall specificity of a bacterium for a particular host is contingent on the presence of definitive oligosaccharide receptors (Mazariego-Espinosa et al., 2010).

Enterotoxigenic *Escherichia coli* is the most problematic bacteria in the piglet's weaning period, leading to large economic losses. Besides its ability to produce enterotoxin (Zhang et al., 2007), this bacterium also expresses F4 fimbrial adhesins (K88), providing a highly specific means for ETEC attachment to receptors located in the brush border of the epithelial mucosa (Jin and Zhao, 2000). Therefore, anti-adhesive strategies have emerged by adhesin analogs, competitively blocking pathogen adherence to receptors (Lane et al., 2010), or by receptor analogs, which resemble host oligosaccharide receptors, thus interrupting the adherence process (Shoaf-Sweeney and Hutkins, 2009). Naturally occurring carbohydrates exhibited fimbriae-blocking activity in a variety of assays (Wang et al., 2010; Roberts et al., 2013) and represent a viable option for anti-adhesive therapies (Sharon. 2006). However, the complex microbial ecosystem of the digestive tract makes it difficult to show the likely positive effects given by a single feed ingredient (FI) in the diet. The use of *in vitro* assays using cell cultures may enhance the knowledge between bacteria-to-cell interactions (Brosnahan and Brown, 2012) and, in this particular case, the specific anti-adhesive role of the candidate products.

The aim of this study is to screen, among different FI, their ability to reduce ETEC K88 attachment to the intestinal porcine epithelial cell-line (IPEC-J2).

5.3. MATERIALS AND METHODS

5.3.1. CELL-CULTURE GROWTH

The IPEC-J2 cell-line (epithelial cells derived from cells isolated from the jejunum of a neonatal pig and maintained as a continuous culture) was kindly donated by Dr. Antony Blikslager, from the College of Veterinary Medicine (North Carolina State University). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (GIBCO®, Ref. nº: 31331-028, Life Technologies; Spain), supplemented with insulin, transferrin, selenium and ethanolamine added as ITS solution

(GIBCO®, Ref. nº: 41400-045, Life Technologies; Spain) and maintained in an atmosphere of 5% CO_2 at 37ºC until confluence. Cells were used between passages 85 and 91 and were routinely tested to be free of mycoplasma contamination.

The IPEC-J2 cells were cultured in 75 cm^2 flasks and 2 x 10^5 cells/well were seeded into 96-well flat-bottom plates (Nunclon Delta Surface, Ref. nº: 167008, Thermo Scientific; Denmark) in 200 µl of culture media. Cells were allowed to adhere for 24h in an atmosphere of 5% CO_2 at 37ºC. When confluence was confirmed after 24h, cells were washed once with PBS and then 200 µl of CO_2-independent medium (GIBCO®, Ref. nº: 18045-054, Life Technologies; Spain) were added. Plates were kept in a heater at 37ºC without providing CO_2 for 24h, until they were ready for the *in vitro* test.

5.3.2. *ESCHERICHIA COLI* STRAINS

Two different *Escherichia coli* strains were used in this experiment. The first one was an ETEC K88 strain isolated from a colibacillosis outbreak in Spain (Blanco et al., 1997), a serotype (O149:K91:H10 [K-88]/LT-I/STb) that was kindly provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The other one was a non-fimbriated (NF) *E. coli* strain (F4 -, F6 -, F18 -, LT1 -, ST1 -, ST2 +, Stx2e -) isolated from the faeces of a post-weaning piglet and kindly provided by the Department of Animal Health and Anatomy of the Universitat Autònoma de Barcelona. ETEC K88 was cultured in unshaken Luria broth mèdium (LBM) at 37ºC with the aim to maximize fimbriae expression (Snellings et al., 1997), while the NF *E. coli* was cultured in shaking media. Bacteria were serially cultured every 48h, at least three times.

Bacterial cells of a 15-ml overnight culture (ON) (1,700 x *g*, 5 min**)** were collected by centrifugation, the supernatants were removed, and PBS buffer was added to the cell pellet to achieve an optical density (OD) at 650 nm of 1 (approximately log 9-8.5 CFU [colony forming units]/ml) and serially diluted to reach log 7-6.5 CFU/ml.

5.3.3. FEED INGREDIENTS EXTRACTION

The FI used are shown in **Table 5.1**, including eight ingredients of vegetal origin, one dairy protein and three microbial by-products. All ingredients were prepared as previously described Becker et al. (2007). Coarse ingredients, like wheat bran (WB), soybean hulls (SO), sugar-beet pulp (SBP) and mushrooms (MSH) were finely ground in an analytical grinder. All products were suspended in

PBS to a solid-to-liquid ratio of 1:100 (w/v). Subsequently, the suspensions were sonicated three times for 30s each and then centrifuged (460 x *g,* 5 min). The supernatants were filter-sterilized (0.2-µm filters, Ref. nº 28145-477, VWR; Spain) and stored at -20ºC until used in the *in vitro* test.

5.3.4. *IN VITRO* TEST WITH IPEC-J2

The *in vitro* test was performed by adapting the methodology described by Hermes et al. (2011). As a first step, IPEC-J2 cells were subjected to a 24h adaptation period in 96-well flat-bottom plates with a CO_2-independent medium. Separately, a mixture (1:1) of each FI extract with each *E. coli* strain suspension was gently mixed in a 1.5-ml eppendorf, and 200 µl were immediately transferred to each well. The mixtures and cells were incubated for 30 min at 37ºC, allowing non-blocked bacteria to attach to cells. Wells were gently washed once with sterile PBS by hand-pipetting so as not to disturb the IPEC-J2 monolayer, in order to remove the non-adhered bacteria. Finally, 200 µl of CO_2-independent medium were added to allow for the growth of the adhered bacteria and to keep the cells alive. Bacterial growth at 37ºC was monitored in a microplate reader (Spectramax 384 Plus, Molecular Devices Corporation; Sunnyvale, California, USA) by measuring the OD_{650nm} at intervals of 10 min for 12h. The FI screening was performed in two independent assays, in triplicate. Feed ingredients that showed the highest specific anti-adhesive properties (CGMP, LB and EPS), and also WB by our previous background, were subsequently submitted to a dose-response experiment in which doses of 1%, 0.1% and 0.01% were evaluated. Again, two independent assays were performed in triplicate.

5.3.5. BACTERIAL CFU COUNTS AND $t_{OD=0.05}$ CORRELATIONS

The perceptibility ranges of ETEC K88 and the NF *E. coli* were determined as correlations between OD_{650nm} values and colony counts on Luria agar. For that, both bacterial strains were serially diluted in LBM and the CFU/ml was determined by plate counting. The growth characteristics were determined at 37ºC for 12h every 10 min by measuring the OD_{650nm} in the microplate reader. The bacterial growth times, $t_{OD=0.05}$ (h), were analysed following the same procedure explained above. For ETEC K88, the fitted equation was $y = -1.682\ x + 13.916$ ($R^2 = 0.989$), and for the NF *E. coli*, the equation obtained was $y = -1.0841\ x + 9.364$ ($R^2 = 0.975$), where "*y*" corresponded to the $t_{OD=0.05}$ and "*x*" to the log of CFU per well.

Table 5.1. Feed ingredients used in the blocking test, specifying the feed ingredient abbreviation, the main active compounds, the provider company and the country of origin.

Product		Abbreviation	Main active compounds	Provider	Country
Controls					
1	Phosphate buffer saline	PBS		Sigma	Spain
2	D-Mannose	MAN	Mannose	Sigma	Spain
Vegetable products					
3	Wheat bran	WB	Arabinoxylans, non-starch polysaccharides	Local Mill	Spain
4	Soybean hulls	SO	Oligomannoside-type carbohydrate chains	Local Mill	Spain
5	Sugar-beet pulp	SBP	Pectins	Local Mill	Spain
6	Locust bean[a]	LB	Galactomannans, non-starch polysaccharides, phenolic compounds	Armengol, S.A.	Spain
7	Locust gum	LG	Galactomannans	Polygal, S.A.	Switzerland
8	Fructooligosaccharides	FOS	Oligosaccharide fructans	Beghin Meiji, S.A.	France
9	Inulin	INU	Fructose polymers	Orafti, S.A.	Belgium
10	Mushroom	MSH	Polysaccharides, glycosides, alkaloids, volatile oils and organic acids	Commercial shop	Spain
Dairy protein					
11	Casein glycomacropeptide	CGMP	Glycoproteins rich in sialic acids	Arla Foods, S.A.	United Kingdom
Microbial products					
12	Exopolysaccharides[b]	EPS	D-glucose, exopolysaccharides	IG-CSIC*	Spain
13	Mannanoligosaccharides	MOS	Mannose, proteins	Alltech, S.A.	Spain
14	*Aspergillus oryzae* fermented extract	AO	Galactomannans, non-starch polysaccarides	Molimen, S.L.	Spain

[a]Product obtained from the fruit of the carob tree (*Ceratonia siliqua*) including a meal mixture of carob pods and carob seeds.

[b]Exopolysaccharides obtained from olive fermentation brines.

*** IG-CSIC: Instituto de la Grasa - Centro Superior de Investigaciones Científicas (Sevilla, España).**

5.3.6. STATISTICAL ANALYSES

All statistical analyses were performed using SAS 9.2 (SAS Inc.; Cary, NC, USA). OD_{650nm} data were processed by non-linear regression analysis using the non-linear P-NLIN procedure (Gauss-Newton method) following the equations described by Becker et al. (2007). From the time at which the bacterial growth reached an OD_{650nm} of 0.05 ($t_{OD=0.05}$, h), the log CFU was calculated for each FI using the previously described linear models. Values are presented as means ± SD. Significant differences among FI were determined by one-way analysis of variance (ANOVA). The linear trends were used to compare the dose-response effect. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons.

5.4. RESULTS AND DISCUSSION

The intestinal porcine epithelial cell-line (IPEC-J2) was isolated from a neonatal suckling piglet (Berschneider. 1989). It conserves the epithelial nature and can serve as a convenient *in vitro* model system for studying porcine-specific pathogenesis (Schierack et al., 2006).

The screening among a variety of FI revealed differences regarding their ability to specifically reduce ETEC K88 adhesion to IPEC-J2 (**Figure 5.1**). Three particular ingredients, LB, CGMP and EPS, showed a clear ability to reduce the number of ETEC K88 adhered to the cells, being more significant in CGMP (P = 0.01) and EPS (P = 0.0004). However, this significant reduction was not observed with the NF *E. coli,* suggesting that their chemical composition is able to disrupt the fimbria F4 and receptor recognition of ETEC K88 (Koh et al., 2008). On the other hand, none of the other FI extracts at 1% were clearly able to significantly reduce ETEC K88 attachment. Nonetheless, interestingly IN reduced (P = 0.03) the NF *E. coli* adhesion to IPEC-J2, as it did similarly on natural porcine mucus (González-Ortiz et al., 2013a). Gibson et al. (2005) briefly reviewed the anti-adhesive properties of this type of oligosaccharides against bacterial pathogens; because of that, it was included in the present study. No reduction in ETEC K88 attachment to the IPEC-J2 cell-line was found, but it is difficult to explain the persistent effect against the NF *E. coli.* It may be hypothesized that interference with other adhesins different from fimbria F4 that are only present in the NF *E. coli* can take part in such response.

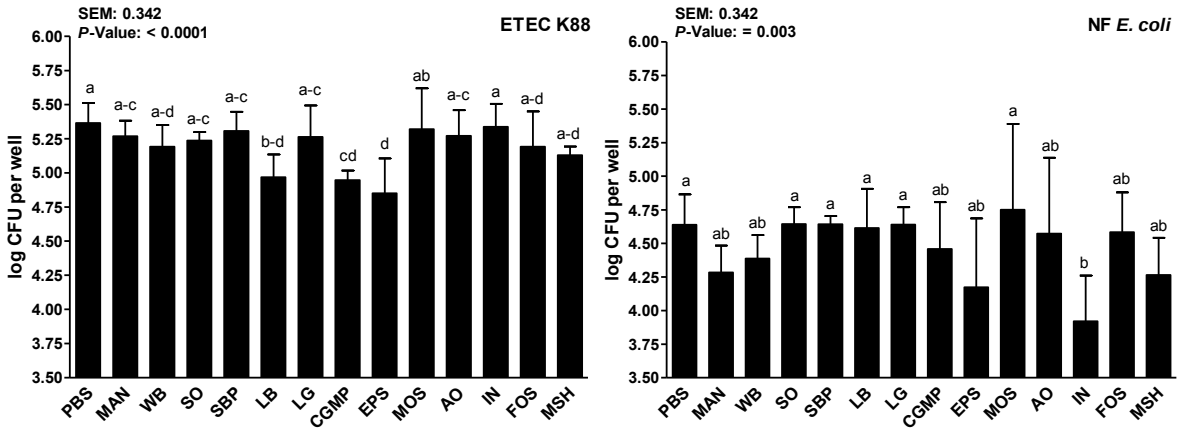

Figure 5.1. Number of bacteria (log CFU per well) that attached to IPEC-J2 cells after being co-incubated for 30 min with different feed ingredients.

Feed ingredients tested: PBS: phosphate buffer saline; MAN: D-Mannose; WB: wheat bran; SO: soybean hulls; SBP: sugar beep pulp; LB: locust bean; LG: locust gum; CGMP: casein glycomacropeptide; EPS: Exopolysaccharides; MOS: mannan-oligosaccharides; AO: fermented extract from *Aspergillus oryzae*; INU: inulin; FOS: fructooligosaccharides; MSH: mushroom extract.

All feed ingredient extracts were tested at 1% of concentration.

Each bar (log CFU per well ± SD) results from the average of three replicates from two independent *in vitro* assays according to the fitted equations.

Different letters indicate statistical differences ($P < 0.05$) among feed ingredients regarding their ability to block bacterial adhesion, when compared to PBS.

Regarding the products of vegetable origin, LB reduced ETEC K88 adhesion, as compared to PBS (P = 0.02). The LB used in this study was obtained by mixing locust pods and seeds from the *Ceratonia siliquia* tree, which has been described to contain a mixture of galactomannans, non-starch polysaccharides and phenolic compounds (Papagiannopoulos et al., 2004). Whereas galactomannans have demonstrated the ability to reduce ETEC K88 adhesion to IPEC-J2 (Badia et al., 2012c), other authors have reported that phenolic compounds play a role in disrupting the bacterial ligand-epithelial receptor (Liu et al., 2008). Nevertheless, the heterogeneous composition of this product makes it difficult to elucidate the specific molecular basis of this interaction.

The dairy compound CGMP also promoted a significant reduction on ETEC K88 adhesion to IPEC-J2 (P = 0.01), which agrees with previous *in vitro* and *in vivo* studies (Hermes et al., 2011; Hermes et al., 2013). In contrast to the results with NF *E. coli*, this specific recognition of CGMP to ETEC K88 was also seen later by using chronoamperometric measurements (Espinoza-Castañeda et al., 2013). Casein glycomacropeptide contains three glycosilation sites with a heterogenous array of

glycans, based on a core of Galβ(1→3)GalNAc- and NeuAc(2→5)Gal-, which could act as a potential receptor analogs (Grange et al., 2002; Rhoades et al., 2005).

The EPS used in this work came from the natural fermentation process of green olive brines in which *Lactobacillus pentosus* and yeasts play a relevant role (Garrido-Fernández et al., 1997). Results showed that EPS was the most efficient at reducing adhesion of the ETEC K88 strain to the IPEC-J2 cell-line among all FI regarding PBS (P = 0.0004). In this sense, similar interferences of EPS in the *E. coli* adhesion to Caco-2 (Alp et al., 2010) and porcine erythrocytes (Wang et al., 2010) have been described. However, previous investigations by our group conversely detected that EPS does not reduce the attachment of ETEC K88 at the porcine mucosal intestine (González-Ortiz et al., 2013a). The recruitment of intestinal pathogens by the mucosal layer is a mechanical way to push out undesirable bacteria, avoiding receptor attachment (Bergstrom et al., 2010). Therefore, it seems that EPS may possess dual function versatility depending on the *in vitro* test environment exposed.

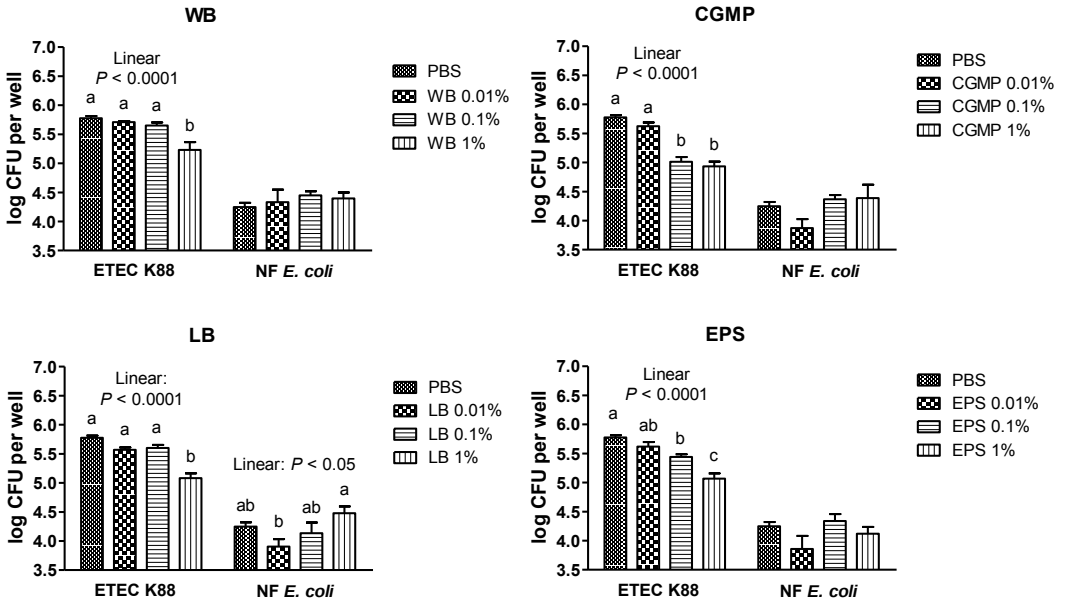

Figure 5.2. Dose-response relationships of the ability of wheat bran (WB), casein glycomacropeptide (CGMP), locust bean (LB) and olive brine exopolysaccharides (EPS) extracts to block the attachment of ETEC K88 and the non-fimbriated (NF) *Escherichia coli* in the IPEC-J2 cell-line.

Log CFU: number of bacteria attached to the intestinal cells that were not blocked by the feed ingredient extract, when compared to PBS. The lower the log CFU counts, the higher the blocking-adhesion ability.

Different letters within each feed ingredient and bacterial strain mean statistical differences between doses (P < 0.05).

Data result from the experiments performed in triplicate in two independent assays. Error bars show the standard deviation of the mean.

The dose-response assay was performed with those feed ingredients (LB, CGMP and EPS) that showed clear anti-adhesive properties (**Figure 5.2**). We also included WB in the dose-response assay because our previous results showed that WB might reduce the ETEC K88 attachment to the mucosa of the ileum (Molist et al., 2009) and to IPEC-J2 (Hermes et al., 2011). WB is the outermost covering of wheat grain, which is rich in carbohydrates (40% of non-starch polysaccharides, 34% of starch) and protein (12%) (Palmarola-Adrados et al., 2005). All of these ingredients reduced linearly ($P < 0.0001$) ETEC K88 adhesion to IPEC-J2, including the WB. About the vegetable products, a concentration of 0.1% of WB and LB did not have any anti-adhesive ability as did 1% ($P > 0.05$). When all FI at 1% were compared in the screening assay, WB resulted in a poorer anti-adhesive capacity. However, the dose-response assay re-confirmed the previous results obtained by our research group. CGMP, as well as EPS, were efficient at 0.1%, but the activity was lost when tested at 0.01%. The dose-response assay allowed for checking the minimum FI concentration able to reduce *in vitro* bacterial adhesion although it is difficult to extrapolate these values under *in vivo* conditions and to recommend a final dietary dose.

5.5. CONCLUSION

In this study, the soluble extracts of CGMP and EPS resulted in the best feed ingredients to reduce ETEC K88 attachment to IPEC-J2, but the vegetable extracts from LB and WB were also able to prevent bacterial adhesion. Their promising results make them good candidates to be included in the piglet's diet with supported functional activity against colibacillosis.

6. INSIGHTS INTO THE MECHANISMS OF WHEAT BRAN INTERFERING IN THE ATTACHMENT OF ENTEROTOXIGENIC *E. COLI* K88 (F4+) TO PORCINE EPITHELIAL CELLS

Under review

6.1. ABSTRACT

Wheat bran (WB) from *Triticum aestivum* has many beneficial effects on human health. To the best of our knowledge, very little has been published about its ability to prevent pathogenic bacterial adhesion in the intestine. Here, a WB extract was fractionated using different strategies, and the obtained fractions were tested in different *in vitro* methodologies to evaluate their interference in the attachment of enterotoxigenic *Escherichia coli* (ETEC) K88 to intestinal porcine epithelial cells (IPEC-J2) with the aim of identifying the putative anti-adhesive molecules by mass spectrometry (MS). It was found that a proteinaceous compound in the >300-kDa fraction mediates the recognition of ETEC K88 to IPEC-J2. Further fractionation of the >300-kDa fraction by size-exclusion chromatography showed several proteins below 90 kDa, suggesting that the target protein belongs to a high-molecular-weight (MW) multi-component protein complex. The identification of some relevant excised bands by MS mostly revealed the presence of various protease inhibitors (PIs) of low MW: Serpin-Z2B, Class II chitinase, endogenous alpha-amylase/subtilisin inhibitor and alpha-amylase/trypsin inhibitor CM3. Furthermore, an incubation of the WB extract with ETEC K88 allowed for the identification of a 7S storage protein globulin of wheat, Globulin 3 of 66 kDa, which seems to be one of the most firmly attached WB proteins to ETEC K88. It is known that many PIs in plants are part of their natural defense mechanism against invading bacteria. This is the first report which gives insights into the possible roles of several PIs and also of Globulin 3 of WB preventing ETEC K88 adhesion to IPEC-J2. Further studies should be performed to gain an understanding of the molecular recognition of the blocking process that takes place. All gathered information can eventually pave the way for the development of novel anti-adhesion therapeutic agents to prevent bacterial pathogenesis.

6.2. INTRODUCTION

The possibility that pathogens can be inhibited by naturally occurring anti-adhesive compounds is especially attractive and has captured significant attention (Shoaf-Sweeney and Hutkins, 2009). Milk sources, plant-derived compounds, and microbial by-products have been the most important dietary products considered for this function (Lane et al., 2010). Vegetable products with anti-adhesion activity are attractive candidates as therapeutic agents, because they are generally abundant or can be engineered to become widely available. Although plant lectins are well represented in human and animal diets and many of these are well characterized (Sharon. 2006), their application to anti-adhesion therapies is a recent strategy that has become an alternative to antibiotics. Theoretically, these lectins could interact with host-cell receptors to block adhesion by competition. But, additionally, they could interact with bacterial adhesins to enhance the clearance of bacteria by exclusion (Slifkin and Doyle, 1990). Numerous investigations involving plant extracts have recently been performed to reduce bacterial adhesion (Becker and Galleti, 2008; Shmuely et al., 2012).

Wheat bran (WB) was first reported to modify intestinal microbiota by reducing the enterobacteria population in feces (Eeckhaut et al., 2008; Molist et al., 2010). Additional investigations showed a reduction of the *E. coli* population in the ileum digesta, and more interestingly, a reduction of the enterotoxigenic *E. coli* (ETEC) K88 attached to the ileum mucosa was observed when piglets received WB in their diet (Molist et al., 2009). Wheat is by far the most important cereal produced worldwide (FAO. 2013). It is estimated that twenty-five per cent of the total wheat production corresponds to bran, a by-product obtained from the milling industry (Hemery et al., 2007). Many beneficial effects on human health have been ascribed to WB, such as cholesterol reduction, prevention of cardiovascular diseases and protection against colon cancer (Mohsin-Javed et al., 2012).

Even though the arabinoxylooligosaccharides of WB were initially considered to promote the anti-adhesive properties against enteropathogens (Eeckhaut et al., 2008), preliminary data suggested the involvement of a proteinaceous compound, which could specifically recognize ETEC K88 (Gonzalez-Ortiz et al., 2013a). Therefore, the initial hypothesis of this work is that a protein or a glycoprotein present in the soluble extract of WB might interfere in the binding of ETEC K88 to the porcine epithelial cells, thus preventing the infectious process. Under this hypothesis, the aim of this study is to zoom in on the responsible molecule or molecules from the WB extract. To this end, a WB

extract was fractionated using different strategies, and the obtained fractions were tested by different *in vitro* methodologies with the aim of identifying the putative candidates by MS.

6.3. MATERIAL AND METHODS

6.3.1. WHEAT BRAN EXTRACTION AND FRACTIONATION PROCEDURES

6.3.1.1. OBTAINING THE SOLUBLE EXTRACT OF WHEAT BRAN

The WB used in the study comes from a local Spanish mill (Moretó, Mollet del Vallès, Barcelona). First, the WB was finely ground in an analytical grinder and was then suspended in demineralized (DEMI) water to a solid-to-liquid ratio of 1:10 (w/v). Subsequently, the suspension was sonicated (J.P. Selecta, S.A.) three times for 30s each and then centrifuged (460 x *g*, 5 min, 20ºC). The supernatant extracted was divided into three aliquots. One aliquot was used just as it was to perform heat treatment and carbohydrate digestion. The second aliquot was freeze-dried and stored at room temperature (RT), and finally the third aliquot was immediately fractionated by MW.

6.3.1.2. HEAT TREATMENT

Wheat-bran extract was heated during 30 min at 90ºC. The sample was cooled and stored at -20ºC until use. The activity of the heated product was tested in the *in vitro* adhesion test.

6.3.1.3. CARBOHYDRATE DIGESTION

O-glycosidase (P0733S, 40,000,000 U/ml, New England BioLabs, Inc.) combined with Neuraminidase (N2876, 1,000 mU/ml, Sigma) was used to catalyze the hydrolysis of *O*-linked saccharides and *N*-acetyl-neuraminic acid from glycoproteins and oligosaccharides, maximizing the disappearance of sugars attached to proteins in the soluble extract of WB. The reaction was performed by mixing 2 mL of the soluble extract of 10% WB with 290 µL 10X G7 Reaction Buffer, 10 µL *O*-glycosidase (180 Units) and 25 µL Neuraminidase (11 Units). Samples were incubated at 37ºC for 1.5h with gentle shaking. A negative control was also included, which consisted of the same volume of the sample mixed with DEMI water, instead of the reaction buffer and enzymes. To stop the enzymatic reaction, the digested samples were heated at 60ºC for 20 min. Samples were stored at -20ºC.

6.3.1.4. FRACTIONATION BY MOLECULAR WEIGHT

One aliquot of the soluble extract of WB was used to fractionate it by MW using Vivaspin® 6 centrifugal concentrators (Sartorious) with a cut-off size of 300,000 Da. The upper part of the tube was filled with the soluble extract and centrifuged (3,000 x *g*, 3.5h, 4ºC). After centrifugation, the upper part was adjusted with DEMI water to the same volume retrieved in the bottom container to achieve the same sample-volume. Two fractions were obtained: 1) >300 kDa and 2) <300 kDa. Finally, these fractions were immediately freeze-dried and kept at RT until further testing.

6.3.1.5. SIZE EXCLUSION CHROMATOGRAPHY

To further fractionate the >300-kDa fraction, size-exclusion chromatography (SEC) was performed on an AKTA Purifier System (GE Healthcare) using a High Load Superdex 200 26/60 column (GE Healthcare). Thirty-five milligrams of the lyophilised >300-kDa fraction were re-suspended in 13 mL of PBS 0.25X and filtered through 0.22-µm filter units. The sample was injected into the column and eluted in a 1.5 column volume (CV) using an isocratic gradient of PBS 0.25X at a flow rate of 2.5 mL/min. The absorbance of the eluted fractions was monitored at 214 nm and 280 nm. Fractions were pooled in eight major fractions (**Figure 6.1**) and were then freeze-dried. They were resuspended at ¼ of their initial volume with DEMI water, so that the final buffer concentration was PBS 1X. The aliquots were stored at -20ºC until use.

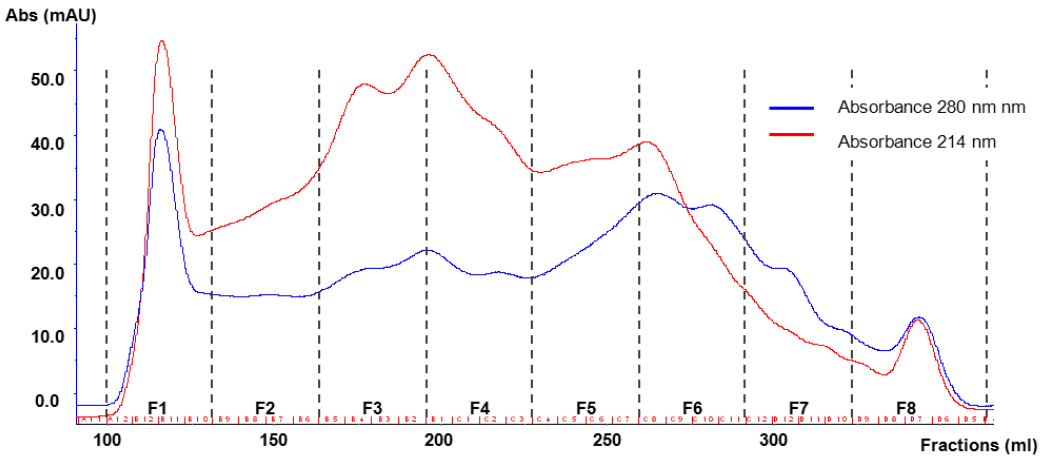

Figure 6.1. Size-exclusion chromatography of the >300-kDa fraction. Distribution of the eight fractions obtained, which corresponded to F1 to F8.

In order to disrupt protein aggregations, the same separation was repeated using acetonitrile (ACN) 20% in PBS 0.25X as chromatographic buffer. The fractions were pooled in six major fractions

(**Figure 6.2**) and after lyophilisation fractions were resuspended at ¼ of the initial volume with DEMI water and kept at -20ºC until their use.

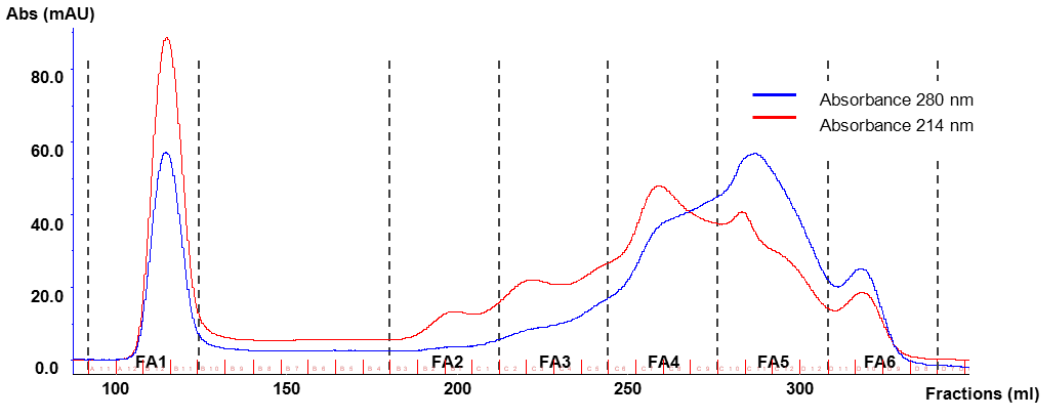

Figure 6.2. Size-exclusion of the >300-kDa fraction treated with acetonitrile (ACN). Representation of the six fractions obtained, which corresponded to FA1 to FA6

6.3.2. EFFICACY VALIDATION TOOLS

6.3.2.1. BACTERIAL STRAINS

An enterotoxigenic *E. coli* (ETEC) K88 strain isolated from a colibacillosis outbreak in Spain (Blanco et al., 1997) (Strain Reference nº: FV12408), serotype (O149:K91:H10 [K-88]/LT-I/STb), which was generously provided by the *E. coli* Reference Laboratory, Veterinary School of Santiago de Compostela University (Lugo), was used. ETEC K88 was cultured in unshaken Luria broth medium (LBM) at 37ºC (Snellings et al., 1997). Bacteria were serially cultured every 24h, at least three times.

Bacterial cells were collected by centrifugation of 15 mL of an overnight culture (ON) (1,700 x *g*, 5 min, 20ºC). Supernatants were removed and PBS buffer was added to the cell pellet to achieve an optical density (OD) of 1 (650 nm) for the bacterial suspension that was used in the adhesion test (approximately log 8.5-9 CFU [colony forming units]/mL). For the blocking test, the bacterial suspension was serially diluted to 6.5-7 log CFU/mL.

To isolate K88 fimbriae, wild-type ETEC strains FV12408 and 5/95 [O149, K88ac, LT+STb+; (Joensuu et al., 2006)] were cultured ON at 37ºC in tryptone soy broth (TSB, 100 mL) with mild shaking (50 rpm), or on Luria agar (tetracycline 12.5 µg/mL), respectively. Bacteria were collected and suspended in PBS. The bacterial suspensions were vortexed for two to four minutes to detach fimbrial filaments from bacterial surfaces (Westerlund-Wikstrom et al., 1997). Next, the bacteria were pelleted, and bacteria-free supernatants were analyzed in 15% sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE gels). After Coomassie staining, the protein concentrations of fimbriae were determined densitometrically by the Tina (v2.0) image analysis program using bovine serum albumin (BSA, Sigma) as protein concentration standards in the gels.

6.3.2.2. ADHESION TEST

The ability of the different fractions to adhere to ETEC K88 was determined by using high-binding polystyrene microtitre plates in the *in vitro* adhesion test (AT) as described by Becker et al. (2007) and adapted by Gonzalez-Ortiz et al. (2013a). After different incubation and rinsing steps, the bacterial growth was monitored as OD at a wavelength of 650 nm at intervals of 10 min for 12h (Spectramax 384 Plus, Molecular Devices Corporation). All readings were performed in two independent assays and in triplicate per trial. The OD data were translated to colony forming units (CFU) by using the linear models fitted by (Gonzalez-Ortiz et al. (2013a).

6.3.2.3. BLOCKING TEST

The cell-line characteristics, maintenance procedure and blocking test (BT) protocol were followed according to Gonzalez-Ortiz et al. (2013b). Briefly, IPEC-J2 cells were subjected to a 24h adaptation period in 96-well flat-bottom microtitre plates with a CO_2-independent medium. Separately, a mixture (1:1) of each WB fraction (previously filter-sterilized) with the bacterial suspension was gently mixed in a 1.5-mL eppendorf, and 200 µL were immediately transferred to each well. The mixtures and cells were incubated for 30 min at 37ºC, allowing non-blocked bacteria to attach to cells. Wells were washed once with PBS and, finally, 200 µL of CO_2-independent medium was added. The bacterial-growth monitoring procedures were managed as in the *in vitro* AT protocol described above. Wheat-bran extract and both fractions obtained by molecular weight (>300 kDa and <300 kDa) were subsequently submitted to a dose-response experiment. Each fraction was tested in triplicate in two independent assays. The OD data were translated to CFU by using the equations proposed (Gonzalez-Ortiz et al., 2013b).

6.3.2.4. K88AC FIMBRIAL BINDING TO WHEAT-BRAN FRACTIONS

Binding of purified K88ac fimbriae to WB and the obtained fractions was tested in a dot-blot assay (Virkola et al., 1993) using K88ac fimbriae purified from strain ETEC FV12408. Moreover, another purified K88ac fimbriae from ETEC strain 5/95, previously used for similar purposes (Hermes et al., 2013), was used to compare the binding ability of these fimbriae extracts. To confirm K88

fimbrial-specific binding, we included casein glycomacropeptide (CGMP) and fetuin (Sigma) in the assay as positive control targets, as well as BSA, as a negative control (Hermes et al., 2013). All target proteins from the WB extract, >300-kDa, <300-kDa, CGMP, BSA (5 µg/dot), and chromatographic fractions (2.5 µl/dot), as well as fetuin (2.5 µg/dot), were immobilized on nitrocellulose membranes. After blocking for 1h at 37ºC in 2% (w/v) BSA/PBS, the membranes were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) and incubated with the purified K88 fimbriae (20 µg/ml in 1% BSA/PBS-Tween) ON at 4ºC with gentle shaking. Dot-blot membranes were washed three times with cold PBS-Tween and incubated with anti-FaeG polyclonal serum [diluted 1:2000; (Joensuu et al., 2006)] for 2h at 4ºC. After washing, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (1:1000; DakoCytomation) for 2h at 4ºC, and the bound proteins were visualized by bromochloroindolylphosphatenitrobluetetrazolium (Sigma).

6.3.2.5. RESCUING WB COMPONENTS THAT BIND TO FIMBRIAE

To identify the possible molecular compounds of WB interacting with fimbriae, an incubation of the bacteria with the whole extract of WB was performed. For the experiment, we used the same bacterial conditions as described for AT. Enterotoxigenic *E. coli* K88 and WB extract (3:1) were incubated for 30 min at 37ºC, and an incubation with PBS was also included as negative control. After the incubation, the bacteria cells were pelleted by centrifugation (1.700 x *g*, 5 min, 20ºC) and washed by hand pipetting with 1 mL of PBS. The washing step was repeated four times. Finally, a treatment with Triton X-100 at 1% for 10 min at RT eluted the WB compounds that interacted with the bacteria. The supernatant was filtered through 0.22-µm pore-size filters and kept at -20ºC.

6.3.3. IDENTIFYING THE WHEAT-BRAN COMPONENTS THAT BIND ETEC K88 FIMBRIAE

6.3.3.1. ONE DIMENSIONAL SDS-PAGE SEPARATION

Samples were treated with the 2D Clean-Up Kit (GE Healthcare) and were resuspended in lysis buffer (8M urea, 2.5% Chaps, 2% ASB-14 and 40mM dithiothreitol [DTT], pH 8.5). Samples were quantified using the Microplate BCA protein assay kit (Thermo Scientific). SDS-PAGE separation of the samples was performed using 12% acrylamide gels (BioRad). Fifteen micrograms of each sample were mixed with 6X loading buffer containing Tris HCl 0.35M pH 6.8, glycerol 30%, SDS 10% and 5%

B-mercaptoethanol and were then boiled for 5 min. Samples were separated at 15mA for 30 min and 20mA for 70 min. Proteins were stained with Instant Blue (Expedeon) for 1h at RT.

6.3.3.2. TWO DIMENSIONAL SDS-PAGE SEPARATION

The proteins rescued after the incubation between ETEC K88 and WB were separated by 2D SDS PAGE. Samples were treated with the 2D Clean-Up Kit (GE Healthcare), resuspended in lysis buffer and quantified using the Microplate BCA protein assay kit (Thermo Scientific). Two-dimensional electrophoresis with immobilized pH gradients was carried out (Bjellqvist et al., 1982). Briefly, first-dimension isoelectric focusing was performed on immobilized pH-gradient strips (7 cm, pH 3–10) using an Ettan IPGphor System. Samples (15 μg) were applied using cup-loading, and after focusing at 14 kVh, the strips were equilibrated for 15 min in 5 mL of equilibration solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS) with 10 mg/mL DTT and then in 5 mL of equilibration solution with 22.5 mg/mL iodoacetamide) for 15 min on a rocking platform. Two-dimension SDS-PAGE was performed by laying the strips on 12.5% precast gels (BioRad). The gels were run at RT at constant amperage (15 mA/gel) until the bromophenol-blue tracking front had run off the end of the gel.

6.3.3.3. IDENTIFICATION OF WHEAT-BRAN PROTEINS BY MASS SPECTROMETRY

The selected protein spots were excised from gels and digested in-gel. Before tryptic digestion, reduction and alkylation was performed by incubating samples with 10 mM DTT in 50 mM of ammonium bicarbonate for 30 min at RT, followed by alkylation with 25 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min at RT and protected from light. Protein digestion was accomplished using 25 ng of trypsin sequencing grade (Promega) for 3h at 37ºC. Peptides were eluted by centrifugation with 50 µL of ACN:H_2O (1:1) + 0.2% trifluoroacetic acid (TFA), evaporated using a speed-vacuum concentrator and resuspended in 5 µL of H_2O + 0.1% TFA. For the mass spectrometry (MS) analysis, all samples were prepared by mixing 0.5 µL of sample with the same volume of a solution of α-cyano-4-hydroxycinnamic acid matrix (10 mg/mL in 30% ACN, 60% water + 0.1% TFA) and were spotted onto a ground-steel plate (Bruker Daltonics) and allowed to air-dry at RT. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were recorded in the positive ion mode on an Ultraflextreme mass spectrometer (Bruker Daltonics). Ion acceleration was set to 25 kV. All mass spectra were externally calibrated using a standard peptide mixture. For

peptide-mass fingerprint analysis, the Mascot search engine (Matrix Science) was used with the following parameters: NCBInr database, 2 maximum-missed trypsin cleavages, cysteine carbamidomethylation and methionine oxidation as variable modifications and 50 ppm tolerance. Positive identifications were accepted with scores over the significance threshold and $P < 0.05$.

6.3.4. STATISTICAL ANALYSIS

The statistical analyses were performed using SAS 9.2 (SAS 9.2. 2010). The OD data from the AT and the BT were processed by non-linear regression analysis using the non-linear P-NLIN (Gauss-Newton method) procedure following the equations described by Becker et al. (2007). From the time at which the bacterial growth reached an OD of 0.05 ($t_{OD=0.05}$, h), the log CFU were calculated for each fraction using the described linear models for each *in vitro* test (Gonzalez-Ortiz et al., 2013a; Gonzalez-Ortiz et al., 2013b). Significant differences in the log CFU among fractions were determined by one-way analysis of variance (ANOVA), by using the GLM procedure. Linear, quadratic and cubic contrasts were performed to analyze the dose-response of WB and the >300-kDa fraction in the BT. Differences between means were tested by the Tukey-Kramer adjustment for multiple comparisons. Differences with P values < 0.05 were considered to be statistically significant.

6.4. RESULTS

6.4.1. INSIGHTS INTO THE POSSIBLE CARBOHYDRATE OR PROTEINACEOUS NATURE OF THE FUNCTIONAL MOLECULE

Since anti-adhesives based on carbohydrate structures are more common than are those based on proteins (Wittschier et al., 2007; Pieters. 2011), we firstly decided to check whether carbohydrates were involved in ETEC K88 binding. A carbohydrate digestion was carried out by using both *O*-glycosidase and neuraminidase in order to detect carbohydrate participation in the ETEC K88 binding. The *O*-glycosidase, also known as endo-α-N-acetylgalactosaminidase, catalyzes the removal of Core 1 (Galβ(1-3)GalNAc-α-O-Ser/Thr) and Core 3 (GlcNAcβ(1-3)GalNAc-α-O-Ser/Thr) *O*-linked disaccharides from glycoproteins. On the other hand, the neuraminidase is an exoglycosidase enzyme which catalyzes the hydrolysis of α2-3-, α2-6-, and α2-8- linked *N*-acetyl-neuraminic acid residues from glycoproteins and oligosaccharides, thus maximizing the disappearance of sugars attached to proteins. The digestion of the soluble extract of WB did not modify its adhesive ability, as compared to

the non-incubated WB (data not shown), indicating that carbohydrates do not play any important role in the recognition of ETEC K88.

Wheat bran is a by-product which is very rich in different components with a protein content of about 151 g/kg to 221 g/kg dry matter (Rosenfelder et al., 2013). Previous investigations have suggested the participation of a protein or a glycoprotein in the recognition of ETEC K88 (Gonzalez-Ortiz et al., 2013a). Therefore, a simple heat treatment of WB extract was conducted to verify the involvement of a protein-derived compound in the recognition of the bacteria. The heat treatment at 90ºC for 30 min reduced the number of ETEC K88 attached to wells to levels similar to those of PBS, as compared to the WB extract, which significantly attached more bacteria (5.42 vs. 5.39 vs. 6.68 log CFU, per well, respectively; $P < 0.0001$). This result is consistent with the involvement of a protein from WB in the attachment of ETEC K88.

6.4.2. IDENTIFICATION OF THE MOLECULAR WEIGHT FRACTION THAT CONTRIBUTES IN THE RECOGNITION OF ETEC K88

Fractionation by MW of the soluble extract of WB using a 300,000-Da cut-off-size filter resulted in two different fractions: >300 kDa and <300 kDa. The sample concentrations included in the *in vitro* AT and BT were fixed according to an equivalent of an extract at 10%. Therefore, the WB extract and the >300-kDa and <300-kDa fractions were tested at 14 mg/mL, 2.7 mg/mL and 17 mg/mL, respectively. Results obtained in the AT as well as in the BT with the different fractions (**Figure 6.3**) revealed that the fraction adhering to and blocking ETEC K88 attachment to IPEC-J2 was the >300 kDa; whereas the <300-kDa fraction did not modify the number of ETEC K88 adhered to or blocked, when compared to PBS in both *in vitro* assays.

A dose-response assay was performed with the WB extract and the >300-kDa fraction in a wide range of concentrations (**Figure 6**). The linear and quadratic responses were significant for different doses in the WB extract ($P < 0.05$) (**Figure 6.4.A**) and also in the >300-kDa fraction (**Figure 6.4.B**), demonstrating their anti-adhesive ability.

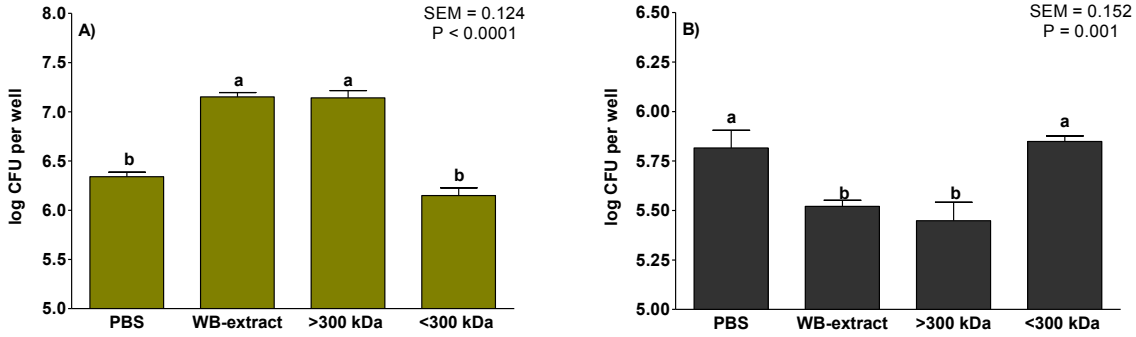

Figure 6.3. The adhesive (Figure A) and anti-adhesive (Figure B) abilities of fractions evaluated using the enterotoxigenic *E. coli* (ETEC) K88. A) Number of bacteria (log CFU per well) attached to wells coated with the different molecular-weight (MW) fractions obtained from wheat bran (WB) in the *in vitro* adhesion test (AT). The higher the log CFU counts than those of PBS, the higher the adhesive ability. The samples tested were the WB extract (14 mg/ml), the >300-kDa fraction (2.7 mg/ml) and the <300-kDa fraction (17 mg/ml). B) Number of bacteria (log CFU per well) that attached to IPEC-J2 cells after being co-incuabated for 30 min with the different fractions obtained from WB. The lower the log CFU counts than those of PBS, the higher the blocking-adhesion ability. Different letters mean significant differences (P < 0.05) between fractions. Data result from the experiments performed in triplicate in two independent assays. Error bars represent the standard error of the mean.

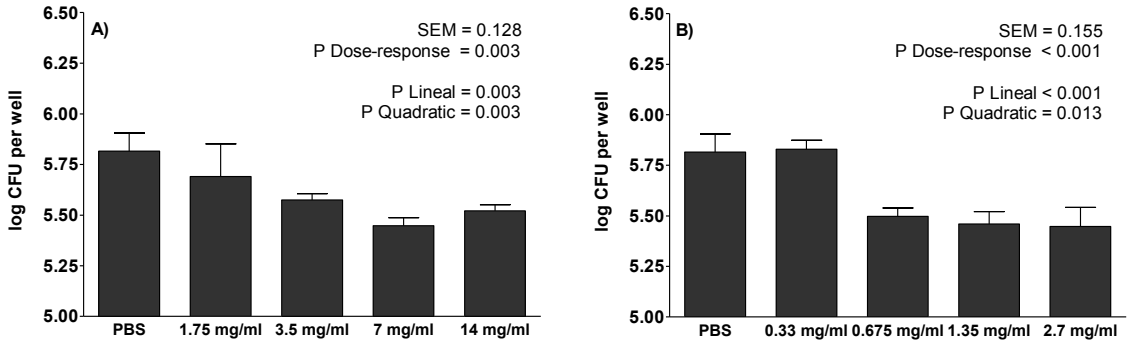

Figure 6.4. Dose-response of WB extract (A) and the >300-kDa fraction (B) to block the attachment of ETEC K88 to IPEC-J2. Log CFU: number of bacteria attached to the intestinal cells that were not blocked, when compared to those of PBS. The lower the log CFU counts, the higher the blocking-adhesion ability. Linear, quadratic and cubic contrasts were performed to analyze the dose-response. Data result from the experiments performed in triplicate in two independent assays. Error bars represent the standard error of the mean.

On the basis of these results, a proteinaceous compound from the >300-kDa fraction seems to mediate the recognition of ETEC K88. Therefore, a 1D SDS-PAGE was performed to compare the protein profile among the WB extract and the >300-kDa and the <300-kDa fractions to detect a specific band that could be involved in the fimbriae recognition process. Different protein profiles were

visualized in the 1D SDS-PAGE (data not shown). However, almost no bands in the <300-kDa fractions were detected. The WB extract and the >300-kDa fraction shared several protein bands, and many of them displayed a MW below 90 kDa. This fact is quite unexpected, since one of the fractions should mostly contain proteins with a MW higher than 300 kDa. These findings could indicate that the target protein belongs to a high MW multicomponent protein complex (>300 kDa) which is disrupted under the denaturing conditions of the SDS gel and renders the individual proteins.

6.4.3. FURTHER CHARACTERIZATION OF THE ACTIVE MOLECULE BY SIZE-EXCLUSION CHROMATOGRAPHY

Size-exclusion chromatography (SEC) was performed to fractionate the >300-kDa fraction and to isolate the target protein. Eight SEC fractions (**Figure 6.1**) were obtained (F1 to F8) and subsequently evaluated in the in *vitro* AT and BT and dot-blot assay. Results obtained from the AT revealed that F1, F2, F3 and F4 had roughly the same number of ETEC K88 cells bound as displayed by the >300-kDa fraction and the WB extract (**Figure 6.5.A**). Fractions from F5 to F8 were not able to bind ETEC K88, as was expected due to the low-molecular weight proteins that were contained in these samples. The binding of F1 to F4 fractions to ETEC K88 cells may suggest the presence of a protein in these samples that is similar to the target protein on the tissue surface, which is recognized by the fimbriae.

When the same fractions were evaluated in the BT assay, the main difference was found in the F1 fraction, which was the only one able to interfere in the ETEC K88 adhesion to IPEC-J2 by reducing the number of attached bacteria (**Figure 6.5.B**). This result shows that the chromatographic separation allowed us to isolate the protein with blocking activity of the >300-kDa sample in a single Fraction, F1. Also, it indicates that there are two distinct functions in the >300-kDa fraction, an adhesive property observed in Fractions F1 to F4 and a blocking function located in Fraction F1. In **Figure 6.6.A**, the results of the dot-blot assay are represented. Both K88ac fimbriae, coming from two different enterotoxigenic *E. coli* strains (FV12408 and 5/95), bind to the WB extract and >300-kDa fraction but not to the <300-kDa fraction. Regarding the eight SEC fractions obtained from the >300-kDa fraction, K88ac fimbriae bind strongly to F2, F3 and F4 fractions, but less intensely to F1. Similarly to the AT and BT assays, no binding signals were detected for F5, F6, F7 and F8 fractions. The eight SEC fractions were further separated by SDS-PAGE and compared (**Figure 6.6.B**).

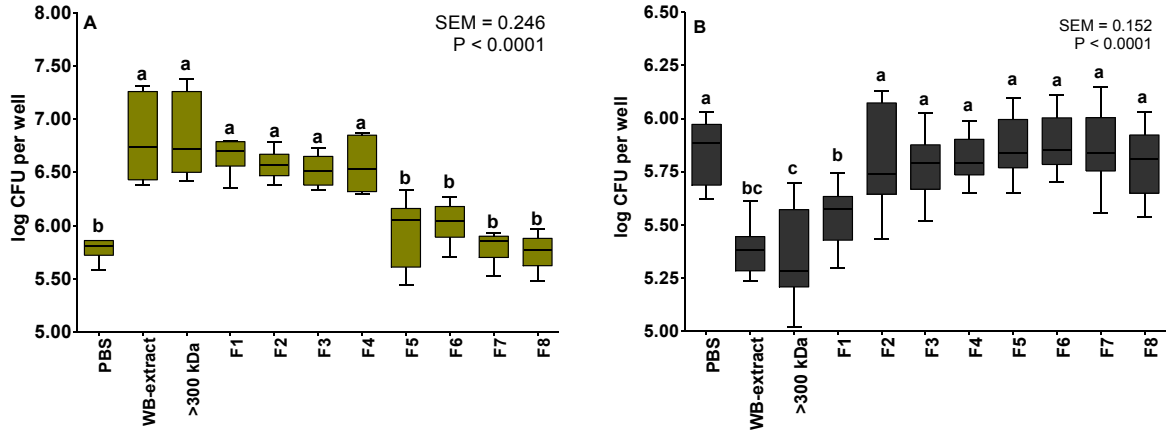

Figure 6.5. A) Number of bacteria (log CFU per well) attached to wells coated with the different fractions in the *in vitro* adhesion test (AT). The higher the log CFU counts than those of PBS, the higher the adhesive ability. B) Number of bacteria (log CFU per well) that attached to IPEC-J2 cells in the *in vitro* blocking test (BT). The lower the log CFU counts than those of PBS, the higher the blocking-adhesion ability. In Figures A and B, different letters mean significant differences (P < 0.05) between fractions. Data result from two independent assays performed in triplicate. Error bars represent the standard error of the mean.

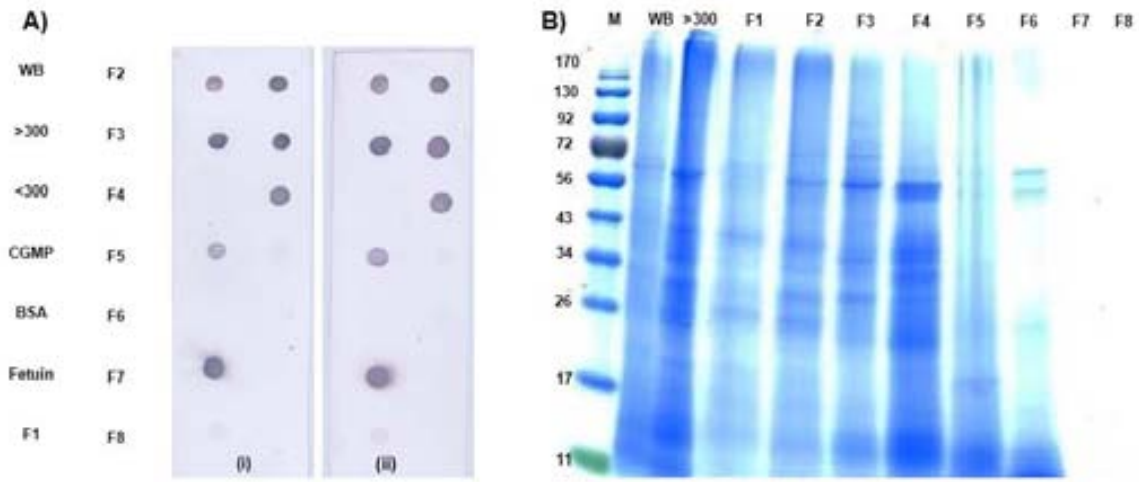

Figure 6.6. A) Dot-blot analysis with purified fimbriae on immobilized WB extract, >300-kDa and <300-kDa fractions, CGMP, BSA, fetuin and the eight fractions (F1 to F8). (i) Binding of purified K88ac fimbriae of ETEC strain FV12408. (ii) Binding of purified K88ac fimbriae of ETEC strain 5/95. B) One-dimension SDS-PAGE of the eight fractions obtained by size-exclusion cromatography.

Surprisingly, F1, F2, F3 and F4 contain several low-MW proteins. The presence of these low-MW proteins in a fraction separated by a filter of 300 kDa could confirm the previous hypothesis regarding the existence of protein complexes in the >300-kDa fraction, which are individualized under the denaturing conditions of the gel. Accordingly, the >300-kDa fraction was treated with ACN and subsequently fractionated by SEC in the presence of ACN to break down the potential complexes

without completely denaturing the proteins, in order to keep their activity. As a result of this chromatography (**Figure 6.2**), six fractions were obtained (FA1 to FA6) and evaluated by both *in vitro* AT and BT and the dot blot assay.

In the AT, SEC fractions FA1 to FA4 showed a similar effect to that of the >300-kDa fraction and WB extract, binding a high log CFU of ETEC K88 (**Figure 6.7.A**) and showing that the ACN treatment did not affect their binding capacity. These results are in accordance with the dot-blot results

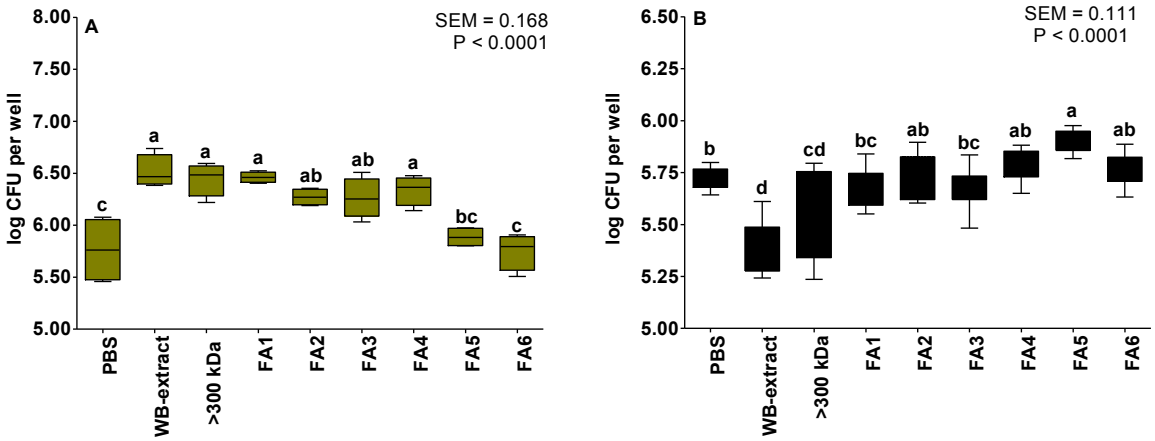

Figure 6.7. A) Number of bacteria (log CFU per well) attached to wells coated with the different fractions in the *in vitro* adhesion test (AT). The higher the log CFU counts than those of PBS, the higher adhesive ability. B) Number of bacteria (log CFU per well) that attached to IPEC-J2 cells in the *in vitro* blocking test (BT). The lower the log CFU counts than those of PBS, the higher the blocking-adhesion ability. In Figures C and D, different letters mean significant differences (P < 0.05) between fractions. Data result from two independent assays performed in triplicate. Error bars represent the standard error of the mean.

(**Figure 6.8.A**), which show that both types of K88ac fimbriae bind strongly to FA2 and FA3, but with lower intensity to FA1 and FA4. In contrast, those active fractions in the AT and the dot-blot assay were not able to interfere in the ETEC K88 attachment to IPEC-J2 (**Figure 6.7.B**). The 1D SDS-PAGE electrophoresis did not reveal a clear, different band pattern among fractions (**Figure 6.8.B**). Six bands (B1, B2, B3, B4, B5 and B6) which were in common among the active fractions in the AT and the dot-blot assay (FA2 to FA4) were excised to identify the proteins by MALDI-TOF MS. **Table 1** sums up the most important information regarding the identification of the excised bands by MS using the Mascot search engine. Bands B1 and B2 shared the same beta amylase and B1 also contained a protein disulphide isomerase 2 precursor of 56 kDa. In FA4, the high intensity of bands distributed at lower MW allowed us to excise more bands for identification. Serpin-Z2B and Class II chitinase were

found to be the most representative proteins in the B3 and B4 bands, respectively. Finally, two protease inhibitors were identified in B5 and B6 with a low-MW of approximately 19 kDa.

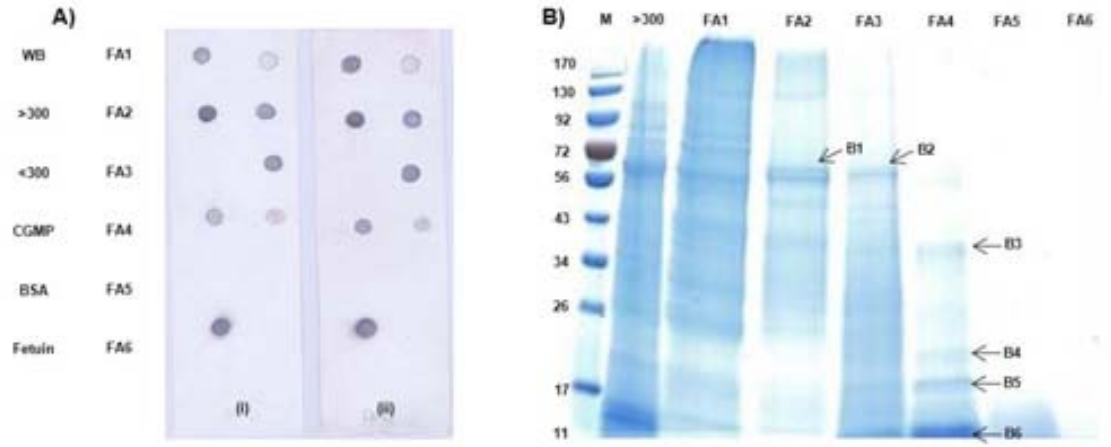

Figure 6.8. A) Dot-blot analysis with purified fimbriae on immobilized WB extract, >300-kDa, <300-kDa, CGMP, BSA, fetuin and the six fractions (FA1 to FA6). (i) Binding of purified K88ac fimbriae of ETEC strain FV12408. (ii) Binding of purified K88ac fimbriae of ETEC strain 5/95. B) One-dimension SDS-PAGE of the six fractions obtained by size-exclusion cromatography treated with solvent. From B1 to B6 are indicated the excised bands which were identified by mass spectrometry.

6.4.4. ISOLATION OF WHEAT-BRAN PROTEINS ATTACHING TO ETEC K88

In parallel, an *in vitro* experiment was conducted which consisted of incubating ETEC K88 cells with the WB extract in order to rescue the WB molecules most firmly attached to the bacteria. The material retrieved after the shaving process was used to perform 1D and 2D gels with both samples and to identify the spots which were only detected in the presence of WB (**Figure 6.9**). In the 1D gel it was not possible to identify any differential bands between the incubation of bacteria with PBS or WB extract due to the high number of proteins in the sample. The two 2D gels were compared using the Progenesis Same Spots software (Nonlinear Dynamics) and, after the alignment of the images, six spots with a fold >2 were detected. Three of the spots belonged to the same train of spots (**Figure 6.9.1,** Spot Number B7) and contained a bacterial protein (**Table 6.1**), as did Spot Number B9. Finally, two spots belonging to the same train of spots (**Figure 6.9.1**, Spot Number B8) were identified as a protein from *Triticum aestivum,* Globulin 3 (**Table 6.1**).

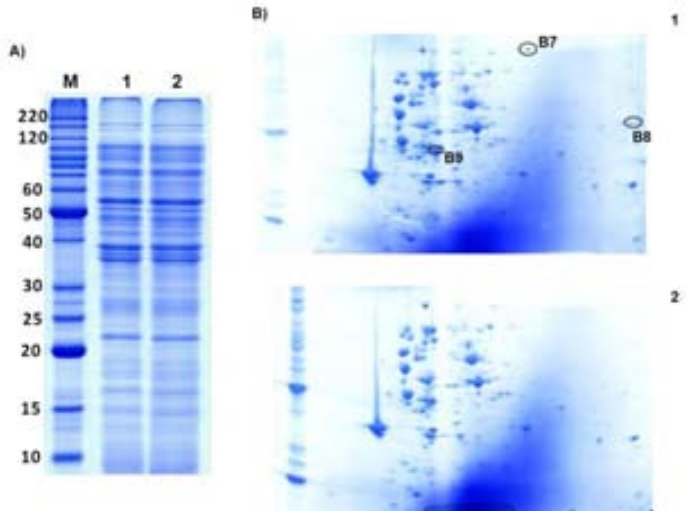

Figure 6.9. Isolation of wheat-bran proteins attaching to ETEC K88. ETEC K88 cells were incubated with PBS (1) and wheat-bran extract (2). The proteins obtained after the shaving process were separated in 1D (A) and 2D (B) gels. The spots with a fold > 2 are labeled as B7, B8 and B9.

6.5. DISCUSSION

6.5.1. NATURE OF THE PUTATIVE COMPOUND OF WHEAT-BRAN INVOLVED IN ETEC K88 BLOCKAGE

To study the anti-adhesive properties of WB, we have focused on the interference of the binding of ETEC K88 cells. This *E.coli* strain expresses F4 fimbriae, which are proteinaceous filament adhesins composed of multiple copies of the major fimbrial subunit FaeG (Devriendt et al., 2010). These strains, also called F4+ ETEC, adhere to F4-specific receptors on the intestinal epithelium, colonize the small intestine and cause diarrhea in neonatal and recently weaned pigs (Fairbrother et al., 2005). *In vivo* experiments have demonstrated the ability of WB to reduce the fecal enterobacteria population in piglets (Hermes et al., 2009; Molist et al., 2010) as well as the ability to reduce the number of ETEC K88 that attach to the ileum mucosa (Molist et al., 2009). Further *in vitro* investigations have shown that WB minimizes the adhesion of ETEC K88 to IPEC-J2 cells (Hermes et al., 2011; Gonzalez-Ortiz et al., 2013b). The fact that WB is a by-product easy to find worldwide makes it an interesting target to work on. Eeckhaut et al. (2008) reported that the dietary addition of arabinoxylooligosaccharides from WB inhibits *Salmonella enteritidis* colonization in broiler chickens. However, the isolation of WB fractions with a high content of arabinoxylans did not prevent ETEC K88 from binding to the porcine mucus, thus suggesting that a protein or a glycoprotein may be involved in the specific recognition of F4 (Gonzalez-Ortiz et al., 2013a). The first approach performed in the

present study was to digest the WB extract with *O*-glycosidase and neuraminidase. This digestion allowed us to confirm that the anti-adhesive effect was not related to the removal of *O*-linked disaccharide and *N*-acetyl-neuraminic acid from glycoproteins. The heating procedure assayed afterwards confirmed that a protein was likely to be involved in the recognition of F4 fimbriae. Some reports have suggested the ability of protein fractions from plants to act as anti-adhesive substrates (Lengsfeld et al., 2004; Wittschier et al., 2007). Nonetheless, WB is also known to contain other components that could positively interfere with the binding of ETEC K88 to epithelial cells. Wheat-bran possesses high quantities of anti-nutritional factors such as phytic acid (42 mg/g), polyphenols (3.20 mg/g), tannins (2.9 mg/g), saponins (2.7 mg/g) and trypsin inhibitors (54.2 U/g) (Kaur et al., 2011), which should also be taken into account for their possible involvement in bacterial adhesion. For example, the proanthocyanidins of cranberry extracts are known to prevent urinary tract infections by disrupting the binding between the bacteria and the uroepithelial receptors and by changing the physicochemical surface properties of *E. coli* (Liu et al., 2008). An extensive literature survey reveals that all of these non-nutritional components are non-protein in nature, which could be discarded in the current identification of the active compound recognizing ETEC K88 due to the loss of activity when the WB extract was heated.

6.5.2. A PROTEIN COMPLEX AS THE RESPONSIBLE FACTOR FOR BACTERIAL ATTACHMENT INHIBITION

The first fractionation step applied to the WB extract enabled us to elucidate that the active proteins remained in the >300-kDa fraction. Further separation by SEC led to the isolation of two distinct activities in common of different fractions, i.e., anti-adhesive activity and blocking activity. The WB extract blocking activity could be isolated in a single chromatographic fraction. This activity disappeared when SEC was performed in the presence of ACN. The fact that the WB adhesive properties were retained under both conditions indicates that the proteins separated by SEC in the presence of ACN were still active. Therefore, the loss of the blocking activity should likely be caused by the disruption of protein complexes produced by the presence of the solvent. On the other hand, the detection of adhesive activity in both SEC conditions points towards the implication of an isolated protein rather than of a protein complex.

Table 6.1. Identification of proteins present in the soluble extract of wheat bran (*Triticum aestivum)* after fractionation by MALDI-TOF (Spot nº from B1 to B6). Identification of proteins rescued after incubation between ETEC K88 and WB extract (Spot numbers from B7 to B9).

Spot No.	Accession number	Protein description	Taxonomy	Mascot Score	Protein MW (Da)	Pep No.[a]	Seq. Cov. (%)[b]
Proteins present in the soluble extract of wheat bran (Triticum aestivum) after fractionation							
B1	gi|474451266	Beta amylase	*Triticum urartu*	93	58,710	14	29
	gi|13925726	Protein disulfide isomerase 2 precursor	*Triticum aestivum*	70	56,406	8	24
B2	gi|474451266	Beta amylase	*Triticum urartu*	131	58,710	14	28
B3	gi|473793747	Serpin-Z2B	*Trititicum urartu*	79	45,112	12	36
B4	gi|62465514	Class II chitinase	*Triticum aestivum*	64	28,200	13	87
B5	gi|123975	Endogenous alpha-amylase/subtilisin inhibitor	*Triticum aestivum*	313	19,621	21	92
B6	gi|123957	Alpha-amylase/trypsin inhibitor CM3	*Triticum aestivum*	85	18,209	6	57
Proteins rescued after incubation between ETEC K88 and WB-extract							
B7	gi|260870699	RNA polymerase, beta subunit	*Escherichia coli*	286	155,048	32	26
B8	gi|215398470	Globulin 3	*Triticum aestivum*	122	66,310	21	41
B9	gi|300946929	Translation elongation factor Tu	*Escherichia coli*	342	44,822	41	94

[a]represent the number of identified peptides by PMF.

[b]represent the percentage of identified peptide coverage in total sequence of protein

The identification of beta amylase in the chromatographic fractions with adhesive activity is probably a consequence of the abundance of this enzyme in cereals. Most likely the successive separation steps were not enough to completely eliminate the protein. The other five protein bands identified belonged to protease inhibitors related to pathogen resistance in plants. Protein disulphide isomerase (PDI) is linked to the formation of disulphide bonds in the endoplasmatic reticulum necessary to fold seed-storage proteins (Kamauchi et al., 2008). This protein has been previously involved in a plant's defense response (Ray et al., 2003). Real-time quantitative reverse-transcription PCR and northern analysis revealed that PDI was induced within 3h of inoculation of wheat with *Mycosphaerella graminicola*, a widespread pathogen of wheat. Plant chitinases, also considered seed proteins, break bonds between C1 and C4 between two consecutive N-acetylglucosamines of chitin, which is a main component of the cell wall in fungi (Kasprzewska. 2003). Class II chitinase possesses anti-fungal activity by hydrolyzing fungal cell-wall constituents (Kitajima and Sato, 1999; Oldach et al., 2001; Shin et al., 2008). Wheat serpins come from the superfamily of serine protease inhibitors and have been identified in almost all organisms, including plants. Most of them possess inhibitory functions resulting in the formation of large complexes between native serpin and protease, usually proteases of the chymotrypsin family (Silverman et al., 2001). Recently, some members of the serpin family have been reported to display antimicrobial activity (Malmstrom et al., 2009; Rehault-Godbert et al., 2013). The lowest MW bands identified belong to two different alpha-amylase inhibitors considered to correspond to stress and/or defense proteins (Guo et al., 2012). Alpha-amylase inhibitors play important roles in protecting starch and protein reserves in the endosperm against degradation, particularly when caused by biotic stresses such as insect attack (Franco et al., 2002). Besides these anti-infective properties, the alpha-amylase/trypsin inhibitors are strong activators of innate immune response, being novel contributors to celiac diseases in human (Junker et al., 2012).

6.5.3. GLOBULIN 3 IS ONE OF THE BINDING PROTEINS TO ETEC K88

Presumably, the incubation of WB extract with ETEC K88 allowed for the identification of the dietary molecule compounds firmly attached to the bacteria. This procedure has been called "surfomics" by some authors and allows for the identification of surface proteins through the shaving of the exposed proteins of living cells (Olaya-Abril et al., 2013). This method enables the exploration of the surface-protein profile of the samples. The comparison of the protein profiles obtained after the

shaving of ETEC K88 cells incubated with WB extract or with PBS led to the identification of three different spots by 2D electrophoresis. One of the excised spots corresponded to Globulin 3, a 7S globulin storage protein of wheat (Guo et al., 2012). Up to now, few studies have sought to characterize 7S wheat globulins because they were thought to be minor storage proteins. However, Teodorowicz et al. (Teodorowicz et al., 2013a; Teodorowicz et al., 2013b) have recently demonstrated the modulation of human-gut microbiota proliferation, survival and adhesion by 7S peanut globulin, thus suggesting that this kind of proteins may have anti-adhesive properties against some bacterial strains.

6.6. CONCLUSIONS

Taken together, our results suggest that protease inhibitors and Globulin 3 from WB extract are involved in the binding to enterotoxigenic *E. coli* K88 cells. Further studies should be performed to validate the results and gain an understanding of the recognition and blocking processes that take place. All of the gathered information would eventually pave the way for the design of novel therapeutic agents to prevent bacterial pathogenesis.

7. DISCOVERING NEW PROPERTIES OF WHEAT BRAN: THE ANTI-BIOFILM ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS* AND THE INTERFERENCE WITH BACTERIAL QUORUM-SENSING SYSTEMS

Under review

7.1. ABSTRACT

Some natural remedies, such as plant extracts, have been demonstrated to interfere in the biofilm formation of several pathogenic bacteria, however, to our knowledge, no study exploring the potential of wheat-bran (WB) soluble extract has been published. The purpose of the present study was to evaluate the anti-biofilm activity of WB against a cow mastitis strain of *Staphylococcus aureus* and also its possible interference with bacterial quòrum-sensing (QS) systems. The potential of inhibition and destruction of the biofilm formed was studied by different *in vitro* assays. Moreover, the ability of WB to interfere in bacterial QS by degrading acyl-homoserine lactones (AHL) was also tested. The soluble extract of WB at 0.5% showed anti-biofilm activity, inhibiting biofilm formation and also destroying the biofilm previously formed, when compared to PBS (P = 0.05 and P = 0.02, respectively). Similarly to what the WB extract did, the >300-kDa fraction had significant anti-biofilm activity in both *in vitro* assays (P < 0.05). The soluble extract of WB also showed a potential to interfere with bacterial QS Systems, as it was demonstrated to contain certain lactanase activity able to reduce AHL concentration in the medium. The present study reveals two additional beneficial properties of WB extract never explored before, which may be related to the presence of defence compounds in the plant extract able to interfere with microbial biofilms and, also, QS systems. Further studies should be performed to explore possible future applications of WB extract to control biofilm infections.

7.2. INTRODUCTION

Bacterial biofilms cause chronic infections that can persist despite the use of antibiotic treatments. Additionaly, in some cases low doses of antibiotics can even enhance biofilm formation, suggesting a natural defence mechanism of bacteria in avoiding the lethal effects of antibiotics (Costa et al., 2012).

As an example, mastitis is one of the most important diseases in dairy cattle, being at the top among diseases related to biofilm production (Melchior et al., 2006b). *Staphylococcus aureus* is the prime aetiological agent causing mastitis in bovines (Bradley et al., 2007). Besides the production of many microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and mediating epithelial attachment (Clarke and Foster, 2006), the formation of biofilms is also recognized as an important virulence factor in *S. aureus* (Otto. 2013). The general therapeutic approach towards mastitis is to use antibiotics to combat underlying infection. However, bacteria in biofilms are significantly less responsive to antibiotics and antimicrobial stressors than are planktonic organisms of the same species (Bryers. 2012), and *S. aureus* may resist many therapeutic regimes (Melchior et al., 2007). Therefore, looking for natural compounds able to prevent biofilm formation may be a feasible alternative to reduce antimicrobial resistance.

Furthermore quorum sensing (QS) is an important regulatory mechanism for many microorganisms to regulate and coordinate the expression of different virulence factors, also including the synthesis and development of biofilms (Boyen et al., 2009). Therefore, the targeting of QS signalling systems might also offer a new strategy to combat bacterial infections. Approaches that interfere with proper microbial QS signalling are called “quorum quenching (QQ)” (Dong et al., 2007). Thus, looking for natural compounds with QQ properties may also be an attractive alternative to antibiotics. This makes sense, considering that in many cases natural compounds in plants have co-evolved to prevent bacterial encroachment and infection or to outcompete microbial counterparts in the environment (Zhu and Kaufmann GF., 2013). In this sense, the hard outer layer of wheat grain, the bran (WB), whose main function is to protect the grain against pathogen invasion, could be a natural source of QQ compounds or other antimicrobial substances. Many beneficial effects have been described for WB (Mohsin-Javed et al., 2012), also including anti-adhesive properties against enteropathogens (Molist et al., 2009). In this regard, it has been previously demonstrated that WB extract has the potential to modify the intestinal microbiota of animals (Molist et al., 2009) and also to

interfere in the binding capacity of enteropathogens such as *Escherichia coli* K88 to intestinal epithelial cells and mucus (Hermes et al., 2011; Gonzalez-Ortiz et al., 2013a).

Taking this into account, it seemed reasonable that a soluble extract obtained from WB could have anti-biofilm properties and could also interfere with the QS system of bacteria. With this hypothesis, the purpose of the present study is to evaluate wether a WB soluble extract could prevent or destroy the biofilm of *S. aureus* using *in vitro* microtitre-based models. Also, its potential to interfere in the activity of acyl-homoserine lactones (AHL), as one of the most studied QS signal molecules, is evaluated.

7.3. MATERIAL AND METHODS

7.3.1. BACTERIAL STRAINS AND MEDIA

The *S. aureus* BMA/FR/0.32/0074 strain was generously provided by Prof. Dr. J. Fink-Gremmels and was isolated from a mastitis episode in cows in The Netherlands. This strain is characterized by high slime production and has previously been used in biofilm antimicrobial susceptibility studies (Melchior et al., 2006a).

One colony from a blood-agar culture was used to inoculate 5 mL of tryptone soy broth (TSB) + 0.25% glucose. The culture was incubated overnight (ON) at 37ºC with gentle shaking. This bacterial culture was used for the biofilm inhibition and the biofilm destruction assays.

Yersininia enterocolitica 057 was used to produce natural AHL. The strain, which was satisfactorily tested to produce AHL (Medina-Martinez et al., 2007), was provided by the laboratory of Food Microbiology and Food Preservation (Ghent University; Ghent, Belgium). The sterile supernatant of a 24h culture of *Y. enterocolitica* grown in Luria broth medium (LBM), thus containing natural AHL, was recovered after centrifugation (6,000 x g, 5 min, room temperature) and filter sterilized (0.22-µm filters, Supor Acrodisc, Pall Life Science).

Two recombinant *Pseudomonas fluorescens* strains, P3/pME6863 and the P3/pME6000 derivatives, were used as degrader and non-degrader reference strains, respectively (Molina et al., 2003). Both strains were grown ON at 30ºC in LBM. Before use, the optical densities of the cultures were adjusted with sterile LBM to an optical density (OD) of 0.5 at a wavelength of 600 nm.

Escherichia coli JB523, containing plasmid pJBA130, responsible for the production of a green fluorescent protein, was used as an AHL biosensor (Andersen et al., 2001).

7.3.2. WHEAT-BRAN EXTRACTION AND FRACTIONATION

The WB used in the study comes from a local Spanish mill (Moretó; Mollet del Vallès, Barcelona). First, the WB was finely ground in an analytical grinder and was then suspended in demineralized (DEMI) water to a solid-to-liquid ratio of 1:10 (w/v). Subsequently, the suspension was sonicated (J.P. Selecta, S.A.) three times for 30s each and then centrifuged (460 x g, 5 min, 20ºC). The supernatant extracted was divided twice. One aliquot was stored at -20ºC until use and the other aliquot was immediately fractionated by molecular weight (MW) using Vivaspin® 6 centrifugal concentrators (Sartorious) with a cut-off size of 300,000 Da (300 kDa) and 100,000 Da (100 kDa). The upper part of the tube was filled with the soluble extract and centrifuged (3,000 x *g*, 3.5h, 4ºC). After centrifugation, the upper part was adjusted with DEMI water to the same volume retrieved in the bottom container in order to achieve the same volume. The <300-kDa fraction was submitted again to a 100,000-Da cut-off size filter, following the same centrifuging protocol and the same filling conditions. Finally, three fractions were obtained: >300 kDa, <300>100 kDa and <100 kDa. These fractions were stored at -20ºC until used.

7.3.3. MINIMAL INHIBITORY CONCENTRATION ASSAY

A minimal inhibitory concentration (MIC) assay was performed for WB soluble extract to discard any possible antimicrobial effect on *S. aureus* 0074. The MIC was determined in 96-well microtitre plates. Briefly, in a sterile flat-bottom plate (Corning® Costar® Ref. nº 3599), a stock sample of WB soluble extract was diluted (1:2) from 1% to 0.016% in TSB + 0.25% glucose (final concentration 100 µL) in duplicate from rows A to G. Columns 3, 6, 9 and 12 were filled with an additional 100 µL TSB + 0.25% glucose medium. One-hundred microliters of the ON, cultured bacteria suspension (1:50 diluted) were added to the other columns. Row H served as a control well, where instead of sample, medium was added. Plates were covered with a breathseal and the lid and kept at 37ºC under static conditions ON. Turbidity was measured by reading the OD at 630 nm in a spectrophotometer reader (Biotek µQuant spectrophotometer). The readings were taken in two independent assays and in duplicate per trial.

7.3.4. BIOFILM INHIBITION ASSAY

A biofilm inhibition assay was employed to test the effect of WB extract, and the different fractions thereof, on inhibition of biofilm formation. This protocol is based according to Hensen's methodology (Melchior et al., 2006a) to stain the biofilm formed with safranin (Figure 7.1). Assays were performed in sterile round-bottom 96-well polystyrene plates (Corning® Costar® Ref. nº 3799). Each sample and dilution was tested in duplicate in two independent assays. A concentration of 1:100 of the samples' soluble extracts were added in the first-row wells. Samples were serially diluted (1:2) from 0.5% to 0.016% (100 µL of final sample volume). Then, the bacterial suspension was prepared by diluting 1:50 in TSB + 0.25% glucose from the ON culture, and 100 µL were added to each well. Plates were closed with breathseal and the lid and were placed in a 37ºC incubator for 24h. The supernatants were removed carefully, the wells were rinsed twice with DEMI water, and the biofilm was fixed with 200 µL 0.1M HCl for 1.5h at room temperature (RT). The fixative was removed directly onto dry paper, and 200 µL of safranin 0.1% were added to stain the biofilm for 1h at RT. Safranin solution was removed and wells washed 4X with DEMI. Finally, 125 µL of 0.2M NaOH was added to all wells and incubated for 30 min at 57ºC to dissolve the safranin. Well contents were mixed, 100 µL were transferred to a non-sterile flat-bottom plate, and absorbance was measured in a spectrophotometer reader at 540 nm. The positive control (medium + bacteria) was considered to show good biofilm formation when the OD was above 0.2 (Biotek µQuant spectrophotometer). Positive controls and negative controls (medium + sample – without bacteria) for sample background colour were included in each assay.

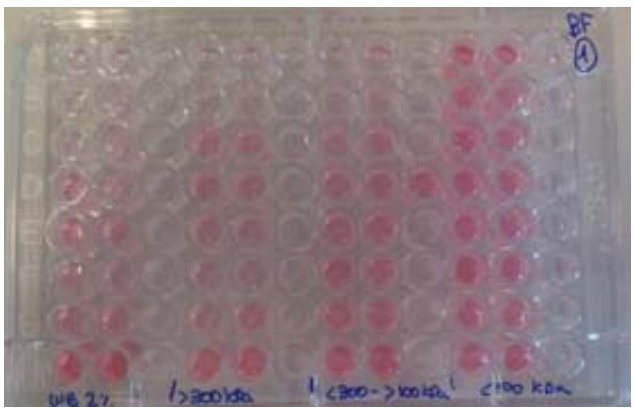

Figure 7.1. Picture of the *in vitro* biofilm inhibition assay showing the already stained biofilm in the positive control wells (row H). This miniaturized test allows the comparison in a dose-response manner of each sample dilution, in this case corresponding to WB extract, the >300-kDa, <300>100-kDa and <100-kDa fractions.

7.3.5. BIOFILM DESTRUCTION ASSAY

The biofilm destruction assay was a modified version of the biofilm inhibition assay in which first the bacteria are allowed to form the biofilm inside the wells for 48h. Next, test samples are included to allow for the destruction of the biofilm previously formed. This protocol is based on quantification of the remaining biofilm as in the biofilm inhibition assay. Assays were performed in sterile round-bottom 96-well polystyrene plates (Corning® Costar® Ref. nº 3799). Each sample and dilution was tested in duplicate in two independent assays. Firstly, the bacterial suspension was prepared by diluting 1:200 in TSB + 0.25% glucose from the ON culture, and 100 µL were added to each well. Plates were covered with breathseal film and the lid and were placed in the incubator at 37ºC for 48h. In a new sterile flat-bottom plate (Corning® Costar® Ref. nº 3599), samples concentration from 1:100 (w/v) were used to prepare serial dilutions (1:2) from 0.5% to 0.016%. The supernatants from the bacteria plates were removed carefully without disturbing the already-formed biofilm. Then, 100 µL of the sample dilutions plate were transferred to the bacteria plate, and extra medium was added to a final volume of 200 µL/well. The plates were covered and incubated ON at 37ºC. The fixation with 0.1 HCL, the staining with 0.1% safranin and the dissolving with NaOH procedures were as described above for the biofilm inhibition assay. The remaining safranin content was measured in a spectrophotometer reader at 540 nm. The positive control (medium + bacteria) was considered to show good biofilm formation when the OD was above 0.2 (Biotek µQuant spectrophotometer). Positive controls and negative controls (medium + sample – without bacteria) for sample background colour were included in each assay.

7.3.6. ABILITY OF WHEAT BRAN TO DEGRADE ACYL-HOMOSERINE LACTONES

The ability of WB extract and its fractions to degrade naturally produced AHL were evaluated *in vitro* (Medina-Martinez et al., 2007). Nine-hundred microliters of WB extract 1:100 (w/v) or the >300-kDa and the <300-kDa fractions were mixed with 100 µL of naturally produced AHL. The recombinant *P. fluorescens* P3/pME6863 strain and the *P. fluorescens* P3/pME6000 derivative were used as degrader (DS) and non-degrader (NDS) control strains, respectively (Molina et al., 2003). The mixtures were incubated for 24h at 30ºC with shaking (170 rpm). Afterwards, the mixtures were centrifuged (6,000 x g, 5 min, RT) and filtre-sterilized. AHL detection was performed using an indirect fluorescence-based method (Medina-Martinez et al., 2007). The green fluorescence was detected by

a microtitre-plate fluorescence reader (FL_x800, Bio-Tek Instruments Inc., USA), where 100 µL of the biosensor strain was incubated with 50 µL of the suspensions for 6h at 30ºC. All samples were tested in triplicate in three independent assays. Positive (natural produced AHL) and negative (LBM) controls were run in parallel. Data are presented as the relative response to AHL detection in positive controls (%).

7.3.7. STATISTICAL ANALYSIS

Values from biofilm inhibition and biofilm destruction assays were analysed with a generalized linear mixed model (GLMM) by using the GLIMMIX procedure of the statistical package of SAS 9.2 (SAS Inc.; Cary, NC, USA). For both parameters, the model was fitted to an inverse Gaussian distribution for the analysis. The relative response to AHL detection in positive controls was analysed with the same procedure fitting the model to a negative binomial distribution. For all analyses, the criterion for significance was $P < 0.05$.

7.4. RESULTS

7.4.1. THE ANTI-BIOFILM ACTIVITY OF WHEAT-BRAN SOLUBLE EXTRACT

First of all, a MIC assay was performed to discard any possible inhibitory effect of WB extract on *S. aureus* growth. It was confirmed that bacterial growth was not inhibited during incubation ON with WB extract nor with the fractions obtained by MW.

Figures 7.2 and **7.3** show the results of the biofilm inhibition and destruction assays for the WB extract and its fractions at different concentrations. At the higher concentration (equivalent to a 0.5% WB suspension), the soluble extract from WB showed a clear anti-biofilm capacity, inhibiting biofilm formation ($P = 0.05$) and also the destruction ($P = 0.02$) of the biofilm previously formed, when compared to PBS (less than 11.52% ± 1.62% and 9.93% ± 1.92% of biofilm formation, respectively). All of the rest of decreasing concentrations tested were also shown to be statistically effective ($P \leq 0.05$) in a dose-response picture. With the lowest concentration assayed (equivalent to 0.016% WB suspension), the biofilm formed represents less than 57.75% ± 2.75% and 36.21% ± 4.18%, as compared to PBS ($P = 0.006$ and $P < 0.0001$), for the biofilm inhibition and destruction assays, respectively.

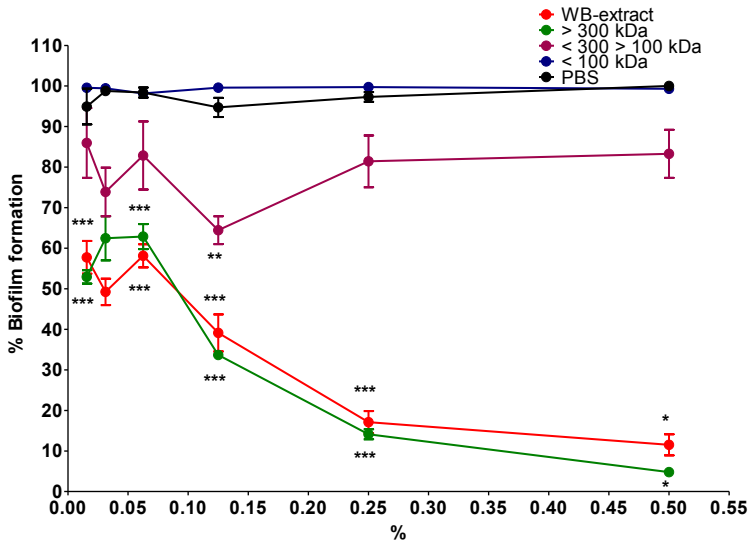

Figure 7.2. Dose-response of the biofilm formation (%) in the biofilm inhibition assay using wheat-bran (WB) extract and the fractions obtained by molecular weight. The tested samples were the WB extract, the >300-kDa fraction, the <300>100-kDa fraction and the <100-kDa fraction. PBS was included as a positive control with which to compare each treatment. Data are results from two independent assays in duplicate. Bars represent standard error of the mean. For each concentration, the asterisks show significant differences regarding PBS: *P < 0.05; **P < 0.01; ***P < 0.001.

Fractionation by MW of the soluble WB extract led to three different fractions to test, the >300-kDa, the <300>100-kDa and the <100-kDa ones. Showing a very similar response as did the WB extract, the >300-kDa fraction had a significant anti-biofilm activity in both *in vitro* assays at all concentrations tested (P < 0.05) (**Figures 7.2 and 7.3**). In the biofilm inhibition assay, the half-maximal inhibitory concentration (IC_{50}) for WB extract and the >300-kDa fraction were 0.06% ± 0.01% and 0.07% ± 0.01%, respectively. On the other hand, in the biofilm destruction assay, the IC_{50} of both samples was lower than 0.016% (**Figure 7.3),** thus suggesting that WB extract and the >300-kDa fraction are even more active in destroying the biofilm at lower concentrations than they are in inhibiting the biofilm formation. No significant anti-biofilm effects were found, regarding the <300>100-kDa and the <100-kDa fractions. These findings confirm that the active molecule or molecules present in WB extract against *S. aureus* biofilm are inside the >300-kDa fraction.

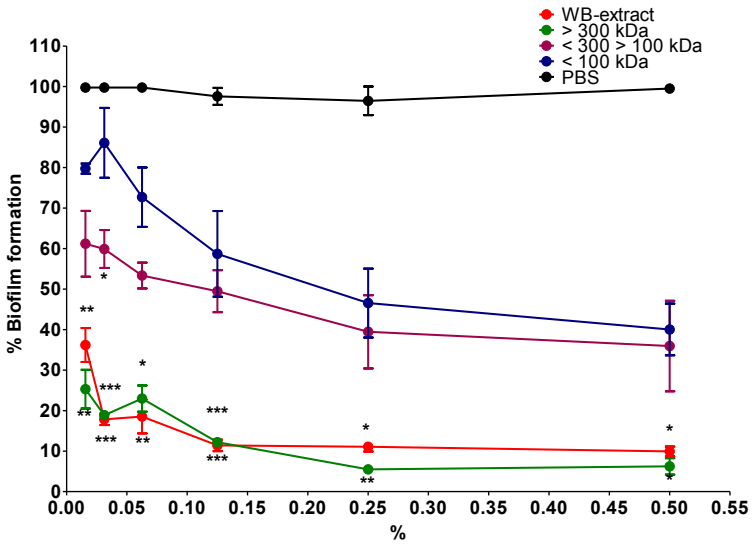

Figure 7.3. Dose-response of the biofilm formation (%) in the biofilm destruction assay using wheat-bran (WB) extract and the fractions obtained by molecular weight. The tested samples were the WB extract, the >300-kDa fraction, the <300>100-kDa fraction and the <100-kDa fraction. PBS was included as a positive control with which to compare each treatment. Data are results from two independent assays in duplicate. Bars represent standard error of the mean. For each concentration, the asterisks show significant differences regarding PBS: *P < 0.05; **P < 0.01; ***P < 0.001.

7.4.2. THE POSSIBLE INTERFERENCE OF WHEAT-BRAN IN THE ACYL HOMOSERINE-BASED QUORUM- SENSING SYSTEM

Additionally, the ability of WB extract and the >300-kDa and <300-kDa fractions to degrade AHL was measured, thus suggesting the interference in QS pathways based on this bacterial signal. In **Figure 7.4**, the relative response of AHL of each treatment compared to the positive control is illustrated. As expected, the AHL degrader control strain (DS) presented a very low relative response, 4.3% ± 0.67%, whereas the AHL non-degrader control strain (NDS) showed a high value of the relative response to AHL, 98.2% ± 2.32%. The WB extract also showed significant reductions in the AHL response with a mean value of 25.0% ± 3.17%, and similar values were also found for both MW fractions that did not show differences between them (26.24% ± 4.00% and 24.26% ± 3.74% values for >300-kDa and <300-kDa fractions, respectively).

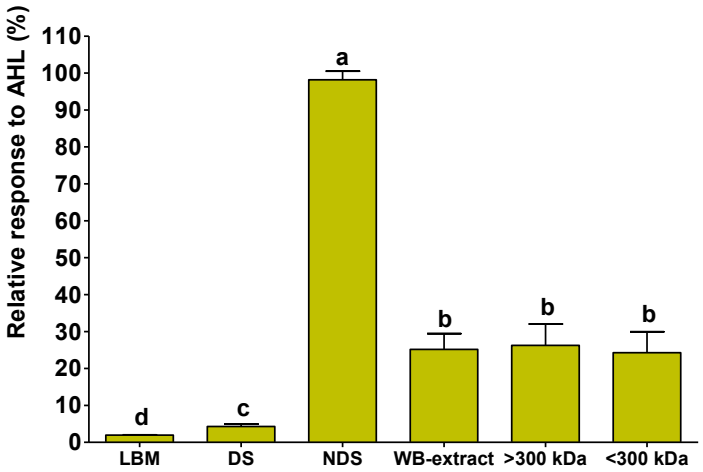

Figure 7.4. Relative response to the natural acyl-homoserine lactone (AHL) present in the positive control sample (%). The Relative Fluorescence Units (RFU) of samples were measured after the incubation of 900 µl of each sample with 100 µl of AHL for 24h at 37ºC in the presence of a biosensor *Escherichia coli* strain after 6h of incubation. The values of samples were converted by giving the maximal percentage (100%) to the positive control sample. LBM: luria broth medium (without AHL added); DS: AHL degrader control strain (*Pseudomonas fluorescens* P3/pME6863); NDS: AHL non-degrader strain (*Pseudomonas fluorescens* P3/pME6000); Wheat-bran extract equivalent to a 1:100 (w/v) suspension; >300-kDa fraction; <300-kDa fraction. Bars represent standard error of the mean. Treatments with unlike letters were significantly different ($P < 0.05$). Results were obtained from the average of three replicates in three independent assays.

7.5. DISCUSSION

To the best of our knowledge, this is the first report demonstrating the anti-biofilm activity of WB soluble extract. Wheat is, by far, one of the most important cereals produced worldwide (FAO. 2013), and, as a consequence, WB is an easy by-product to find worldwide in contrast to other natural products that are not easily available (Quave et al., 2008). Wheat bran is rich in carbohydrates (60%), protein (12%), fat (0.5%), minerals (2%) and several bioactive compounds and vitamins (Mohsin-Javed et al., 2012). Even though in this study the active compound responsible of the anti-biofilm activity was not elucidated, it was demonstrated that the putative compound are mostly retained in the >300-kDa fraction. Moreover, the heating of this fraction eliminated the activity, suggesting that a protein could be involved (data not shown). Previous studies of our group, studying the possible interference of WB soluble extract in the *E. coli* adhesion to IPEC-J2 cells, have shown how this fraction is rich in protein compounds, and also that it was the only fraction able to interfere with bacterial adhesion (González-Ortiz et al., 2013; In preparation). In that study, several low MW proteins in this >300-kDa fraction were identified, some of them belonging to seed-storage proteins, others to

protease inhibitors (PI) and the beta-amylase enzyme. Some of these proteins, or others, can also be held responsible for the anti-biofilm effects described in this study. The role of proteases of *S. aureus* in biofilm formation and dispersal is undoubtedly important and complex. Over 10 proteases have been described to be secreted by most *S. aureus* strains (Dubin. 2002), and some of them have been suggested to have a role in biofilm remodelling (Mootz et al., 2013). In this regard, an inhibition of some of these proteases could impair *S. aureus* to fully develop a functional biofilm. Previous works have reported the possible involvement of PI in the inhibition of biofilms. It has been described how a rice protein extract containing PI against cysteine proteases known as gingipains can suppress the growth and biofilm formation of *Porphyromonas gingivalis*, one of the most important periodontal pathogens (Taiyoji et al., 2013). However, whereas proteases can be an endogenous tool of *S. aureus* to remodel its own biofilm structure, they can also become a way to destroy its biofilm. Actually, recent strategies for controlling biofilm growth are based on degradation of the matrix using enzymatic treatments (Kiedrowski and Horswill, 2011). Some externally added proteases could even have a role promoting an overexpression of endogenous proteases in *S. aureus* (Park et al., 2012). The wheat grain is rich in proteases (Kaminski and Bushuk, 1969), and particularly different cysteine proteases have been well described (Fahmy et al., 2004; Yang et al., 2011). It would therefore be plausible that endogenous proteases in WB could be related to the inhibition and destruction of biofilm.

On the other hand, efforts have been underway to devise more creative approaches for biofilm treatments or other bacterial virulence factors by targeting QS systems (Otto. 2004). The potential of plant extracts to interfere with QS of bacteria is not new and it has been previously described by other authors (Musthafa et al., 2010; Nazzaro et al., 2013). Common examples such as the algae *Delisea pulchra* (Hentzer et al., 2002), cranberry extract (LaPlante et al., 2012) and vanilla extract (Choo et al., 2006) have proved quorum-quenching (QQ) activity, thus resulting in anti-biofilm properties. Furthermore, the gallic acid present in tea leaves, oak bark and grape seeds has demonstrated an anti-biofilm capacity against a wide range of different pathogens, both Gram-positive and Gram-negative, by interfering in the QS system (Nazzaro et al., 2013). Therefore, the purpose of the present study is to study the lactonase activity of the WB soluble extract and the fractions obtained by MW. It is really interesting to see that the soluble extract was able to decrease AHL activity. AHL is one of the most studied QS signals in the last recent years (Boyen et al., 2009). The synthesis of AHL by Gram-negative bacteria is dependent on the LuxI/LuxR-type QS. The LuxI-like enzymes produce a specific

AHL which is freely diffuse across the cell membrane and increases in concentration in proportion to cell density. The transcriptional regulator, a LuxR-like receptor protein, binds to the diffusing AHL, thus activating transcription of target genes (Miller and Bassler, 2001). Many virulence genes have been described to be regulated by AHL (Winzer and Williams, 2001), and between them also biofilm formation (Ali and Benitez, 2009).

A number of structural AHL analogs, but also autoinducing peptides analogs and other small molecules, have been shown to inhibit the expression of QS-controlled genes and biofilm formation (Schuster et al., 2013). Some plant extracts can act as QQ due to the similarity of their chemical structure to those of QS signals and/or their ability to degrade signal receptors (Nazzaro et al., 2013). In our study, lactanase activity was equally found in the >300-kDa and <300-kDa fractions, suggesting that the putative active compounds related to this activity are in both fractions equally.

7.6. CONCLUSION

In conclusion, the present study demonstrates new beneficial effects related to WB. The anti-biofilm ability of WB extract against a cow mastitis strain of *S. aureus* may be related to the presence of defence compounds in the plant that could inhibit different bacterial enzymatic processes or catalyse synthesized biofilm components. Moreover, some compound in WB seems able to interfere with AHL activity, opening the possibility of a new anti-pathogenic effect through interference with the QS of bacteria. Further studies should be performed to validate these results and to realize the application of WB to control biofilm infections

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8. GENERAL DISCUSSION

The aim of this Thesis was to investigate whether it is possible to reduce bacterial attachment, in particular enterotoxigenic *Escherichia coli* (ETEC) K88 through natural feed ingredients (FI). Also, it was afforded extra beneficial properties which have never been previously published about wheat bran (WB): the anti-biofilm activity against a cow mastitis strain of *Staphylococcus aureus* and the interference with bacterial quòrum-sensing (QS) systems.

The ability of natural FI to interfere with bacterial adhesion was studied using different *in vitro* model systems, such as a model to adhesion to the natural ileal porcine mucus and to intestinal porcine epithelial cells (IPEC-J2). The screening allowed identifying WB as one of the most promising candidates with anti-adhesive properties against ETEC K88. For that reason, WB was fractionated with the ultimate goal of understanding the molecules involved in the specific recognition to ETEC K88.

8.1. NEW APPROACHES TO COMBAT BACTERIAL ADHESION: LOOKING FOR NATURAL COMPOUNDS

8.1.1. DISRUPTING BACTERIAL ADHESION

Blocking or inhibiting the attachment of bacterial lectins by suitable carbohydrates or their analogs for the prevention and treatment of microbial diseases (Sharon and Lis, 1989) is the aim of anti-adhesion therapies (Shoaf-Sweeney and Hutkins, 2009). There are numerous reports describing anti-adherence activities from natural sources, such as milk, plant compounds, and other foods (Signoretto et al., 2012). Most of these natural sources are safer than antibiotics because they may not induce bacterial resistance, are not toxigenic and may be are cheaper than synthetic compounds.

For these reasons it was performed two *in vitro* independent screenings where it was investigated the ability of different natural products to interfere with ETEC K88 binding to the natural ileal porcine mucus (Chapter 4; (Gonzalez-Ortiz et al., 2013a) and to intestinal porcine epithelial cells (IPEC-J2) (Chapter 5; (Gonzalez-Ortiz et al., 2013b). The natural products were carefully selected by its likely anti-adhesive properties against pathogenic bacteria. Among the FI evaluated, casein glycomacropeptide (CGMP), exopolysaccharides (EPS), locust bean (LB) and WB exhibited the highest blocking activity.

8.1.1.1. CASEIN GLYCOMACROPEPTIDE

The ability of CGMP to block the adhesion of ETEC K88 to the ileal mucus and to IPEC-J2 showed a lineal dose-response in the range of 0.01-1%. The CGMP is a glycoprotein originating from the C-terminal portion of κ-casein during cheese manufacture. After the enzymatic treatment of milk, CGMP (containing 106-169 aminoacid residues) is removed and is the most abundant protein in whey (20 – 25%). There are evidences that CGMP binds to *E. coli* toxins, promotes bifidobacterial growth, modulates the immune system response and inhibits viral and bacterial adhesion (Brody. 2000). The anti-adhesive properties of CGMP against *E. coli* is related with the presence of glycoprotein structures, which may act as potential receptor analogs (Grange et al., 2002; Rhoades et al., 2005). Our research group has recently led some investigations regarding the anti-adhesive properties of CGMP to ETEC K88 (Hermes et al., 2013). Casein glycomacropeptide was able to reduce *in vitro* the attachment of ETEC K88 to porcine intestinal epithelial cells (IPEC-J2) (Hermes et al., 2011) and also to reduce *in vivo* diarrheic incidence and *E. coli* attachment to intestinal villi in weaned piglets challenged with ETEC K88 (Hermes et al., 2013). In the same study, the recognition of CGMP by the fimbria F4 was also confirmed in dot-blot assay, which agrees with *in vivo* evidences. In this sense, searching for new theranostic systems, the specific recognition of CGMP to ETEC K88, instead of to the non-fimbriated *E. coli*, was seen by using chronoamperometric measurements (Espinoza-Castañeda et al., 2013), thus confirming the specificity of CGMP for ETEC K88.

The application of CGMP has also been proposed to alleviate the inflammatory response in patients with inflammatory bowel diseases (IBD) (Requena. 2009). Even though the IBD are still of an unknown etiology, a huge list of microorganisms including the commensal intestinal microbiota have been involved in the pathogenesis of IBD (Fiocchi. 2005). During inflammation the incidence of bacterial adherence to the ileal wall increased in a model of indomethacin-induced ileitis in rats (Terán-Ventura. 2009). Therefore, given the anti-adhesive properties of CGMP together with the modulation of the immune response and the prebiotic activities, it seems a good candidate that encompasses different etiological factors in the disease (de Medina et al., 2010).

8.1.1.2. MICROBIAL EXOPOLYSACCHARIDES

Microbial exopolysaccharides are long chain polysaccharides consisting mainly of branched repeating units of sugars, mainly glucose, galactose and rhamnose, in different ratios (Welman and Maddox, 2003). They are exocellular molecules excreted during bacterial growth. The product tested

in this Thesis was obtained from the natural fermentation process of green olives and generously donated by Dr. Jimenez-Diaz (Instituto de la Grasa, Consejo Superior de Investigaciones Científicas, Sevilla; Spain) in which *Lactobacillus pentosus* and yeasts have had a relevant role (Garrido-Fernández et al., 1997; Arroyo-López et al., 2008). Among all the FI tested, EPS showed actually promising results. Even though EPS was not able to promote significant reduction of the adhesion of ETEC K88 to the ileal mucus (Gonzalez-Ortiz et al., 2013a), it demonstrated the ability to recognize specifically ETEC K88 instead of the non-fimbriated *E. coli* in the same study. It seems as if the adhesion of fimbria F4 to EPS would not be enough to interfere with the blocking process in the mucus. If EPS could attach not only to fimbria F4 but also to other molecular structures in the mucus, EPS could even favor the attachment of the bacteria. In this regard, the potential of different EPS to adhere to probiotics or enteropathogens to the mucus have been previously described (Ruas-Madiedo et al., 2006a; Ruas-Madiedo et al., 2006b). The lack of ability, as had been found in the first screening, to reduce the attachment to the mucus does not fully exclude the potential of EPS to interfere with the adhesion of bacteria to the epithelial cells. Nevertheless, adhesion to the mucus can be considered as a way to maintain bacteria far away from the epithelium borderline (Variyam. 2007). Results from (Gonzalez-Ortiz et al., 2013b), showed that EPS was the most efficient at reducing adhesion of the ETEC K88 strain to IPEC-J2 cell-line among all feed ingredients tested. Other authors have also described how EPS can reduce adhesion of *E. coli* to Caco-2 cells (Alp et al., 2010) and to porcine erythrocytes (Wang et al., 2010). To our knowledge these are the first results published about the ability of EPS to prevent specifically ETEC K88 adhesion to porcine epithelial cells. These findings could have consequences with a positive economic impact in the Spanish agriculture and livestock area. Spain is the worldwide leader in olive production accounting the 36% of the total production (FAO. 2011). The 90% of the total olive production is intended for oil production and the 10% is for table olives production (ASEMESA. 2013). Spain is also in the top of worldwide producers of table olives corresponding to the 22% of the global production, followed by Egypt (17%), Turkey (16%) and Syria (6%). Therefore, it could be plausible to propose rescuing the EPS produced by several microorganisms involved during the fermentation of table olives to revalue a product that by the moment has none interest. However, further studies must be performed to confirm results including *in vivo* trials to evaluate until what extent EPS coming from the fermentation of green olives possess anti-adhesive properties against pathogenic bacteria in animals and likely humans.

8.1.1.3. LOCUST BEAN

The LB used in the present study comprised the milled mixture of carob pods and part of their seeds. The carob pod or "locust bean" is the fruit of carob tree (*Ceratonia siliqua*) and is commonly used as non-conventional feed for ruminant feeding (Vasta et al., 2008). Its seed is highly rich in galactomannans, a neutral polysaccharide consisting of a β-(1→4)-mannan backbone to which single D-galactopyranosyl residues are attached via α-(1→6) linkages. The locust pod is especially rich in insoluble dietary fibre and diverse polyphenolic compounds (83 mg/kg) (Papagiannopoulos et al., 2004).

Regarding galactomannans, they have been demonstrated to inhibit fimbrial adhesins of *E. coli* and *Salmonella* (Swanson et al., 2002). Previous studies using plant-rich products have demonstrated anti-adhesive properties against *P. aeruginosa* (Zinger-Yosovich and Gilboa-Garber, 2009) and *Salmonella* (Badia et al., 2012b). Previous studies of our group using a micronized and partially dehulled carob seed also demonstrated its efficacy in the context of an ETEC K88 challenge model (Guerra et al., 2013). In that study, a significant reduction of more than 2 log CFU in the number of total enterobacteria and *E. coli* adhered to ileum and a decrease in diarrhoea incidence was reported.

Other *in vitro* studies of our group using the same locut bean product tested here also demonstrated its ability to reduce the number of ETEC K88 to IPEC-J2 cells (Hermes et al., 2011). In the same study, locust bean was able to reduce the relative expression of cytokines and chemokines in cells challenged with the bacteria. Considering the heterogeneous composition of the LB, the functional activities found in this Thesis could have been due to its content in galactomannans but also other components like polyphenolic compounds. Phenolic compounds have been reported to be involved in the blockage of bacterial adhesion (Huttunen et al., 2011; Kurek et al., 2011; Riihinen et al., 2011) and specifically in the recognition of ETEC K88 (Wittschier et al., 2007). Thus, it could be hypothesised that the anti-adhesive response given by LB could have been mediated by the galactomannans but also by phenolic compounds able to interfere in the recognition of the type1 fimbriated *E. coli*.

Finally, because the WB soluble extract yielded positive results in all the *in vitro* assays and has starred in more than half of the Thesis, deserves a more detailed discussion.

8.2. THE INTERFERENCE OF WHEAT BRAN TO PREVENT BACTERIAL ADHESION

8.2.1. THE NUTRITIONAL VALUE OF WHEAT

Wheat is by far the most important cereal produced worldwide (FAO. 2013). This year is expected to produce around 704.6 million of tones, an average change of +7.7% over the last year. Wheat grain is mainly used as food ingredient in human nutrition provided as bread, but also in diets for pigs, wheat is primarily used as an important energy component due to its high starch content (Lin et al., 1987; Sauer et al., 2012).

The carbohydrate fraction of wheat includes low molecular weight sugars, starch and various cell wall and storage non-starch polysaccharides (NSP). Major cell wall polysaccharides contain pentoses such as arabinose and xylose and hexoses including glucose, galactose and mannose. Among NSP, arabinoxylans represent the main cell wall polysaccharides of the starchy endosperm. Arabinoxylans are linear polymers consisting of D-xylose joined with β-(1-4) linkages and residues of arabinose. As arabinoxylans represent the major NSP fraction in wheat, the contents of arabinose and xylose follow the total NSP content. It is well accepted that NSP have a major role, both on intestinal microbiota composition and the complex processes of digestion and absorption in the digestive tract of pigs (Rosenfelder et al., 2013). They stimulate the secretory output from salivary glands, stomach, liver, pancreas and intestinal walls, and increase the viscosity of the digesta by aggregating into large networks or mesh-like structures as a result of entanglement of many very large polymers.

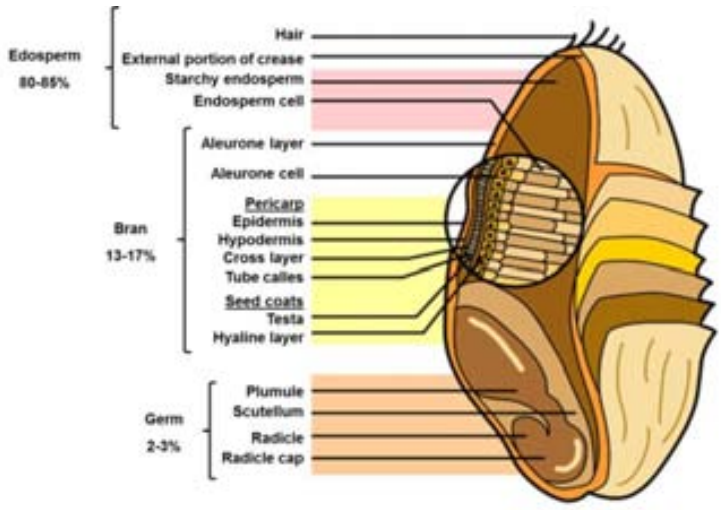

Figure 8.1. Histological composition of wheat grain. Percentage of each area corresponding to grain.

The crude protein (CP) content of wheat is rather low compared to protein ingredients such as soybean meal, but due to its high dietary inclusion level in the diet, wheat delivers significant amounts of indispensable amino acids to the pig. The soluble proteins are concentrated in the seed coats, the aleurone cells and the germ of wheat. They account for approximately 25% of the total protein grains (Belderok et al., 2000).

8.2.2. WHEAT BRAN CHARACTERISTICS

Several by-products of wheat processing such as WB, wheat middlings and wheat distillers grains with solubles are being used in livestock feeding (Rosenfelder et al., 2013). By-products of wheat milling are of considerable economic significance as they represent 25% of the original grain (Hemery et al., 2007). They can reduce feed costs considerably but vary in nutritional value which, in turn, may limit the efficient use of wheat by-products both in poultry and pig nutrition (Slominski et al., 2004) (see the wide range values in **Table 8.1**).

In particular, WB is frequently used as ingredient in diets for gestating pigs (Loisel et al., 2013). It is composed of the pericarp and the outermost tissues of the seed, including the aleurone layer with variable amounts of remaining starchy endosperm (**Figure 8.1**). Compared to wheat, WB has higher amounts of CP, in particular due to the removal of starch during flour milling (**Table 8.1**). However, the high NSP content of WB may lead to a reduced digestibility of aminoacids and energy in monogastric animals (de Blas et al., 2010). In this sense, the high level of insoluble NSP in WB is known to be highly resistant to degradation in the GIT (Noblet and Le Goff, 2001).

8.2.3. THE INTERFERENCE OF WHEAT BRAN TO REDUCE ETEC K88 BINDING

Previous investigations of our group showed a reduction of *E. coli* population in the ileum digesta and more interestingly a reduction of ETEC K88 attached to the ileum mucosa when piglets received WB in their diet (Molist et al., 2009). These results also agree with other findings (Hermes et al., 2009; Molist et al., 2011) reporting that WB in the diet reduce the ubiquitous *E. coli* in the intestinal tract. The positive results found in several *in vivo* trials regarding the anti-adhesive activity of WB encouraged us to go deeper in the study of the molecular interaction between WB and ETEC K88. In the first *in vitro* assays (Gonzalez-Ortiz et al., 2013a), it was demonstrated that WB was able to bind ETEC K88 and block its attachment to intestinal mucus. Later, it was showed that WB might reduce

the ETEC K88 attachment to IPEC-J2 in a linear dose-response manner (Gonzalez-Ortiz et al., 2013b).

Even though the arabinoxylooligosaccharides of WB were initially considered as responsible for the anti-adhesive properties against enteropathogens (Eeckhaut et al., 2008), the present study has demonstrated the involvement of a proteinaceous compound inside a multicomponent protein complex above 300 kDa, which specifically recognizes ETEC K88. The investigations encompassed in Chapter 6 revealed the presence of different protease inhibidors (PI) and Globulin 3 from WB extract which might be involved in the binding to ETEC K88. It is known that many PI in plants are part of their natural defence mechanism against invading bacteria (Franco et al., 2002). On the other hand, Globulin 3 is a storage protein 7S globulin of wheat (Guo et al., 2012). Although few studies have characterized this kind of proteins, Teodorowicz et al. (2013b) have recently demonstrated the modulation of gut microbiota proliferation, survival and adhesion by peanut 7S globulin. The authors suggested that this protein may have anti-adhesive properties against some bacterial strains. To our knowledge this is the first study which gives insight into the possible roles of several PI and Globulin 3 of WB preventing ETEC K88 to IPEC-J2. Although these are very promising results, additional investigations should be performed to gain understanding of the molecular recognition of the blocking process that takes place or other reported effects against colibacillosis *in vivo*.

Another mechanism that could explain the interference of WB in the attachment of ETEC K88 to the intestine is by a possible degradation of acyl-homoserine lactones (AHL), the typical QS signals excreted by Gram negative bacteria. Labbate et al. (2007) demonstrated the necessity of AHL presence for *Serratia marcescens* for surface colonization. The current thesis has demonstrated the ability of WB to reduce AHL concentrations maybe by hydrolysing it or by interfering with its functionality. This activity was found in both fraction, >300 kDa or <300 kDa, of the soluble extract from WB whereas the anti-adhesive activity was only found in the >300-kDa fraction of WB extract. This would demonstrate that the molecules implicated in both would be independent. It is not strange that natural sources would provide quòrum-quenching (QQ) agents since they might have co-evolved to prevent bacterial encroachment and infection or to outcompete microbial counterparts in the environment (Zhu and Kaufmann GF., 2013). In the recent years, the screening and evaluation of health promoting foods and other natural products for their QQ activities have gained increased

popularity (Nazzaro et al., 2013), and in this view plant extracts have demonstrated to have some roles (Musthafa et al., 2010).

Table 8.1. Comparison of wheat grain and wheat bran composition

	Wheat grain	Wheat bran
Proximate nutrients (g/kg DM)		
Dry matter	881.0	828-945
Crude ashes	19.0	34-65
Crude protein	122.0	151-221
Ether extract	22.0	37-65
Energy content (MJ/kg DM)		
Gross energy	18.1	18.6-19.4
Digestible energy	16.3	10.1-14.1
Metabolizable energy	15.5	10.1-13.3
Net energy	11.1	6.6
Amino acid content (g/kg DM)		
Indispensable aminoacid		
Arginie	5.8	5.5-16.0
Histidine	2.9	4.1-6.8
Isoleucine	4.2	4.9-7.7
Leucine	7.8	7.5-13.2
Lysine	3.4	5.8-9.9
Methionine	1.8	2.0-4.0
Phenylalanine	5.2	4.1-9.0
Threonine	3.4	4.4-8.3
Tryptophan	1.5	2.4-3.3
Valine	5.2	6.7-9.7
Dispensable aminoacid		
Alanine	4.3	7.7-12.1
Aspartic acid	6.1	11.1-16.7
Cystine	2.8	2.9-3.7
Glutamic acid	33.3	14.5-37.3
Glycine	4.9	8.4-10.3
Proline	11.7	-
Serine	5.7	6.7-9.4
Tyrosine	2.9	2.6-5.3
Carbohydrate content (g/kg DM)		
Starch	663.0	139-343
Arabinose	25.0	88-98
Xylose	40.0	158-188
Mannose	3.0	-
Galactose	7.0	7
Glucose	11.0	4-30
Neutral detergent fiber	140.0	278-482
Acid detergent fiber	35.0	84-150
Acid detergent lignin	9.0	29-114
Non-starch polysaccharides	108.0	183-416

Winter wheat data were only considered. The WB composition range declared. From Rosenfelder et al., (2013)

8.2.4. IMPLICATIONS OF WHEAT BRAN IN PIGLET NUTRITION TO PREVENT COLIBACILLOSIS

Previous studies of our group demonstrated that the addition of WB at 4% in the diet of piglets decreased the *E. coli* and coliform counts in faeces (Hermes et al., 2009; Molist et al., 2011).

Moreover, WB supplementation in piglets orally challenged with ETEC K88 showed significant reductions in the *E. coli* (- 2.2 log CFU[colony forming units]/g) and ETEC K88 (- 4 log CFU/g) counts in the ileal mucosa and digesta respectively, compared with control animals (Molist et al., 2009). Just to give some practical data, providing 40 g of wheat bran per kilogram of diet represent an equivalent of 0.56 g/kg of the active soluble extract. Considering the piglet's daily feed consumption in the first two weeks after weaning can range between 200 to 300 g (Molist et al., 2009), thus representing a total consumption of the anti-adhesive active compund of 0.11-0.17 gr/day. We have reported that 14 mg/ml of WB extract is able to significant reduce ETEC K88 adhesion to IPEC-J2 under our *in vitro* conditions. However, these estimations may not be far from the *in vivo* reality (Hermes et al., 2009; Molist et al., 2011), because if the 20% of this soluble extract reamins undigested, this would means about 22 mg are available in the intestine to exhert anti-adhesive activity. Despite of bacterial adhesion is highly complex and seems to be a multifactorial event, it is plausible to think that not only the anti-adhesive effect of a particular proteinaceous compounds inside a complex above than 300 kDa from WB is playing a role inside the pig's intestine. The inclusion of moderate amounts of insoluble fibre sources, especially in low-dietary fibre diets, may have a positive effect in the weaned piglet (Molist et al., 2013). These positive effects can also be associated with the modification of the physicochemical properties of digesta, an enhanced fermentation capacity, together with the physical effect of the fibre particles on the intestinal function and finally, leading to a different microbial profile thus reducing pathogenic adhesion to the intestinal mucosa, which benefit animal health.

8.3. BIOFILM CONTROL STRATEGIES AND THE RELEVANCE OF WHEAT BRAN TO COMBAT BIOFILM

Bacterial biofilms cause chronic infections that can persist despite the use of antibiotic treatments. Bacteria in biofilms are significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species (Bryers. 2012). Moreover, in some cases sub-lethal doses of antibiotics can even enhance biofilm formation suggesting a natural defence mechanism of bacteria in avoiding the lethal effects of antibiotics (Costa et al., 2012). Due to these problems, in veterinary practice as well as in human medicine, alternatives to antibiotic therapies are increasingly required. The screening and evaluation of health promoting foods and other natural

products for their anti-biofilm activities have gained increasing popularity (Quave et al., 2008; Sandasi et al., 2010; LaPlante et al., 2012; Nazzaro et al., 2013).

In the present Thesis we observed that WB extract had anti-biofilm activity against a cow mastitis strain of *S. aureus a* well known strain in veterinary medicine which is characterized to produce biofilm-related infections. This strain would serve us as an excellent *in vitro* model to study the anti-biofilm activity of WB-soluble extract (Chapter 7).

It was observed that WB-soluble extract at 0.5%, and that fraction above 300 kDa had anti-biofilm capacity, inhibiting the biofilm formation and also destroying the biofilm previously formed. Even though we were not able to identify the precise mechanism of such anti-biofilm activity, it seems to be related with the presence of defence compounds in the plant extract able to interfere with the synthesis of microbial biofilms.

Mastitis is one of the most important diseases in dairy cattle being on the top among diseases related with biofilm production (Melchior et al., 2006b). The general therapeutic approach towards mastitis is to use antibiotics to combat underlying infection. The present findings could be considered by the pharmaceutical industry to include WB extract as co-adjuvant with an antibiotic which together would gain effectiveness: the natural product preventing or destroying biofilm formation and the antibiotic killing the planktonic bacteria after dispersion of the biofilm.

Looking for alternatives to antibiotics has given rise to research natural products. Sometimes the promising properties of natural products are restricted under *in vitro* conditions because are not easily found in nature anywhere in the world. Here we have demonstrated the anti-adhesive properties of four natural FI: CGMP, EPS, LB and WB that could turn them on a viable alternative to be used in the feed industry. In particular, and by the previous background of our research group, we have attempted going further in the knowledge and understanding of the functionality of WB blocking the adhesion of the bacteria to the intestine. Moreover, in this Thesis we have found two additional properties for WB extract: anti-biofilm and quorum quenching. The recently proposed properties may open new ways to formulate piglet's diets to prevent colibacillosis, as well as to prevent or treat biofilm-related diseases, such as mastitis in dairy cows.

9. CONCLUSIONS

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

1) The soluble extract of the following feed ingredients: casein glycomacropeptide (CGMP), locust bean (LB) and wheat bran (WB) at 1% exhibit anti-adhesive activity against enterotoxigenic *Escherichia coli* K88, when they were coincubated with porcine intestinal mucus (-1.40, -1.23 and -1.26 log CFU, respectively) and porcine intestinal epithelial cells (IPEC-J2) (-0.84, -0.696 and -0.54 log CFU, respectively).

2) The exopolysaccharides obtained from table olives fermentation (EPS) at 1% is the most efficient tested ingredient reducing the adhesion of enterotoxigenic *Escherichia coli* K88 to the porcine intestinal epithelial cells (IPEC-J2), when compared to PBS (-0.71 log CFU) even considering it is not able to promote significant reductions on adhesion to the intestinal mucus.

3) Fractionation of the soluble extract of WB by size-exclusion chromatography revealed that adhesion and blocking activity is retained in the >300-kDa fraction. Electrophoresis showed several proteins below 90 kDa in this >300-kDa fraction suggesting that the target protein which recognises enterotoxigenic *Escherichia coli* K88 belongs to a high molecular weight multicomponent protein complex. The identification of some relevant bands in this fraction by mass spectrometry revealed the presence of various protease inhibitors that could be implicated in the reported blocking effects.

4) Isolation of proteins able to bind specifically enterotoxigenic *Escherichia coli* K88 allow to identify Globulin 3 as another possible responsible molecule involved in the interference of the bacteria binding to the ileal mucus and porcine intestinal epithelial cells (IPEC-J2).

5) The soluble extract of WB at 0.5% has anti-biofilm activity against a cow mastitis strain of *Staphylococcus aureus*. The putative compound of this activity was mostly retained in the >300-kDa fraction.

6) The incubation of the soluble extract of WB at 1% or its >300-kDa or <300-kDa fractions with acyl-homoserine lactone (naturally produced by *Yersinia enterocolitica*) reduce its concentration in the medium as reveal the biosensor strain. This suggests that WB extract contains certain lactanase activity that could interfere with the quorum sensing of Gram-negative bacteria.

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- ✓ Technical research support (Department of Animal Science and Food Research (UAB). From 2007-until present
- ✓ Technical forthightly writer (Department of Animal Science and Food Research (UAB). 2012-2013
- ✓ Training practices (Nestlé Petcare S.A - Castellbisbal, Barcelona, Spain). 2008

ATTENDING POST-GRADUATE COURSES

- ✓ Food & Health Entrepreneurship Program. Parc de Recerca UAB. 2013
- ✓ Management system of health and safety at the work position. Ingecal SL. 2013
- ✓ Dealing with extinguishant and fire extinctions. UAB. 2011
- ✓ Training researchers using experimental animals for experimental purposes. UAB. 2009
- ✓ Risk prevention at lab. UAB. 2008

SCIENTIFIC PUBLICATIONS

1. **González-Ortiz et al.,** NMR based metabonomic study of the liver, muscle and abdominal fat of chickens fed diets with different degrees of unsaturation. The Journal of Nutritional Biochemistry (In preparation)
2. **González-Ortiz et al.,** Discovering new properties of wheat bran: the anti-biofilm activity against *Staphylococcus aureus* and the interference with bacterial quorum sensing systems. Veterinary Microbiology (Submitted).
3. **González-Ortiz et al.,** Insights into the mechanisms of wheat bran interfering the attachment of enterotoxigenic *E. coli* K88 (F4+). Plos One (Submitted).
4. **González-Ortiz et al.,** Screening the ability of natural feed ingredients to interfere with the adherence of enterotoxigenic *Escherichia coli* (ETEC) K88 at the porcine mucosal intestine. British Journal of Nutrition (http://dx.doi.org/10.1017/S0007114513003024)
5. **González-Ortiz et al.,** Screening the ability of natural feed ingredients to reduce *Escherichia coli* K88 adhesion to the porcine intestinal epithelial cell line IPEC-J2. Veterinary Microbiology (http://dx.doi.org/10.1016/j.vetmic.2013.07.035)
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7. **González-Ortiz et al.,** Consumption of dietary n-3 fatty acids decreases fat deposition

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10. Hervera et al., Prediction of digestible protein content of dry extruded dog food: comparison of methods. Journal of Animal Physiology and Animal Nutrition (2009), 93: 366-372.

CHAPTER BOOK

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CONTRIBUTION IN RESEARCH, DEVELOPMENT AND INNOVATION PROJECTS (Nationals & Internationals)

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2. **Project title**: Zn AND SUBSTRATES BLOCKING INTESTINAL MICROBIAL ADHESION IN THE NUTRITION OF POST-WEANED PIGLETS
3. **Project title**: ESTERIFIED ACID OILS UTILIZATION IN MONOGASTRIC ANIMALS FEED. COMPARATIVE NUTRITION AND IMPACT ON MEAT LIPID QUALITY
4. **Project title**: MODIFICATION OF THE FAT QUANTITY AND ITS DISTRIBUTION IN BROILERS AND PIGS BY NUTRITION STRATEGIES
5. **Project title**: *Bacillus amyloliquefaciens* (CECT 5940) AND *Enterococcus faecium* (CECT 4515) INCLUSION IN DOG'S FED
6. **Project title**: THE EFFECTS OF A TRYPTOPHAN ENRICHED DIET AND PHYSICAL ACTIVITY ON BEHAVIOR AND BIOMARKERS IN DOGS
7. **Project title**: EFFECT OF DIETARY INCLUSION OF *Bacillus cereus* VAR. *Toyoi* (TOYOCERIN®) IN DOG'S
8. **Project title**: PREFERENCE, FITNESS AND TOLERANCE OF SNAKS FOR DOGS
9. **Project title**: DIGESTIBILITY TRIALS OF EXTRUDED DIETS AND FAECAL CONSISTENCY EVALUATION IN DOGS

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TEACHING COLLABORATION

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