Universitat Autònoma de Barcelona

Natural feeding strategies to reduce enteric disorders and improve adaptation of young pigs to weaning.

MEMÒRIA PRESENTADA PER: RAFAEL GUSTAVO HERMES

PER ACCEDIR AL GRAU DE DOCTOR DINS EL PROGRAMA DE DOCTORAT DE PRODUCCIÓ ANIMAL DEL DEPARTAMENT DE CIÈNCIA ANIMAL I DELS ALIMENTS

BELLATERRA, SEPTEMBRE DE 2011

FACULTAT DE VETERINÀRIA DE BARCELONA

Susana M. Martín Orúe i José Francisco Pérez Hernandez, investigadors del departament de Ciència Animal i del Aliments de la Facultat de Veterinària de la Universtitat Autònoma de Barcelona,

certifican:

Que la memoria titulada **"Natural feeding strategies to reduce enteric disorders and improve adaptation of young pigs to weaning"**, presentada per Rafael Gustavo Hermes per optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritzan la seva presentació per que sigui jutjada per la comissió corresponent.

I per que consit als efectes oportuns, signa la present a Bellaterra 11 de Juliol de 2011.

Dra. Susana M. Martín Orúe

Dr. José Francisco Pérez Hernandez

The author was in receipt of a grant from the Agència de Gestió dÀjuts Universitaris i de Recerca of the Generalitat de Catalunya for this study.

DEDICATED TO TISCIANE

“Love is the answer at least for most of the questions in my heart.

Why are we here? And where do we go? And how come it's so hard?

It's not always easy and sometimes life can be deceiving.

I'll tell you one thing, it's always better when we're together.”

Jack Johnson, Better together

ACKNOLEDGEMENTS

Me gustaría agradecer a todos que hicieran parte de la realización de este gran sueño que ha empezado desde hace 5 años cuando llegué a Barcelona.

Agradezco a todos los compañeros y amigos que he tenido el placer de conocer, compartiendo intensamente todos los momentos.

Me gustaría de dejar aquí mi gratitud a todos los profesores que con toda la dedicación y cariño me han enseñado todo lo que he necesitado desde mis primeros enseñamientos hasta la conclusión de esta tesis. Vuestro ejemplo de comprometimiento, humildad y pasión por lo que hacen seguramente estará para siempre junto conmigo. En especial, agradezco a Susana y Francisco por ser mis verdaderos mentores, por la incesante disposición en ayudar en los momentos difíciles, por la orientación cuando todo parecía confuso y por los lazos de amistad que me ha dado mucha ilusión por seguir luchando para tirar adelante. Agradezco también a los profesores Josep, Ana Cris, Mariola, Roser, Alfred y Sergio.

Un párrafo especial de agradecimiento lo dedico a Olga. Gracias por la paciencia conmigo (o falta, a veces) por repetir muchas y muchas veces hasta aprender todo lo necesario sobre el complejo funcionamiento del laboratorio de nutrición y sobretodo de sus equipos. Por las "ultimas" veces que he entrado en el laboratorio y todas las charlas sobre el cine, viajes y cosas de la vida. Me acuerdo que una vez me dijiste que lo triste de tu trabajo era saber decir adiós a los llegan y rápidamente se van de la Uni. Pues si te sirve de consuelo, que sepas que te queremos mucho y que todos hemos aprendido mucho contigo.

Ahora toca los agradecimientos a todos los compañeros, becarios y sobretodo amigos (ojala no me deje a nadie) que hemos tenido oportunidad de trabajar, estudiar, ir al SAF o compartir una comida. Agradezco primero a los más "antiguos": Muzzafer, Juanca, Hugo, Antonio, Alba, Choque,

Walquiria, Gabriele, Marta, André, Eva Creues, Eva Mainau, Alejandra, Diego, Paul, Feliu, Bladmir, Joseane y Leo. Y luego a los más "recientes": Ramonet, Victor, Ester, Lorena, Cecilia, Yuliaxis, Sandra, Sergio. En especial a mis últimos compañeros de despacho: Rogerinho y Jaimito; a Rosa y Gemma por la incontestable disposición en ayudar a todos y los lazos de amistad, a David por los consejos y todo lo que me ha enseñado y a Alexei, el "fichaje estrella" directo de Cuba y que mucho nos ha alegrado.

Obrigado aos brasileiros da UEL que tive o prazer de conviver graças ao professor Caio, como a Sabrina, a Mara e a Luiza, e o Pierão e o Maurera. Foram bons momentos que passamos, onde muitas vezes a vontade de comer algo que lembrava o Brasil, a saudade de casa e de falar português e foram supridas. Espero não perdermos o contato quando todos estiverem no Brasil.

Obrigado também a empresa Duas Rodas Industrial Ltda, que me apoiou no inicio desta conquista, seja como abrindo as possibilidades de ir a Europa, como me apoiando como consultor. Em especial, agradeço ao meu ex-chefe Roberto Alvarez Pereira; ao grande companheiro Péricles e tantos outros que tive o prazer de trabalhar no início de minha carreira profissional como a Dorotéia, Tati, Mario, Silvana, entre outros...

Otro párrafo en especial para aquellos que más han dedicado tiempo para compartir conmigo todo lo saben. Para que sepan que os admiro mucho y soy muy grato a vosotros. A Francesc, gracias por todo lo que me permitiste enseñar en el trabajo y en la vida catalana; a Edgar por abrir todas las puertas, el interminable conocimiento de la ciencia y por su dedicación a lo que haces, a Montse Anguita, por tu amistad, confianza y su modo tranquilo de hacer las cosas; y a Arantza por todo lo que eres y lo que ha dedicado a enseñar a los veterinarios manazas del laboratorio.

I would like to thanks Dr. Klasing (Kirk as he prefers) for the opportunity to learn and experience the Californian way to do the things. Your simplicity to live and kindly behavior inspired me to focus in what is really important and essential to move forward. Special thanks for Vanessa, Cailin and Laura.

Thanks to Dr. Alexander Swidisinski for the FISH training in your lab in Berlin and to professor Paolo Bosi (and your team: Trevisi, Davide and Stefano) for the genotype test training in Reggio Emilia, Italy.

Agradeço a todos meus amigos do Brasil que fazem parte do que sou. Obrigado pelo apoio e por nunca se esquecerem de mim.

À minha família (Antonio, Neide e Renan) pelo amor incondicional e pela superação alcançada. Hoje somos mais fortes que nunca, pois soubemos aprender com os erros. Também aos meus sogros (Murilo e Lisiane) pelo carinho e apoio e todos os bons momentos que passamos nesta idas e vindas. E aos meus tios Helder e Zélia, primos Elian e Felipe por estarem sempre presentes.

Um agradecimento especial a minha futura esposa Tisciane. Foram muitas e muitas horas de Skype nestes 5 anos de distância, por isso te agradeço por todo teu amor, por acreditar em mim e por me dar forças quando mais precisei. Emociono-me ao lembrar-se da saudade, mas certamente hoje é o sentimento que mais nos une e nos faz saber que tudo valeu à pena!

Finalmente me gustaría agradecer a las dos becas que he disfrutado y que me han dado el soporte financiero indispensable para llegar hasta aquí. La del programa europeo Alban (código de becário: E06M100404BR) y la del Ministerio de Cultura y Educación de la Generalitat de Catalunya, beca de formación de investigadores (FI-RH050417). También a los proyectos públicos de investigación financiados por el Ministerio de Educación de España (AGL2005-07438-C02-01/GAN 2004 - 2007; AGL2007-60851/GAN 2007-2010; and AGL2009-07328/GAN 2009 – 2012), que he hecho parte y que posibilitaron la realización de los experimentos de esta tesis.

RESUMEN

El objetivo de la presente memoria de esta tesis doctoral fue evaluar el papel de diferentes ingredientes en la dieta para reducir las patologías digestivas en los lechones tras el destete, incrementando así su bienestar y productividad.

Para alcanzar estos objetivos, propusimos una serie de 5 experimentos (capítulos de 4 a 8). En el **Experimento I** estudiamos la interacción entre dos diferentes niveles de proteína y fibra en el rendimiento productivo y el estado de salud de los lechones. Para ello, trabajamos con noventa y seis animales de 35 d de edad, colocados en 32 corrales de 3 animales cada uno y asignados a cuatro tratamientos dietéticos durante 21 d. Las cuatro dietas (a base de arroz, productos lácteos y harina de soja) fueron ordenadas en un diseño factorial 2 × 2, con dos niveles de CP (15,4 vs 19,4%, con base en la materia fresca) y dos niveles de fibra [baja en fibra (LF) 5,3% FDN y alto contenido de fibra (FC) NDF 7,15%, con base en la materia fresca]. La dieta HF fue alcanzada a través de la complementación de la dieta basal con 40 g / kg de salvado de trigo y 20 g / kg de pulpa de remolacha azucarera. El rendimiento de los animales se obtuvo semanalmente. Muestras de heces fueron recogidas para análisis microbiológico en el primer y último el día experimental y luego puntuadas de acuerdo con su consistencia, desde un valor de 1 (líquida) a 4 (dura). En el último día, se tomaron muestras para los análisis de sangre de la proteína de fase aguda (Pig-Map) de un cerdo de cada corral, posteriormente sacrificados para registrar el peso del tracto digestivo (incluyendo el contenido) y la histología del colon. Los cerdos alimentados con la dieta HF presentaron una ganancia diaria de peso (ADG, 390 vs 457 g, $P \leq 0,001$) y peso relativo del intestino grueso (4,4 vs 5,4% del peso corporal; $P \leq 0,05$) mayor que los cerdos LF. Esto coincidió con una mayor ($P \leq 0,05$) concentración de ácidos grasos de cadena corta (especialmente de ácido acético y ácido butírico), una disminución en el recuento de *Escherichia coli* (7,77 vs 6,86 log de UFC / g de heces,

$P \leq 0,05$), y un aumento en la proporción de lactobacilos: enterobacterias (0,76 vs 1,37, $P \leq 0,05$) con la suplementación de fibra. El aporte de niveles diferentes de CP no modificó los resultados productivos. Si bien, el 20% de CP aumentó $P \leq 0,05$) el peso relativo (% del peso corporal) de intestino delgado (6,5 frente a 7,7) y grueso (3,8 frente a 4,3). En el intestino grueso, el 20% de CP en la dieta aumentó el número de células caliciformes (4,6 vs 5.4/100 µm; $P \leq 0,05$) y redujo el número de linfocitos intraepiteliales (1,8 vs 1.3/100 µm; $P \leq 0,05$). En relación con el estado de salud, los efectos del nivel de fibra fueron dependientes del contenido de proteína bruta en la dieta. La suplementación con DF en la dieta con 16% CP redujo la puntuación fecal y aumentó la intervención con antibióticos, mientras que con un 20%CP fueron necesarias menos intervenciones con antibióticos ($P \leq 0,05$). Los lechones alimentados con las dietas de 20% de CP presentaron un descenso en la concentración de Pig Map comparados con las dietas de 16% de CP. En su conjunto, CP mostró efectos importantes en el peso gastrointestinal e integridad de la barrera intestinal, mientras que el DF aumentó el rendimiento productivo y promovió cambios importantes en la colonización microbiana y las variables de la fermentación.

En el **Experimento II**, trabajamos con un total de 144 lechones recién destetados (7,6 ± 1,7 kg) distribuidos en dos tratamientos dietéticos en base a un (60%) de arroz (R) o cebada (B), con 18 réplicas de 4 animales por tratamiento. En el día 14 después del destete, 96 animales (48 de cada dieta) se reasignaron a 32 corrales, respetando la dieta del mismo cereal (R o B) que habían recibido previamente. A partir de entonces pasaron a recibir 4 dietas experimentales con la adición o no (HF vs LF) de 4% de salvado de trigo (WB) y 2% de pulpa de remolacha azucarera (SBP), en un diseño factorial de 2 × 2 hasta el día 35 después del destete. No se observaron diferencias significativas en la productividad animal entre los tratamientos experimentales en el primer período. En el segundo período, los lechones alimentados con la dieta R comieron más (785 vs 677 g / día, $P = 0,03$) y tendieron ($P = 0,067$) a tener un mayor peso corporal final (19,6 vs 18,5 kg) en comparación con los

animales alimentados con la dieta B. La adición de fibra no afectó al rendimiento. Tanto las dietas B y HF redujeron ($P < 0.05$) la concentración de amoníaco en la digesta del colon proximal. Dieta B también redujo la concentración relativa de isoácidos ($P = 0,007$) y tendió ($P < 0.10$) a tener un menor número de coliformes que la dieta R, lo una reducción en la fermentación de proteína. La adición de fibra aumentó el número de enterococos (5,39 vs 4,31 log UFC / g de heces, $P = 0,015$). Los resultados confirman que los lechones alimentados con arroz alcanzaron mejores rendimientos productivos que los alimentados con cebada, si bien también mostraron una mayor fermentación de proteína en la digesta del colon. Una suplementación moderada con WB y SBP permite reducir estos efectos, que pueden estar asociados a un incremento en el riesgo de patologías digestivas, por la reducción de la concentración de amoníaco y el aumento del número de enterococos.

El **Experimento III** fue planeado para llevar a cabo una selección *in vitro* de los alimentos o sustratos que se pueden adherir a la ETEC K88, y de esa manera inhibir o bloquear potencialmente su adhesión al tracto gastrointestinal de los lechones. En este estudio evaluamos más de 30 fuentes naturales. El procedimiento in vitro mostro una elevada capacidad de identificar sustratos con el potencial de adherir bacterias. Entre las bacterias evaluadas, encontramos también una clara diferencia asociada a la presencia o no de adhesina y fimbrias (*Escherichia coli* enteropatogena vs *Escherichia coli* no fimbriada). Los alimentos más prometedores identificados en este estudio fueron los extractos solubles de salvado de trigo, glicomacropéptido de caseína y exopolisacáridos de lactobacilos para ser utilizado como una estrategia de prevención anti-adhesiva contra ETEC K88 en lechones precozmente destetados.

En el **Experimento IV**, una cepa de *E. coli* enterotoxigénica (ETEC) K88 fue utilizada como modelo para estudiar el proceso de adhesión sobre células intestinales. En este estudio evaluamos la adhesión de la ETEC fimbrilada y una *E. coli* no fimbrilada (NFEC) a las células

intestinales y la activación del sistema inmune innato mediante una línea de células intestinales de porcino (IPEC-J2). Con este método estudiamos el impacto de algunos ingredientes alimentarios [salvado de trigo (BM), la caseína glicomacropéptido (CGMP), manano-oligosacáridos (MOS), extracto de algarroba (LB) y extracto de la fermentación del *Aspergillus oryzae* (AO)] en adhesión de ETEC y la respuesta inflamatoria. Los ingredientes estudiados fueron diluidos previamente en PBS 0,1; 0,2, 0,4 y 0,8% (p / v), ultrasonicados 3 veces y centrifugados para obtener el sobrenadante que fue utilizado. La expresión de los genes de TLR-4, TLR-5, IL-1β, IL-8, IL-10 y TNF-α, se cuantificaron utilizando la ciclofilina A, como gen de referencia, en relación con un tratamiento no desafiado enfrentado a la bacteria. La cepa ETEC presentó una mayor adherencia a las células intestinales e inducción de la respuesta inflamatoria que la cepa NFEC. Todos los ingredientes estudiados fueron capaces de reducir la adhesión de ETEC, pero con un mayor descenso con los tratamientos de CGMP o MOS al 0,8%. En cuanto a la respuesta inflamatoria, el WB promovió una menor expresión relativa de citocinas y quimiocinas, mientras el tratamiento AO dio la mayor expresión. Los resultados de este estudio estimulan el interés por caracterizar mejor las fracciones químicas involucradas en los bloqueos o actividades mostradas por el WB y el AO.

Finalmente, en el **experimento V**, probamos la utilización de la caseína glicomacropéptido (CGMP), una glicoproteína de leche de vaca, como sustrato anti-adhesivo para inhibir o disminuir la adherencia de las bacterias patógenas al epitelio intestinal. Se realizaron dos experimentos. En primer lugar, concentraciones crecientes de CGMP (0, 0,5, 1,5 o 2,5 mg / ml) fueron probadas *in vitro* contra el desafío de ETEC en la mucosa ileal. Se observó que cuanto mayor era la concentración de CGMP menor era el número de ETEC adheridos a la superficie epitelial. En el segundo experimento se realizó un experimento *in vivo* con 72 lechones, incluyendo CGMP o no en la dieta de los animales, y se desafió o no con una cepa de ETEC K88. El desafío con ETEC incrementó los recuentos de enterobacterias y *E. coli* K88 en el íleon y digesta de colon, y también

aumentó la adhesión de *E. coli* en el epitelio del íleon. Como consecuencia, el desafío con ETEC produjo una diarrea leve, con cambios en la histología del íleon que incluyeron un aumento de la profundidad de la cripta y el número de linfocitos intraepiteliales. La inclusión de CGMP provocó una reducción en la concentración de nitrógeno ureico en el suero y el aumento de las concentraciones de N, amoníaco e isoacidos en la digesta del colon. Por otra parte, la inclusión de CGMP aumentó el número de lactobacilos en el íleon y digesta de colon y redujo el número de enterobacterias en la digesta ileal de los animales desafiados. CGMP demostró también la capacidad de reducir el recuento de enterobacterias en contenidos de rascado de mucosa y en la adhesión de *E. coli* a la mucosa del íleon. Los resultados presentados confirman el efecto inhibidor del CGMP en la adhesión *in vitro* e *in vivo* de *E. coli* a la mucosa intestinal, y la capacidad del CGMP para reducir el crecimiento excesivo de enterobacterias en el tracto digestivo de los lechones después de un desafío oral por ETEC K88.

Los resultados de esta tesis doctoral apoyan el interés por incluir niveles moderados de fibra en la dieta de animales jóvenes, para estimular la función intestinal y de reducir la proliferación de patógenos intestinales. Nuestro trabajo también da los primeros pasos hacia el desarrollo de estrategias de alimentación para mejorar la resistencia animal a patógenos, a través del uso de ingredientes con un potencial anti-adhesivo, en frente a otras alternativas como pueden ser las antimicrobianas. Entre los ingredientes valorados, los extractos solubles de salvado de trigo, la caseína glicomacropéptido derivado de la leche de vaca y los extractos de exopolisacáridos de lactobacilos obtenidos de salmuera de aceituna han sido algunos de los que han presentado resultados más prometedores y que merecería la pena ser estudiados con mayor profundidad.

SUMMARY

The main objective of this thesis was to evaluate different feed strategies to reduce enteric disorders in young pigs, in order to help their adaptation to weaning and enhance productivity and health.

In order to achieve this main objective, a set of five trials (chapter 4-8) were designed. **Trial I** was designed to study the interaction between the level of dietary protein and fiber on the productive performance and health status of early weaned piglets. The 4 diets were based on rice, dairy products, and soybean meal in a 2 × 2 factorial arrangement of treatments, with 2 levels of crude protein (CP), low (LP) and high (HP) and 2 levels of dietary fiber (DF), low (LF) and high fiber (HF). The HF diet was developed by supplementing the basal diet with 40 g/kg of wheat bran and 20 g/kg of sugar beet pulp. Pigs fed the HF diets had greater average daily gain (390 vs. 457 g; $P \leq 0.001$) and large intestine weight (4.4 vs. 5.4% of BW; $P \leq 0.05$). This coincided with a greater ($P \leq 0.05$)short-chain fatty acid concentration (especially of acetic and butyric acids), a decrease in *Escherichia coli* counts(7.77 vs. 6.86 log of cfu/g of feces, $P \leq 0.05$), and an increase in the ratio of lactobacilli:enterobacteria (0.76vs. 1.37, $P \leq 0.05$). Protein level did not modify the productive performance, but HP increased $P \leq 0.05$) the relative weight (% of BW) of the small (6.5 vs.7.7) and large intestine (3.8 vs. 4.3). In the large bowel, the HP diet increased the numbers of goblet cells (4.6 vs. 5.4/100 µm; $P \leq 0.05$) and reduced the numbers of intraepithelial lymphocytes (1.8 vs. 1.3/100 µm; $P \leq 0.05$). In relation to the health status, the effect of DF was dependent on the dietary CP content. Supplementing the LP diet with DF reduced the fecal score and increased the antibiotics interventions, whereas the opposite was the case in the HP diet. Pigs fed HP diet showed lower ($P \leq 0.05$) Pig-Map concentrations than pigs fed LP diets. As a whole, CP showed major

effects on the gastrointestinal weight and gut barrier integrity, whereas DF increased the productive performance and promoted major changes in the microbial colonization and fermentation variables.

In **Trial II**, a total of 144 piglets (7.6±1.7 kg) were distributed after weaning into two dietary treatments based (60%) on rice (R) or barley (B), with 18 replicates of 4 animals per treatment. On day 14 after weaning, 96 animals (48 from each diet) were reallocated in32 pens, and assigned to a diet of the same cereal (R or B), either containing (high fiber, HF) or not (low fiber, LF) 4% of wheat bran (WB) and 2% of sugarbeet pulp (SBP), in a2×2 factorial design until day 35 after weaning. No significant differences were observed on the productive performance between experimental treatments in the first period. In the second period, the piglets fed on R diet ate more (785 vs. 677 g/day, P=0.03) and tended (P=0.067) to have a higher final body weight (19.6 vs. 18.5 kg) compared to animals fed on diet B. Fiber supplementation did not affect performance. Both, B and HF diets reduced (P<0.05) the ammonia concentration in the proximal colon digesta. Diet B also decreased the relative isoacid concentration (P=0.007) and tended (P b0.10) to have a lower number of coliforms than diet R, which may indicate a reduction in protein fermentation. Fibre supplementation increased the number of Enterococci (5.39 vs. 4.31Log CFU/g faeces, P=0.015). The results confirm that piglets fed on rice performed better than those fed on barley, but showed higher colon protein fermentation. A moderate supplementation with WB and SBP attenuated these effects by reducing ammonia concentration and increasing the number of Enterococci.

Trial III was planned to perform an *in vitro* screening of feedstuffs which could adhere to the K88 ETEC, and in that way inhibit or block its attachment to the intestinal tract of piglets. The adhesion study was planned to be performed with more than 30 natural sources of feedstuffs, being the best ones chosen for further studies of likely blockage of the intestinal ETEC adhesion. The most promising feedstuffs identified in this study were the soluble extracts of wheat bran, casein

glycomacropeptide and exopolysaccharides from lactobacilli to be used as an anti-adhesive preventive strategy against ETEC K88 in early weaned piglets.

In **Trial IV**, an enterotoxigenic *E. coli* (ETEC) K88 strain was used as model to study the adhesion process. In this study, the adhesion of a fimbriated ETEC and a non-fimbriated *E. coli* (NFEC) to the intestinal cells and the activation of the innate immune system were evaluated using a porcine intestinal cell line (IPEC-J2). The impact of several feedstuffs [wheat bran (WB); casein glycomacropeptide (CGMP); mannan-oligosaccharides (MOS); locust bean extract (LB) and *Aspergillus oryzae* fermentation extract (AO)] on ETEC attachment and the inflammatory response were also studied. Feedstuffs were diluted in PBS at 0.1; 0.2; 0.4 and 0.8% (w/v), sonicated 3 times, and centrifuged. The gene expression of TLR-4; TLR-5; IL-1β; IL-8; IL-10 and TNF-α were quantified using Cyclophilin-A, as a reference gene, and related to a non-challenged treatment. The fimbriated strain showed a higher adherence to the intestinal cells and induced a higher inflammatory response than the non-fimbriated strain. All the studied feedstuffs were able to reduce the adhesion of ETEC, with the greatest decrease being observed with CGMP or MOS at 0.8%. Regarding the inflammatory response, WB promoted the lowest relative expression of cytokines and chemokines and the AO treatment promoted the highest. All tested feedstuffs were able to reduce the adhesion of ETEC to IPEC-J2 and interfere on the innate inflammatory response; however WB should be further studied according to the beneficial results on the intestinal inflammatory process evidenced in this study.

In **Trial V**, casein glycomacropeptide (CGMP), milk glycoprotein was tested as an anti-adhesive substrate to inhibit or diminish the adhesion of pathogenic bacteria to the intestinal epithelium and contribute to the animal health. Two experiments were performed. Firstly, increasing concentrations of CGMP (0, 0.5, 1.5 or 2.5 mg/ml) were tested *in vitro* against the ETEC

challenge on ileal mucosa tissues. It was observed that the increased concentration of CGMP resulted in a gradual decrease in the number of ETEC attached to the epithelial surface. The second experiment was performed *in vivo* using 72 piglets, including CGMP or not in the diet, challenged or not by an ETEC strain. ETEC challenge increased the enterobacteria and *E. coli* K88 in the ileum and colon digesta, and increased the *E. coli* attachment to the ileum epithelium. As a consequence, produced a mild diarrhea; caused changes on ileum histology, including an increase of crypt depth, and the number of intraepithelial lymphocytes; and on serum immunological factors. The inclusion of CGMP affected protein metabolism, as indicated by a reduction in the serum urea nitrogen concentration and the increase in the crude protein, ammonia and isoacids concentrations. Moreover, the CGMP inclusion increased the lactobacilli numbers in ileum and colon digesta and reduced enterobacteria in the ileal digesta of challenged pigs. CGMP demonstrated also ability to reduce the enterobacteria counts in the mucosa scrapes and the attachment of *E. coli* to the ileum mucosa. Our results suggest that the inclusion of 1.5% of CGMP in piglet diets resulted in a higher amount of protein reaching and fermenting in the large intestine of piglets. Present results also confirm the inhibition effect of CGMP on the *E. coli* attachment to the intestinal mucosa *in vitro* and *in vivo*, and the ability of CGMP to reduce the overgrowth of enterobacteria in the digestive tract of piglets after an ETEC oral challenge.

The results of this doctoral thesis support the interest of including moderate levels fiber in the diet of the young animals, in order to stimulate the gut function and reduce the proliferation of gut pathogens. Our work also gives the first steps for the development of feeding strategies to improve the animal resistance to the intestinal pathogens, through the use of anti-adhesive feedstuffs rather than antimicrobial properties. Among the evaluated ingredients, soluble extracts of wheat bran, casein glycomacropeptide derived from cow's milk and extracts of lactobacilli exopolysaccharides obtained from olive brine were some with the most promising results and worth to be studied further.

ABBREVIATIONS USED

AA: amino-acids

ADF: acid-detergent fiber

ADFI: average daily feed intake

ADG: average daily gain

ADL: acid-detergent lignin

AED1: alkali extractable after dialysis 1

AED2: alkali extractable after dialysis 2

ANOVA: analysis of variance

AO: *Aspergillus oryzae* fermention extract

AOAC: Association of Official Analytical Chemists

BSA: bovine serum albumin

BSAS: British society of animal science

BW: body weight

CFU: colony formed units

CGMP: casein glycomacropeptide

CMC: carboxymethylcellulose

CP: crude protein

CRA: cranberry

CTFR: cystic fibrosis transmembrane conductance regulator

Cu: copper

Cy3: carbocyanite-3

DAPI: 4,6-diamidino-2-phenylindole

DC: dendritic cells

DF: dietary fiber

DM: dry matter

DMEM: Dulbecco's modified eagle medium

EMB: eosin methylene blue agar

EPS: Lactobacilli exopolysaccharide

ETEC: enterotoxigenic *Escherichia coli*

EU: European Union

FISH: fluorescent *in situ* hybridization

FITC: fluorescein isothiocyanate

FOS: fructo-oligosaccharides

GALT: gut associated lymphoid tissue

GE: gross energy

G:F: gain feed efficiency rate

GG: guar gum

GLM: general linear model

GIT: gastrointestinal tract

HCl: hydrochloric acid

HF: high fiber

HP: high protein

IEL: intraepithelial lymphocytes

IFN-γ: interferon gamma

IgA: Immunoglobulin A

IL: interleukin

INU: inulin

IPEC-J2¨intestinal porcine epithelial cell

ITS: insulin, transferring selenium supplement

LB: locust bean

LF: low fiber

LG: locust gum

LP- low protein

LPS: lipopolysaccharides

LT: heat labile toxin

MAN: Mannose

ME: metabolizable energy

MOS: mannanoligosaccharides

MUC: natural porcine mucus

NC: negative control

NDF: neutral-detergent fiber

NDO: non-digestible oligosaccharides

NFEC: non-fimbriated *Escherichia coli*

NSP: non-starch polysaccharide

OD: optical density

PBS: phosphate buffer solution

PC: positive control

PCR: polymerase chain reaction

PGE2: prostaglandin E2

PKA: protein kinase A

Pig-MAP: Pig major acute-phase protein

PWC: post-weaning colibacilosis

RT-PCR: real time PCR

RS: resistant starch

SAS: statistical analysis software

SEW: segregated early weaning

SBP: sugar beet pulp

SCFA: short chain fatty acids

SDPP: spray-dried plasma protein

SO: soybean hulls

ST: heat stable toxin

Stx2e: Shiga toxin type 2e

TLR: toll-like receptor

TNF-α: tumor necrosis factor alpha

TRITC: tetramethyl rhodamine

VFA: volatile fatty Acid

VIP: vasoactive intestinal peptide

WB: wheat bran

WC: water content

WEM: water extractable material

WGA: wheat germ agglutinin

WRC: water retention content

ZnO: zinc oxide

INDEX

Figures index

Tables index

Chapter 1

General introduction

Due to the huge reduction promoted in the weaning age of piglets in intensive breeding conditions, from 17 weeks in a natural environment (Jensen and Recén, 1989) down to 1-5 weeks, weaning is considered as the most critical period for the development and growth of young animals in the swine industry. Early weaning implies complex social changes in piglets, including separation from their mothers and littermates and exposure to unfamiliar counterparts, as well as environmental and nutritional changes (Weary *et al*., 2008). Main dietary changes are the abrupt change from suckling to a new dry diet, mostly with vegetable ingredients, which helps promote a variable period of anorexia or insufficient feed intake (Buininx *et al*., 2001). All these changes, aggravated by the immaturity of their immune system and digestive capacity (Lallès *et al*., 2007), lead to a reduction in the integrity of the intestinal mucosa (Vente-Spreeuwenberg *et al*., 2003) and imbalance in the intestinal microbiota (Konstantinov *et al*., 2004) that predisposes the growth of pathogenic bacteria (Pluske *et al*., 1997) and the occurrence of digestive pathologies (Montagne *et al*., 2003).

One of the pathogens that take advantage of this situation is the enterotoxigenic *E. coli* K88 (ETEC K88), a leading cause of post weaning diarrhea, which possesses mechanisms of adhesion to intestinal cells such as the expression of F4-type fimbriae (Fairbrother *et al*., 2005).

Until now, the main and most successful strategy of the swine industry to reduce intestinal diseases has been the use of a large number of in-feed antimicrobials, which have shown efficacy in reducing intestinal diarrhea and promoting the growth of animals. However, those antibiotics have been also referred to as leaving residues in animals' meat, causing bacterial resistance to antibiotics and residual contamination of the food chain (Dibner and Richards, 2005). For this reason, the European Union banned the use of antibiotics as growth promoters (sub-therapeutics dosage) and has regulated their clinical utilization since 2006. This regulation has caused a growing interest in developing alternative products and strategies capable of achieving the same results as those did by antibiotics (Lallès *et al*., 2009).

From a nutritional point of view, designing well balanced diets are considered a crucial step to support growth and the immune system requirements of the animals (Klasing 2007) and to control diseases and mortalities in the weaning period (Pluske, 2006). However, due to the anorexia, piglets usually fell in undernutrition and the inclusion of "high quality" feed ingredients and additives are proposed to improve the animals' health. Some reports have suggested the interest of including a higher amount of dietary fiber (Lange *et al*., 2010) and some specific glycoproteins, known as lectins (Sharon, 2009) in the diet, which may serve as a substrate for the growth of lactic acid beneficial bacteria, or act as "decoys" for fimbriated pathogenic bacteria (*Salmonellae* and *E. coli* spp.), likely blocking their growth and attachment to the intestinal epithelium (Shoaf-Sweeney and Hutkins, 2009) and for that reason it was recently called as "anti-adhesion therapy" (Lane *et al*., 2010). In a near future, it expected that, the feed industry will face new designed products with active molecules able to interact specifically with pathogens (Candela *et al*., 2010; Laparra and Sanz, 2010; Szarc vel Szic *et al*., 2010).

The objective of this thesis was to assess the influence of some fibrous feedstuffs and different functional ingredients on the health and growth of the young pigs after weaning. The study will try also to focus the interest, based on *in vitro* and *in vivo* approaches, on understanding the intimate mechanisms that may account for these effects, such as their likely effects on fermentation and the attachment of the microbial pathogens to the intestinal mucosa.

Literature cited

Bruininx EM, van der Peet-Schwering CM, Schrama JW, *et al*. (2001) The IVOG feeding station: A tool for monitoring the individual feed intake of group-housed weanling pigs. *J Anim Physiol Anim Nutr* **85**, 81-87.

Candela M, Maccaferri S, Turroni S, *et al.* (2010) Functional intestinal microbiome, new frontiers in prebiotic design. *Int J Food Microbiol* **140**, 93-101.

Dibner J and Richards J (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poult Sci* **84**, 634-643.

Fairbrother JM, Nadeau E & Gyles CL (2005) *E. coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis and prevention strategies. *Anim Health Res Rev* **6**, 17-39.

Jensen P and Recén B (1989) When to wean — observations from free-ranging domestic pigs. *Appl Anim Behav Sci* **23**, 49-60.

Klasing KC (2007) Nutrition and the immune system. *Br Poult Sci* **48**, 525-537

Konstantinov SR, Favier CF, Zhu WY, *et al*. (2004) Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. *Anim Res* **53**, 317-324.

Lallès JP, Bosi P, Smidt H, *et al*. (2007) Weaning — A challenge to gut physiologists. *Livest Sci* **108**, 82-93.

Lallès JP, Bosi P, Janczyk P, *et al*. (2009) Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: A review. *Animal* **3**, 1625.

Lane JA, Mehra RK, Carrington SD, *et al.* (2010) The food glycome: A source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol* **142**, 1-13.

Lange CFM, Pluske J, Gong J, *et al.* (2010) Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livest Sci* **134**, 124-134.

Laparra JM & Sanz Y (2010) Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research* **61**, 219-225.

Montagne L, Pluske JR and Hampson DJ (2003) A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim Feed Sci Tech* **108**, 95-117.

Pluske, JR. 2006. New thoughts on nutrition of newly weaned pigs. *Proceedings of London Swine Conference,* vol. 1, pp. 63-73 [JM Murphy and TM Kane, editors]. London, Ontario, Canada

Pluske JR, Hampson DJ and Williams IH (1997) Factors influencing the structure and function of the small intestine in the weaned pig: A review. *Livest Prod Sci* **51**, 215-236.

Sharon N (2009) Lectins. In: *Encyclopedia of Life Sciences,* pp- 1-11. Chichester, UK: John Wiley & Sons, Ltd.

Shoaf-Sweeney KD and Hutkins RW (2008) Adherence, anti-adherence, and oligosaccharides: preventing pathogens from sticking to the host. Advances in Food and Nutrition Research [SL Taylor, editor], chapter 2, pp. 101-161. Academic Press

Szarc vel Szic K, Ndlovu MN, Haegeman G, *et al.* (2010) Nature or nurture: Let food be your epigenetic medicine in chronic inflammatory disorders. *Biochem Pharmacol* **80**, 1816-1832.

Van der Meulen J, Koopmans SJ, Dekker RA, *et al*. (2010) Increasing weaning age of piglets from 4 to 7 weeks reduces stress, increases post-weaning feed intake but does not improve intestinal functionality. *Animal* **4**, 1653.

Vente-Spreeuwenberg MAM, Verdonk JMAJ, Verstegen MWA, *et al*. (2003) Villus height and gut development in weaned piglets receiving diets containing either glucose, lactose or starch. *Br J Nutr* **90**, 907-913.

Weary DM, Jasper J and Hötzel MJ (2008) Understanding weaning distress. *Appl Anim Behav Sci* **110**, 24-41

Chapter 2

Literature review

2.1. The weaning period

2.1.1. The stressful factors of weaning and the feed intake.

The weaning period is one of the most critical phases in the pig industry. At weaning, piglets must abruptly deal with numerous changes that in natural conditions would gradually happen. The sudden separation from the mother and littermates, the change of barns and environmental conditions; and specially, the sudden interruption on the maternal milk determine an anorexia period of variable length (van der Meulen *et al*., 2010). Up to 10% of the animals do not take the first meal until 40h after weaning, keeping the animals on underfeeding bellow maintenance during the first week (Bruininx *et al*., 2001). The consequence is a deterioration of the integrity and functionality of the digestive tract, with frequent malabsorption and dehydration (Hampson *et al*., 2001; Spreeuwenberg *et al*., 2001).

As a consequence, the early weaning of piglets is usually related with intestinal disorders and often accompanied by a severe growth check and diarrhea. It is well established that this process is multi-factorial (see Table 2.1), being post-weaning anorexia and under-nutrition major etiological factors (Pluske *et al*., 1997; Lallès *et al*., 2004). Gastrointestinal disorders in weaned piglets result not only from alterations in GIT architecture and function but also from major changes in the enteric microbiota (Konstantinov *et al*., 2004) and immune system (Stokes *et al*., 2004; Bailey *et al*., 2005). A number of consequences on GIT physiology have been elucidated thus far but influences on the local immune system are still incompletely understood (Lallès *et al*., 2007).

Considering these multifactorial causes, the first goal during the weaning period is to estimulate the feed intake of the animals. Geary and Brooks (1998) described that an increment in the feed intake of about 50g/day, during the first days after weaning may promote an increase in the body weight of 870g , 28 days after weaning, having a clear repercussion in the growth of the animals.

Table 2.1. Weaning pig's context: intestinal disorders and main risk factors (from Lallès *et al*., 2004).

Context: weaning = immaturity + stress

- ♦ Immature animal for:
  - ● Behavior (general and feeding)
  - ● Gut functions (secretions, motility, digestion, absorption, defense, etc.)
  - ● Immune system (intestinal and general)
- ♦ Psychological stress:
  - ● Abrupt separation from the mother
  - ● Mixing with pigs from other litters
  - ● New environment (room, building, farm, etc.)

Induced intestinal disorders

- ♦ Alterations in intestinal architecture and function:
  - ● Morphology : villus atrophy followed by crypt hyperplasia
  - ● Reduced activities of intestinal digestive enzymes
  - ● Disturbed intestinal absorption, secretion and permeability
- ♦ Associated enteric pathogens:
  - ● Bacteria (*Escherichia coli*, enterotoxigenic or enteropathogenic)
  - ● Viruses: rotavirus

Main risk factors:

- ♦ Dietary factors:
  - ● Low or erratic feed intake
  - ● Presence of antinutritional factors (antitryptic factors, lectins, antigens, etc.)
  - ● Diets with high complexity and low digestibility (protein, carbohydrates)
  - ● High level of protein (buffering capacity)
- ♦ Rearing factors:
  - ● Large litter size / low weaning weight
  - ● High density of piglets post-weaning
  - ● Low level of hygiene
  - ● Unsuitable environment (low temperature, low air quality, etc.)

It is widely accepted that the voluntary feed intake of the animals of less than 20 kg is limited by the physical capacity of the intestine (Lallès *et al*., 2004). As lower the digestibility of feed is, the higher is the fulfillment of the intestine, and lower the voluntary feed intake. However it does not seem to be this one the case during the first days after weaning when animals rarely achieve the levels of intake that were normal before weaning (Le Dividich and Sève, 2000). As stated above, post-weaning anorexia is a process that is not dependent on the composition of the diet (McCracken *et al*., 1995), rather than other stressful factor. However, it has been observed that the weaning pig (as well as older pigs) may present neofobia for some ingredients and prefers some feed components than others. Thus, the palatability or acceptance to new ingredients may have a higher effect on the early feed intake than their current digestibility values (Solà-Oriol *et al*., 2007).

The start of feed consumption during the 24 hours post weaning may be also influenced by other animal factors. It has been observed that, after weaning, some animals initiates the feed consumption earlier than others: e.g. animals with lower body weights (Bruininx *et al*, 2001), or those eaters of "creep feed" during the lactation period (Bruininx *et al*., 2002; van de Meulen *et al*., 2010). The above reports reveals a high number of factors, of dietary, management and animal origin, involved on the early feed intake and adaptation of piglets after weaning

2.1.2. Gastrointestinal development

2.1.2.1. Digestive physiology of young pigs.

The development of the gastrointestinal tract is a very sophisticated process, which starts during prenatal life and continues after birth (Barszcz and Skomial, 2011). Diet is considered the most important factor modulating structure and functions of the intestine (Boudry *et al*., 2002).

The digestive and metabolic processes are of great importance to understand the influence of nutrition on growth and health. The growth of an animal depends in part on its capacity to digest and assimilate ingested macromolecules (King *et al*., 2000), but also on the maturation of a proper inmunitary response to the likely microbial pathogens (Barszcz and Skomial, 2011).

Considering that the key to digestion is the absorption of nutrients through the gut wall in a metabolisable form, mechanisms of digestion can be summarised on hydrolysis and fermentation (Ewing and Cole, 1994). But, in early weaned piglets, this capacity is compromised temporally (Adeola and King, 2006). For example, the digestive system of piglets, since born to weaning, is adapted to secrete enzymes that digest milk, but not other ingredients, such as those presented in the dry compound feed mostly of vegetal origin (Yen, 2001). Thus, the digestive tract of young pigs must go through important modifications after weaning that lead to the digestion of vegetal compounds (ie. starch, non starch polysacharides, vegetable proteins, etc). The first one is to augment the pancreas and hepatic enzymes production, but the endogenous enzyme production it is age-related and highly dependent on contact with feed substrates (Corring *et al*., 1978).

Several changes have been described in the gastrointestinal tract morphology. Makkink *et al*. (1994) reported a gradual increase in relative stomach mass (g/kg live weight) over the 10 days after weaning but a decrease in that of small intestine during the first three days that was not recovered until day 10. Similar small intestinal responses have been demonstrated by other workers (Cera *et al*., 1988; Kelly *et al*., 1991; Jiang *et al*., 2000) and appear to be positively related to feed intake (Makkink *et al*., 1994). In contrast, the relative mass of the large intestine increases rapidly during the early pot-weaning period (van Beers-Schreurs *et al*., 1998), an effect that is independent of age of weaning (Kelly *et al*., 1991).

This dynamic process that passes through the adaptation of the gastrointestinal physiology to the constant changes exerted by the the diet consumed, inevitably it also cause shifts on the intestinal microbiota composition (Hopwood and Hampson, 2003).

2.1.2.2. Gastrointestinal-related microbiota.

Just before birth, the digestive tract of all animals is bacteria free (Kenworthy and Crab, 1963). However, from the moment that fetal membranes are ruptured, the piglet is exposed to a huge variety of microbes. In a short period of time, contact with the vagina, feces and skin of the

mother, as well as with the environment starts the gastrointestinal colonization of the piglet's gut (Conway, 1997; Mackie *et al*. 1999). Comparisons of bacteria metabolic fingerprinting determined by Katouli *et al*. (1997) demonstrated that there was a high similarity among the flora of piglets and their dams during the early stages of the animals' life. In particular, the mother's feces might be a key factor in this acquisition and future microbiota development, where the piglets can consume up to 85g of feces per day (Sansom and Gleed, 1981). However, in a few days, microbiota patterns change in the piglet and become more different from sow and characteristic for each individual (Katouli *et al*., 1997).

Swords *et al*. (1993) also studied the pig fecal microbiota evolution within the first four months of life, and concluded that the establishment of the adult fecal flora is a large and complex process with three different marked phases in the bacterial succession. The first phase corresponds with the first week of life, the second one, from the end of the first week to conclusion of suckling, and the third phase from weaning to final adaptation to dry food. They defined weaning as the start of the third phase in pig gut colonization process; being the introduction of more complex solid feed ingredients, with carbohydrates as the main energy source instead of lipids, the key factor in the microbiota change.

Microbiota remains fairly stable in terms of species composition during the second phase when the piglets receive milk from their mother. Lactobacilli and streptococci (which are well adapted to utilize substrate from the milk diet), become the dominant bacteria at the end the first week of life and will be maintained for the whole suckling period with counts of around $10^7 – 10^9$ CFU/g digesta (Swords *et al*., 1993). The diversity of anaerobic bacteria increases in this period (Inoue at al., 2005) and a progressive replacement of aerobic and facultative anaerobic bacteria by anaerobic bacteria become almost completed in this phase. *Clostridium Bacteroides*, bifidobacteria, and low densities of *Eubacterium*, *Fusobacterium*, *Propionibacterium* and *Streptococcus* spp. are also usually found in this second phase (Swords *et al*., 1993).

After weaning, the main feature is a decrease in total culturable bacteria (Franklin *et al*., 2002), with marked changes in some characteristics groups. Using a species-specific real-time PCR approach, Konstantinov *et al*. (2006) demonstrated that the populations of *L. sobrius*, *L. reuteri* and *L. acidophilus*, colonizing the porcine intestine in the early post-natal period, were significantly diminished during weaning. On the other hand, other groups increased, such as the gram-negative genus *Bacteroides* which will represent one of the main bacteria populations in the adult pig (Swords *et al*., 1993). This agrees with Jensen and Jorgensen (1994) who found that immediately after weaning, the main part of culturable bacteria from the large intestine were gram-negative. There is also described a decrease in lactobacilli population parallel with an increase in enterobacteria as a consequence of commercial weaning (Franklin *et al*., 2002).

However, our knowledge of this complex ecosystem is still limited. Until recently, the major part of the studies of intestinal microbiology have been based on traditional methods, which disregard an important percentage of bacteria due to failure of many of them to grown in a given culture medium. In this regard, the development in the last years of high resolution molecular techniques based on 16S ribosomal RNA gene has revolutionized our knowledge of complex microbial population such as the pig gut microbiota. Those studies have showed that the complexity of microbial community is much greater than previously thought. Among the diversity of methods, quantitative polymerase chain reaction (PCR) and some fingerprinting techniques like denaturing gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH) and terminal-restriction fragment length polymorphism (t-RFLP) have been extensively used to study pig gut bacteria (Akkermans *et al*., 2003; Konstantinov *et al*., 2004; Castillo *et al*., 2006ab; Castillo *et al*., 2007; Pluske *et al*.,2007).

The microbial fermentation in the digestive tracts is necessary for the animal, as it constitutes a natural defense barrier against the colonization of intestinal epithelia by opportunistic pathogens (Hampson *et al*., 2001). These health benefits have been extensively described in relation to the lactic bacteria proliferation promoted by oligosaccharides and lactose from the milk. With dry

feed, mostly of vegetal origin, the complexity of the microbiota is higher, making difficult to establish what exactly means a health-promoting microbiota. However is generally accepted that an increase in the biodiversity of the intestinal ecosystem can be considered as an index of stability against pathogen colonization (Konstantinov *et al*., 2004) as well as play important roles in gut morphology (Coates *et al*., 1963), immunity development (Pabst *et al*., 1988), nutrient digestion (Wostmann, 1996) and even in modulating gene host expression (Hooper *et al*., 2001).

Carbohydrates are the main energy substrate for bacteria. The digestion of those compounds depends totally on the activity of different bacteria that produce saccharolytic enzymes, cellulases, hemicellulases, pectinases and xylanases (Varel and Yen, 1997). The fermentation of carbohydrates in the pig colon results in the production of high concentrations of volatile fatty acids (VFA), lactic acid and gases (hydrogen, carbon dioxide, methane), varying in concentration and relative proportions depending of the gastrointestinal section and in lower extent of the substrate fermented. Whereas lactic acid is the main organic acid in the stomach and small intestine, VFA predominate in the colon and cecum. A typical ratio of 60 acetate: 25 propionate: 15 butyrate is described in the lower pig gastrointestinal tract (Bach Knudsen and Jensen., 1991).

Diarrhea is limited by fermentative activity as VFA stimulate the reabsorption of water and sodium (Roediger and Moore, 1981), and because, especially in acidic conditions, high concentrations of VFA inhibit the growth of certain opportunistic pathogens (Mroz, 2005).

Degradation of protein by bacteria in the small intestine seems to be scarce. However, proteolytic fermentation in the large intestine may be very important. As carbohydrate sources become depleted due to fermentation by bacteria, the fermentation changes and become more proteolytic (Piva *et al*., 1996) leading to the formation of potentially toxic metabolites such as ammonia, amines, phenols and indols (Williams *et al*., 2001). Ammonia production has been related to an impaired development of the mucosa of the intestine, with a reduced villus height, and may also affect pig metabolism thus reducing animal performance (Visek, 1984).

The presence of bacteria in the gastrointestinal tract also affects its motility. In germ-free animals, the rate at which the digesta is moved by peristalsis along the upper gastrointestinal tract is slower (Falk *et al*., 1998). One possible cause of this effect may be related with end-products of microbiota fermentation. Similarly, the presence of lactobacilli, described as one of the main bacteria in the pig gastrointestinal tract particularly in the gut upper sections (Hill *et al*., 2005), has also been related to the microbiota effect on gut motility. Moreover, *in vitro* studies have demonstrated that lactic acid (which is produced by these genera) is able to stimulate intestinal motility (Tannock *et al*., 1999).

Besides the contribution of indigenous microbiota to gut maturation and development, there is another direct effect that is essential for the protection of the host against pathogenic invaders. The indigenous microbiota suppresses colonization of incoming bacteria by a process named "colonization resistance" that is a first line of defense against invasion by exogenous, potential pathogenic organisms or indigenous opportunists (Rolfe *et al*., 1996; Hooper *et al*., 2001). This process involves several different complex interacting mechanisms of both, the bacteria and the host.

2.1.2.3. Development of gut mucosal defenses

Weaning also affects the systemic development of innate and adaptive immunity, largely as a consequence of the withdrawal of milk, which has important implications for passively modulating immune responses through both suppressive and enhancing pathways (Gallois *et al*., 2009). Young animals begin to develop or mature several mechanisms of defense to deal with the oral delivery of antigens, which could be divided in:

2.1.2.3.1. Passive innate immunity: a) the excretion of salivary enzymes (with hydrolytic activity); b) the epithelial desquamation (that prevent local bacterial adherence); c) the secretion of intestinal enzymes and stomach hydrochloric acid (which has a antimicrobial effect); the luminal flow and ciliary action of the abundant intestinal villi (also help prevent bacterial adherence); d) the

mucus layer (contains mucins that provide both a loosely adherent surface layer and a firmly adherent underlying layer, conferring a medium for protection against pathogens, lubrication, and transport between the lumen and the epithelial cell surface); e) the intimate union of the epithelial cells conferred by the apical and basal tight junctions (which restrict the passage of even very small molecules), as well as; f) the secretion of anti-microbial peptides and/or the production of volatile fatty acids by the commensal bacteria. These are only some nonspecific mechanisms that in a young animal are maturing and play a crucial role to defend it from the pathogens (Gil and Rueda, 2002; Pearson and Brownlee, 2005; Bauer *et al*., 2006; Gallois *et al*., 2009).

2.1.2.3.2 Active innate immunity: a) the evolutionary development of transmembrane toll-like receptors (TLRs), which recognize a wide variety of microbial pathogens (see Fig. 2.1) and start an inflammatory cascade through the production of cytokines and chemokines; b) the activation of lymphocyte cells in the gut-associated lymphoid tissue (GALT, see Fig. 2.2), represented by T-cells – intraepithelial lymphocytes, B-cells and dendritic cells (which act as antigen-present cells and do the phagocytosis); c) the production of non-specific immunoglobulins, such as IgA (which are secreted in the lumen and identify the damaged or antigen cells to be phagocytized). These are only some mechanisms that the GIT use to deal with pathogens.

Figure 2.1. TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in DNA recognition. TLR3 is implicated in the recognition of viral RNA, whereas TLR7 and TLR8 are implicated in viral-derived RNA recognition. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of microbial components (from Takeda and Akira, 2005).

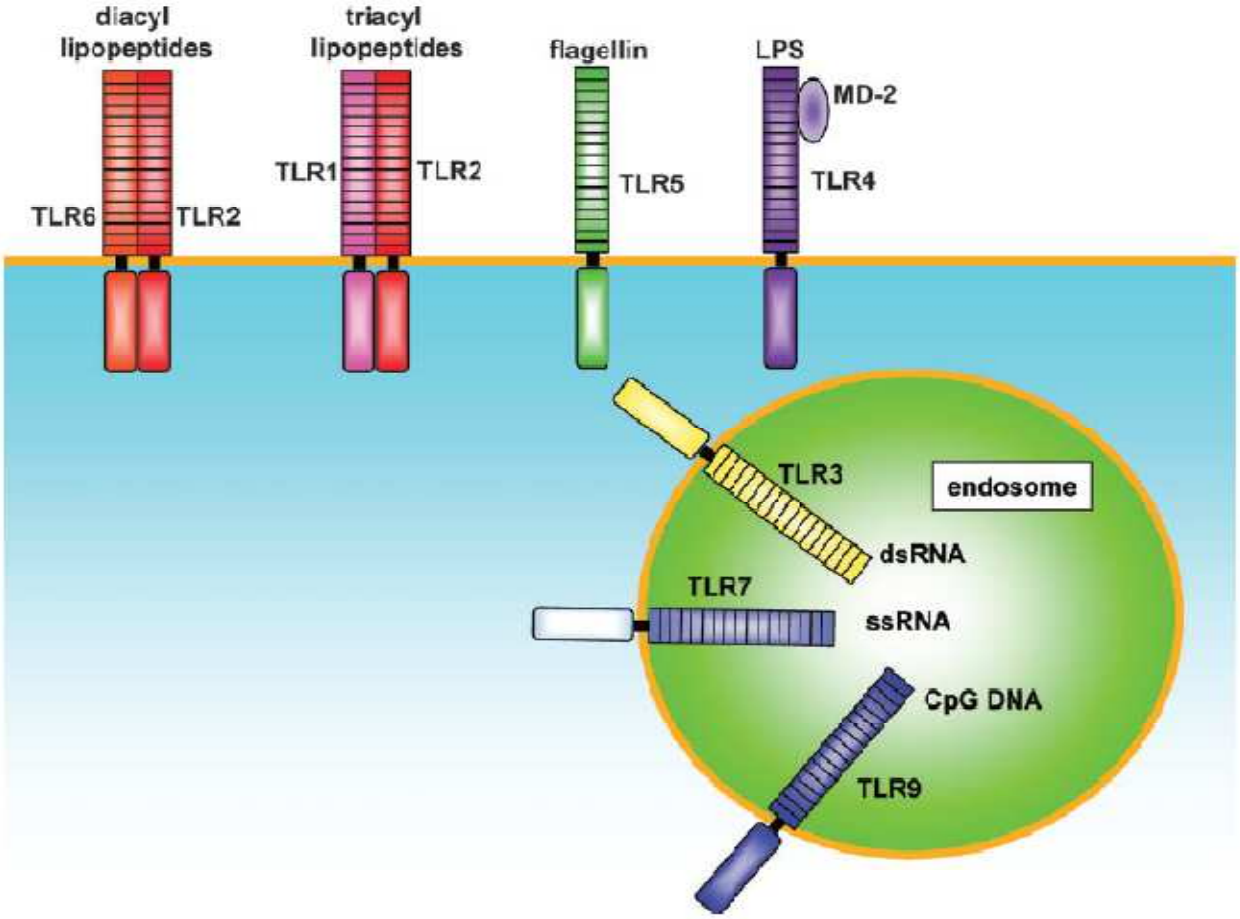
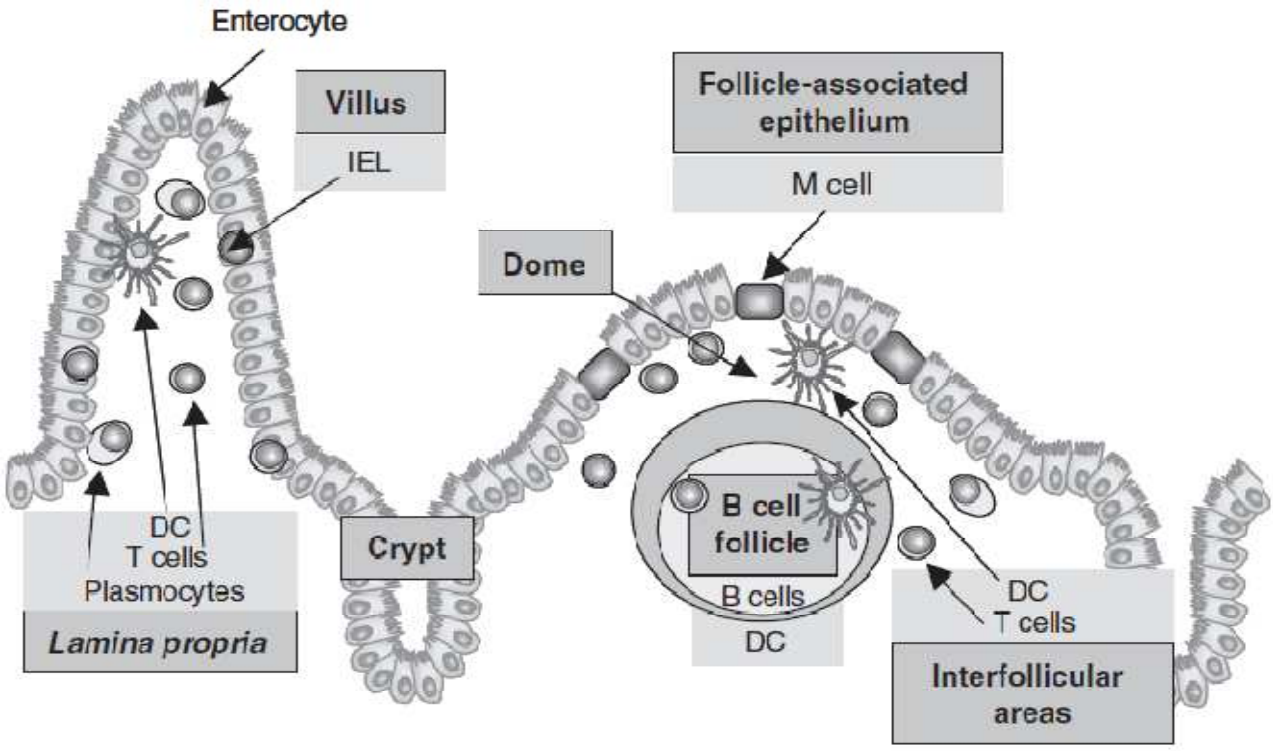


Figure 2. Schematic representation of the distribution of 'immune cells' in the small intestinal tract. Abbreviations: IEL = intraepithelial lymphocyte; DC = dendritic cell (from Gallois *et al*., 2009)



2.1.2.3.1) Adaptive immunity: Adaptive immunity is stimulated by the generic actions of innate immunity. Once a foreign organism is identified by the innate immune system, circulating T-cells begin interacting with foreign antigen. Based on their encounter, they can do one of three things: they can kill infected cells directly, they can boost the actions of macrophages to kill infected cells, or they can return to lymph tissue to incite a B cell response. Stimulated B cells will proceed to produce neutralizing antibody, which can then circulate to fight the infection. However, the intestinal mucosal adaptive immunity is an extremely complex network of cellular and molecular interactions aiming to ensure its ability to mount active immune responses against pathogen and tolerance at least to food antigens and probably commensal flora (Bailey, 2009).

2.1.3. The most common enteric pathologies in the weaning period.

Digestive disorders constitute a common problem worldwide among young pigs, since they are very susceptible to these enteric pathologies due to the multiple factors already described. They are of significant economical importance for the producer due to the increased mortality and delayed growth of the animals; but also due to indirect losses from the increased use of antibiotics and management practices to control or reduce disease (Andersson, 2010). Other aspects of infectious diarrhea outbreak in pigs are the risk of transmitting foodborne diseases and zoonoses to humans (Thomson, 2006), the likely generation of antimicrobial bacterial resistance (Aarestrup *et al*., 2001) and residual contamination of food chain (Dibner and Richards, 2005; Doyle and Erickson, 2011).

Salmonella spp. infections represent also a challenge to swine producers. But its importance is greater as food-borne pathogens and human health issues, than to animal production and health (Callaway *et al*., 2006). Steinback and Hartung (1999) reported that up to 20% of human cases of salmonelosis were due to contaminated pork. Members of the genus *Salmonella* are notorious for their ability to infect a broad range of hosts (virtually all vertebrates) and being the intestinal tract the reservoir of this pathogen (Griffith *et al*., 2006). It has been estimated that between 25% and 48% of the USA swine herd is colonized by *Salmonella* on the farm (Davies *et al*., 1997; Funk *et al*., 2001) and around 43% in Spain (Creus, 2007). Transmission of *Salmonella* is primarily fecal-oral, however respiratory and other environmental transmission routes have been implicated in the spread of *Salmonella* in lairage pens and during transport (Rostagno *et al*., 2003; Winfield and Groisman, 2003). Several intervention strategies have been implemented across the swine industry in a concerted attempt to reduce the incidence of *Salmonella* in herds and consequently in pork products (Wales *et al*., 2011). One of these strategies it is the segregated early weaning (SEW) of piglets, where the piglets are weaned with less than 14 days of age and it is used to produce pigs free from certain swine pathogens (Alexander *et al*., 1980). The use of SEW in swine has been

shown to reduce the incidence of the food-borne pathogenic bacteria *Salmonella*. Because of the health and resultant economic benefits of SEW, this process has been adopted throughout the swine industry (Wales *et al*., 2011). However, the logistical procedures involved with SEW, especially the mixing of pigs originating from different farms at large grower facilities, may have inadvertently introduced social stress that may, contradictorily, lead to increase on fecal shedding of *Salmonella* Typhimurium on early weaned piglets (Callaway *et al*., 2006). Another strategy that may reduce the *Salmonella* prevalence in pigs is the diet acidification adding organic sources of acid directly in the feed (Creus *et al*., 2007) or the drinking water offered to animals (van der Wolf *et al*., 2001).

Clostridium perfringens types A and C and *Clostridium difficile* are the main enteric clostridial pathogens in young pigs (Songer and Taylor, 2006). It occurs worldwide, since, in most cases, it is a member of the normal flora of the swine intestine. Affected piglets become weak, collapse and die. The infections are also characterized by frequently hemorrhagic, often fatal, necrotic enteritis in neonatal piglets and occasionally cause an enteric disease in weaned piglets (Songer and Uzal., 2005).

Another important etiology of post-weaning diarrhea it is the viral infections, caused by *Coronavirus* and *Rotavirus*. *Coronavirus* is the etiology of Porcine Epidemic Diarrhea and the Transmissible Gastroenteritis, both characterized by an acute diarrhea, dehydration with high mortality rates in pigs under 2-3 weeks of age (Pensaert and Yeo, 2006; Saif and Sestak, 2006). *Rotavirus* is an important cause of gastroenteritis and acute diarrhea in suckling and post-weaning piglets, due to the replication of the virus in the villus enterocytes (Yuan *et al*., 2006).

However, the post-weaning colibacilosis (PWC) is probably the most common cause of diarrhea in young pigs and the responsible of great economic losses (Nagy and Fekete, 2005). Due to its great importance for the swine industry, the PWC it will be discussed separately in the following subchapter of this literature review.

2.2. Post-weaning colibacillosis (PWC)

2.2.1. Bacterial strains and the virulence factors

The PWC is mainly caused by enterotoxigenic *E. coli* (ETEC) strains and several factors, such as the multiple stressful factors of weaning, contribute to the severity of this disease. Enteric *E. coli* infection in weaned piglets usually occurs in the first week postweaning and often is manifested by a hypersecretory diarrhea through the specific enterotoxins release and/or other virulence factors such as the F4-type fimbria or F18-type fimbria (Fairbrother *et al*., 2005).

The ETEC strains most frequently implicated in the PWC of pigs are presented on Table 2.2, where the different types of ETEC could be distinguished by several physicochemical characteristics, such as the type of fimbriae, the O-serogroup and the type of enterotoxins secreted.

Zhang *et al*., 2007 presented the prevalence of virulence genes in ETEC strains isolated from young pigs with diarrhea in USA, where 64.4% of strains expressed F4 gene and 34.3% F18. These results are in great accordance of other prevalence studies in Europe (Osek *et al*., 1999; Frydendahl, 2002); Australia (Do *et al*., 2005); China (Chen *et al*., 2004) and Brazil (Vidotto *et al*., 2009). But, besides the great variety of serogroups, the most prevalent one associated with PWC in pigs worldwide is the O149, variant F4ac (Fairbrother *et al*., 2005), and probably the most severe (Francis, 2002).

The other ETEC virulence factor, it is the two major classes of enterotoxins and include: heat labile enterotoxin (LT), which is inactivated at 60ºC for 15 minutes; and heat stable enterotoxin (ST), which is resistant to heat treatment at 100ºC for 15 minutes and divided basically in: type A (STa); heat stable enterotoxin type B (STb) and Shiga toxin type 2e (Stx2e). The STb exterotoxin it is probably the most prevalent (Fairbrother *et al*., 2005), as pointed also by Zhang *et al*., (2007) in 72.6% of the ETEC isolated, followed by LT (57.7%). However, the LT seems to be a greater contributor to the ETEC F4ac virulence than STb (Erume *et al*., 2008). STa appears to be particularly associated with ETEC that cause disease in neonatal animals and is also produced by

some ETEC implicated in PWC in pigs, but rarely as the sole enterotoxin (Fairbrother *et al*., 2005). While the enterotoxin Stx2e, also known as edema disease factor, is the cause of lesions associated with edema disease in pigs (Francis, 2002).

Table 2.2 ETEC strains most frequently implicated in the PWC in pigs (Adapted from: Nagy and Fekete, 1999, Francis, 2002; Fairbrother *et al*., 2005; Nagy and Fekete, 2005).

Fimbria	**Variants**	**Associate O-type**	**Diameter (mean nm)**	**Subunit size (kDa)**	**Mannose sensitivity**	**Entero-toxins**
F4 (K88)	ab ac ad	O8, O141, O149	2.1	27.6	R	LT, STb, ± STa
F5 (K99)	-	O8, O20, O101	5	16.5	R	STa
F6 (987P)	-	O9, O20	7	17.2	R-NH	STa
F17 (Fy)	-	O101	3.4	20	R	
F18	ab (F107) ac (2134P) (8813)	O139, O141, O147, O157	3.5	17	R-NH	LT, STb, ± Stx2e
F41	-	O101	3.2	29	R	
F42	-	?	?	32	R	
F165	-	O115	4-6	17.5, 19	R	

R: mannose resistant - 0.5% of D-mannose do not block the adhesion and the hemagglutination of red blood cells, S: sensitive; NH: non-hemagglutinating.

2.2.2. Pathogenesis- adhesion, a prerequisite step for infection

Enterotoxigenic *E. coli* causing PWC usually attack pigs 4 to 14 days post-weaning, being the oral-fecal and aerosol the main routes of infection (Thomson, 2006). After entering the animal it may colonize the small intestine following bacterial attachment to receptors on the small intestinal epithelium or in the mucus coating the epithelium, by means of specific fimbrial adhesins. These bacteria then proliferate rapidly to attain massive numbers to the order of 10^9 in the mid-jejunum to the ileum. Fimbriae adhere to specific receptors on the cell membrane of intestinal epithelial cells and to specific receptors or non-specifically in the mucus coating the epithelium. ETEC producing fimbriae F5, F6 and F41 mostly colonize the posterior jejunum and ileum, whereas F4-positive ETEC tends to colonize the length of the jejunum and the ileum (Fairbrother *et al*., 2005). F4 fimbria mediate bacterial adherence to the intestinal epithelium throughout most the small intestine and occur mostly in pigs. However, the F4 specific receptors are age-related, since these receptors are found on intestinal cells of newborn piglets, but it disappears when the animals develop and grow (Conway *et al*., 1990). On the other hand, it was found that the F18 intestinal receptor expression levels rise with increasing age during the first 3 weeks after birth and is maintained in older pigs until 23 weeks old (Coddens *et al*., 2007).

Certain pigs do not have receptors for the F4 and F18 adhesins on intestinal epithelial cells and are thus resistant to infection. Several investigators tried to isolate and characterize these receptors and indicated that the sialoglycoproteins were likely the biologically relevant receptor for F4ab and F4 ac (Francis *et al*., 1998; Grange *et al*., 1999, Grange *et al*., 2002). The F18 receptor has not yet been fully characterized, whereas it has been demonstrated in adherence inhibition studies, using specific monoclonal antibodies, that the F18 receptor contains the blood group H-2 (Snoeck *et al*., 2004) and A-2 (Coddens *et al*., 2007), mainly composed by fucose-glycoprotein as major constituents.

Once reached the intestinal epithelium the pathogenic ETEC strains start to produce the enterotoxin, in this case LT and ST, which present a common feature in the mechanisms of action.

They do not produce pathological lesions or morphological alterations on the mucosa, they only produce functional changes such as an increased secretion of water, Na^+ and Cl^-, concomitant decrease of fluid absorption (Nagy and Fekete, 1999), and a great increase on the fluid secreted into the bowel (Fairbrother and Gyles, 2006). LT, leads to an accumulation of cAMP in the enterocytes; which stimulates the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CTFR by the protein kinase A (PKA), thereby causing chloride secretion from the apical region of enterocytes (Thiagarajah and Verkman, 2003; de Haan and Hirst, 2004) as well as the stimulation on the releases of prostaglandin E2 (PGE2), vasoactive intestinal peptide (VIP), and loosening of tight junctions (de Haan and Hirst, 2004). These activities all contribute to increased chloride secretion, reduced sodium absorption, and a concomitant massive entry of water into the intestinal lumen. STa also causes excessive levels of cAMP in enterocytes, while the STb mechanisms of induction of diarrhea are not yet fully understood, but Dreyfus *et al*., (1993) suggested that STb acts by opening a G-protein-linked calcium channel, leading to elevated intracellular Ca^{2+} that would contribute to the formation of prostaglandins (Nagy and Fekete, 2005).

Pigs typically have watery diarrhea that lasts from 1 to 5 days. They usually present an accumulation of fluid in the intestine, signs of dehydration, metabolic acidosis, depression and decrease of the feed consumption, all leading to a peak of mortality 6-10 days after weaning. In certain cases, particularly in young animals, the infection may be so rapid that death occurs before the development of diarrhea (Fairbrother and Gyles, 2006).

2.2.3. Experimental models.

2.2.3.1. In vitro models.

The *in vitro* techniques are useful tools in order to elucidate the adhesion process and the interactions that may play a role in the pathogenesis of intestinal pathogens. It is also useful to test different feed strategies aiming the search for inhibitors of the intestinal colonization by pathogenic bacteria. Accordingly to this, many studies tried to miniaturize the intestinal environment when challenged by a pathogenic ETEC and allowed to new discoveries in the colibacilosis pathogenesis and its prevention strategies.

Naughton *et al*. (2001) described an *in vitro* study using jejunal organ cultures of 18-20kg pigs (4 weeks after weaning) where they could evaluate if the incubation of different types of prebiotics (nondigestible oligosaccharides) where capable to reduce the numbers of *E. coli* in pigs. Becker *et al*. (2007) presented an *in vitro* technique using high-binding 96-well plates, where it was further employed (Becker and Galletti, 2008) to test different ingredients sources with capability to inhibit or reduce the adhesion of certain pathogens such as *Salmonella enterica* and ETEC K88. Van den Broeck *et al*. (1999) presented an *in vitro* test using the mid jejunum villi of weaned piglets, conserved in Krebs-Henseleit buffer with 1% formaldehyde and it was a useful approach to directly observe by phase contrast microscopy the interactions between the ETEC K88 and the brush border of the villi. Jin *et al*. (1998) immobilized the piglet intestinal ETEC-K88-positive mucus in multi-well polystyrene plates and were able to confirm the inhibition effect of egg-yolk antibodies on the ETEC K88 adhesion.

But a promising *in vitro* model has been established by the isolation and culture of two promising intestinal cell lines: a non transformed cell line isolated from jejunum of neonatal piglets, called IPEC-J2; and a transformed cell line called IPI-2I cell line, established from the ileum of an adult boar and immortalized by transfection with an SV40 plasmid (pSV3) (Kaeffer *et al*., 1993). Both of them seem to be reliable *in vitro* models for pig-intestinal pathogens interactions, besides differing in the gene expression response (Mariani *et al*., 2009, Arce *et al*., 2010). The IPEC-J2

have been extensively characterized (Schierack *et al*., 2006), likely express the F4 intestinal receptors (Geens and Niewold, 2010), present a specially high adherence capability to ETEC K88 strains (Koh *et al*., 2008) and it has been successfully used for the study of a *Lactobacillus sobrius* protection effects against an ETEC K88 infection (Roselli *et al*., 2007).

2.2.3.2. In vivo models.

The *in vivo* reproduction of a disease it is not an easy task. Numerous trials which have been undertaken to investigate the etiology of postweaning diarrhea have shown that the process is complex and multifactorial (Hoblet *et al*., 1986; Mezoff *et al*., 1991; Nabuurs *et al*., 1993). In particular, the contributory role of enterotoxigenic *E. coli* in the full range of post-weaning digestive disorders is difficult to assess. Pathogenic strains of *E. coli* can be isolated from pigs on farms affected by diarrhea, as well as on farms without any history of such disorders. Furthermore healthy pigs can harbor large numbers of enterotoxigenic strains of *E. coli* in their small intestines after weaning. To an extent these findings could be explained by the degree to which pathogenic strains of *E. coli* are able to proliferate in the intestinal tracts of affected pigs, with large numbers normally being required to establish themselves before they can initiate diarrhea. The observations, however, also cast some doubts about the exact role of *E. coli* in post-weaning problems as encountered in the field.

Madec *et al*. (2000) presented a large study elucidating the multifactorial points to develop a reproducible experimental model for porcine postweaning colibacillosis. They carried out 6 experiments trying to respond questions like which strain, the best way to challenge, the bacterial load, the frequency of doses and the parameters to control. They reached a 50% of diarrhea incidence (which 14.5% of the cases presented severe illness and died) in the challenged piglets, but it concluded that the response of all pigs depended primarily on the inoculum used, and especially on the challenge load. Moreover, although ETEC are clearly important in the etiology of post-weaning diarrhea, other factors, such as poor environmental conditions, unsuitable dietary

composition and inadequate health status prior to weaning (commonly encountered in commercial piggeries), are also required for the appearance of a chronic post-weaning digestive disorder. This might help to explain the difficulty in the development of a reliable and repeatable experimental model of post-weaning diarrhea, which is realistic in terms of its relationship to the clinical conditions which are encountered in the field.

After this study, a promising molecular tool was developed to help scientists to reproduce a reliable PWC model. Since, it is known that some pigs genetically lack the intestinal receptors for the ETEC and a PCR test is capable to successfully identify the susceptible piglets for F4 (Jensen *et al*., 2006) and F18 (Frydendahl *et al*., 2003), being interesting approaches to select F4 (Erume *et al*., 2008; Sargeant *et al*., 2010; Trevisi *et al*., 2010) and F18 (Cutler *et al*., 2007) positive animals prior to an *in vivo* ETEC challenge.

2.3. Dietary prevention strategies.

2.3.1. Major ingredients used in the weaned period

The feeding industry is currently supplied by a great volume of scientific information accumulated along the years of agricultural research. Therefore, the animal nutrionists are able to design complex weaning diets containing cereals technologically treated, animal products with a high biological value (e.g., milk, dried whey, fish meal, blood meal, etc), synthetic aminoacids, vitamins, minerals and a variety of feed additives aiming a greater growth rate, improve the health status and providing the foundation for the complete adaptation to this transitory phase. In these complex diets some major points are further remarked.

2.3.1.1. Sources of carbohydrates

The carbohydrate source is one of the key points on the dietary formulation for early weaned piglets, since the young pigs have insufficient pancreatic, amylase and intestinal disaccharidases apart from lactase (Maxwell and Carter, 2001).

In this way, lactose rich products are greatly used to feed weaning pigs and its sources include milk by-products like spray dried whey, whey permeate, deproteinized whey, or crystalline lactose. These kinds of ingredients have been shown to be very effective sources of carbohydrates in the initial nursery diet (Nessmith *et al*., 1997) with recommended levels of inclusion between 15 and 25% in early-weaned pigs (Maxwell and Carter, 2001). However, other non-lactose carbohydrates have been evaluated as replacements for lactose or dried whey. Cane sugar is a good energy source, but it is primarily used as a flavoring agent with a typical inclusion level of around 5% and as relatively standard ingredient in traditional pig weaner diets. However, it should be used with caution to avoid diarrhea, because young pigs lack the digestive enzyme sucrase during the first week of life (Tokach *et al*., 2003).

Undoubtedly, cereals are the main source of energy in piglet diets and they may play an important role in the palatability of feed (Solà-Oriol, 2008). In most parts of the world, the major cereal grains used in diets fed to weanling pigs are corn, wheat or sorghum. Barley and oats (naked) are also frequently used, whereas rye and triticale are not very popular because they are rich in anti-nutritional factors. Rice, especially broken rice, is used mainly in Asia (Maxwell and Carter, 2001).

Although oat products have long been considered an excellent energy source for young pigs it was not observed better results in performance of early-weaned pigs compared to corn-based diets (Maxwell and Carter, 2001). Additional ingredients tested with improvements in performance include cooked cereal, sorghum-based distiller's grains, high-oil corn, naked oat, and potato chips scraps. Extrusion of corn or replacing corn with naked oat or hard red winter wheat improved performance of early-weaned pigs (Maxwell and Carter, 2001).

A series of studies carried out in Australia indicated that pigs fed diets based on cooked white rice and animal proteins are less susceptible to infections than are pigs fed diets based on other cereal grains with higher fiber contents (Pluske *et al*., 1996, 1998; McDonald *et al*., 2001a,b). The effect on the reduction of the incidence of diarrhea and on the improvement of pig performance was also studied in Spain (Mateos *et al*., 2007; Vincente *et al*., 2008; Hermes *et al*., 2010). The mechanisms by rice-based diets can cause these positive effects it could be related with the reduction of *E. coli* population (McDonald *et al*., 2001b) or the ability to induce the production of antisecretory factor (Ulgheri *et al*., 2010), suggesting that components in boiled rice also inhibit electrolyte secretions in the small intestine, and hence reduce the magnitude of secretory diarrhea (Mathews *et al*., 1999)

However, differences have been also described on the palatability and feed intake among ingredients. Solà-Oriol *et al*. (2009a) presented a large study where it tested the piglet preference between several cereal based diets and with different presentations in a two-way choice (Solà-Oriol *et al*., 2009b) preference experiments. It concluded that when compared to a control diet, the rice and naked oat-based diet were preferred, and the extrusion of the diets improved the preference

value. When compared barley and oat-based diet, barley were preferred. Moreover, they observed that pelleted form diets were preferred than mash form.

It is evident that the choice of cereal grain in diets fed to weanling pigs does influence feed intake and growth performance, but it also can interfere on intestinal growth of microbiota due to the specific content of each cereal in non-starch polysaccharides (NSP) that could have prebiotic effects in the hind gut of pigs (Stein and Kil, 2006). The effect of dietary fiber inclusion it will be more extensively presented further.

2.3.1.2. Protein source and level of inclusion

Early weaned piglets are sensitive to the source of dietary protein; moreover many of them can produce allergic reactions, reduce growth, and increase mortality (Maxwell and Carter, 2001). The British Society of Animal Science (2003) recommends the protein inclusion of approximately 19% to weaning piglets, but this level can be reduced with the inclusion of synthetic amino-acids. The reduction of protein level in the diet of young pigs may be beneficial, because it was observed that the protein metabolism in the GIT may increase potentially toxic substances such as ammonia, ammines, indoles, phenols and isoacids, which have been implicated in the pathogenesis of PWC and to reduce the environmental contamination (Pluske *et al*., 2002; Bikker *et al*., 2006; Nyachoti *et al*., 2006; Houdijk *et al*., 2007; Heo *et al*., 2008, 2009, 2010; Hermes *et al*., 2009; de Lange *et al*., 2010).

The traditional source of protein for the young nursery pig is dried skim milk. This is not surprising since milk makes up the diet of the pig prior to weaning. Early-weaned pigs fed milk-based diets have generally performed better than those fed other protein sources, due the higher digestibility and the excellent balance of the essential amino acids. In addition, dried skim milk is also high in calcium, phosphorus, and many other essential minerals and vitamins. However, dried skim milk is a very expensive ingredient and usually is not available for animal feeding, so the feeding industry had to search for alternatives (Tokach *et al*., 2003). A good alternative is the whey

protein concentrate (78% of protein), because is a very high quality and it is a cheaper source than dried skim milk (Grinstead *et al*., 2000).

One of the most accepted protein source for weaner pigs is the spray-dried plasma protein (SDPP). Thus, numerous of experiments have been conducted in the last 15 years and a consistent observation in most all of them is an increase in daily gain and feed intake (van Dijk *et al*., 2001, Bosi *et al*., 2004), lower activation of the immune system (Nofrarias *et al*., 2007; Gao *et al*., 2011), and also protection against *E. coli* K88-induced inflammatory status (Owusu-Asiedu *et al*., 2003; Bosi *et al*., 2004) compared to other sources of protein. Different modes of action for SDPP have been proposed: improved palatability (Ermer *et al*., 1994), immune protection provided by its immunoglobulin fraction (Coffey and Cromwell, 1995; van Dijk *et al*., 2001) or prevention of pathogen bacterial adhesion to the gastrointestinal mucosa by the glycoproteins present in SDPP (Nollet *et al*., 1999). However, much like dried skim milk, SDPP is an expensive feed ingredient. It has been recommended that pigs weaned at 21 days of age or less be fed a diet containing plasma proteins for a period of only 7 to 10 days immediately after weaning (Tokach *et al*., 2003).

Recent research has been directed to identify other protein sources that can effectively substitute the more expensive milk proteins, SDPP, or which can be fed in combination with milk proteins to improve performance. Other protein sources, such as high quality fish meal, spray-dried blood meal, and spray-dried whole egg, have been able to replace a portion of the SDPP in the starter diet. However, none of these protein sources is a viable replacement for the entire plasma fraction of the diet (Tokach *et al*., 2003). Good results were observed recently with the inclusion of dried porcine soluble; a product resulting from processing porcine mucosa and small intestine, which produces a liquid peptone product rich in amino acids. This protein product potentially can be also used to effectively replace a portion of the more expensive proteins in diets for early-weaned pigs (Martinez-Puig *et al*., 2007).

The last and cheaper option it is the use of plant proteins such as soybean meal, soybean protein products, wheat gluten, potato protein, peas, lupins, sunflower meal (decorticated), fava

beans and lentils, depending on price and local availability. The inconvenient is that soybean and most all other plant protein sources are rich in anti-nutritional factors and thus heat treatment is needed to make these ingredients suitable for young pigs. Plant proteins are frequently restricted in the first 2 weeks PW to avoid inflammatory reactions to antigenic proteins that are usually found in these ingredients. However, plant protein sources supply the majority of amino acids in practical diets for young pigs after the initial PW phase (Tokach *et al*., 2003).

2.3.1.3. Sources of fats

In the past, it was recommended that diets for newly weaned pigs should contain 8 to 10% of a high quality fat to increase the energy density of the diet and improve fat utilization by the pig (Maxwell and Carter, 2001). However, researches indicate that young pig has only a limited ability to utilize fat. In fact, rather than being needed for use by the young pig, fat is primarily needed to aid in pelleting diets (5-6%), usually established by technological mixing considerations. Diets with more than 5-7% fat tend to not flow well in feed handling equipment (Tokach *et al*., 2003).

The value of added fat to the early-weaned pig diet has been variable. Pettigrew and Moser (1991) summarized data from 92 comparisons of fat addition for pigs from 5 to 20kg and found that fat addition reduced growth rate and feed intake, whereas feed efficiency was improved. The number of positive (37) and negative responses (38) were similar. It seems that utilization may be dependent on pancreatic enzymes. Lindemann *et al*. (1986) and Cera *et al*. (1990) observed up to a 60% decrease in pancreatic lipase after weaning. This decrease is consistent with the observations that, although performance can be improved in pigs fed high-fat diets over the entire nursery phase, the response to fat addition during the first weeks post weaning (PW) is very small or even negative (Mahan, 1991).

Utilization of fat in the early-weaned pig is enhanced with lipid sources with a high concentration of medium-chain fatty acids or long-chain unsaturated fatty acids compared with sources rich in long-chain saturated fatty acids (Maxwell and Carter, 2001). Medium-chain

triglycerides have been shown to increase growth performance substantially in the PW pig (Maxwell and Carter, 2001), likely due to a higher absorption and antimicrobial properties (Decuypere and Dierick, 2003). But there are some other factors that have become interested in specific fatty acids for their potentially beneficial physiological effects on metabolism in the intestinal mucosa, anti-inflammatory and immunomodulatory activity, observed with the inclusion of omega-3 fatty acids and conjugated linoleic acid (Rossi *et al*., 2010).

When fat is included in the diet for weanling pigs, only high quality fat sources such as white grease, soybean oil, or corn oil should be used. Lower quality fats like tallow, restaurant greases, and yellow grease should not be used (Tokach *et al*., 2003).

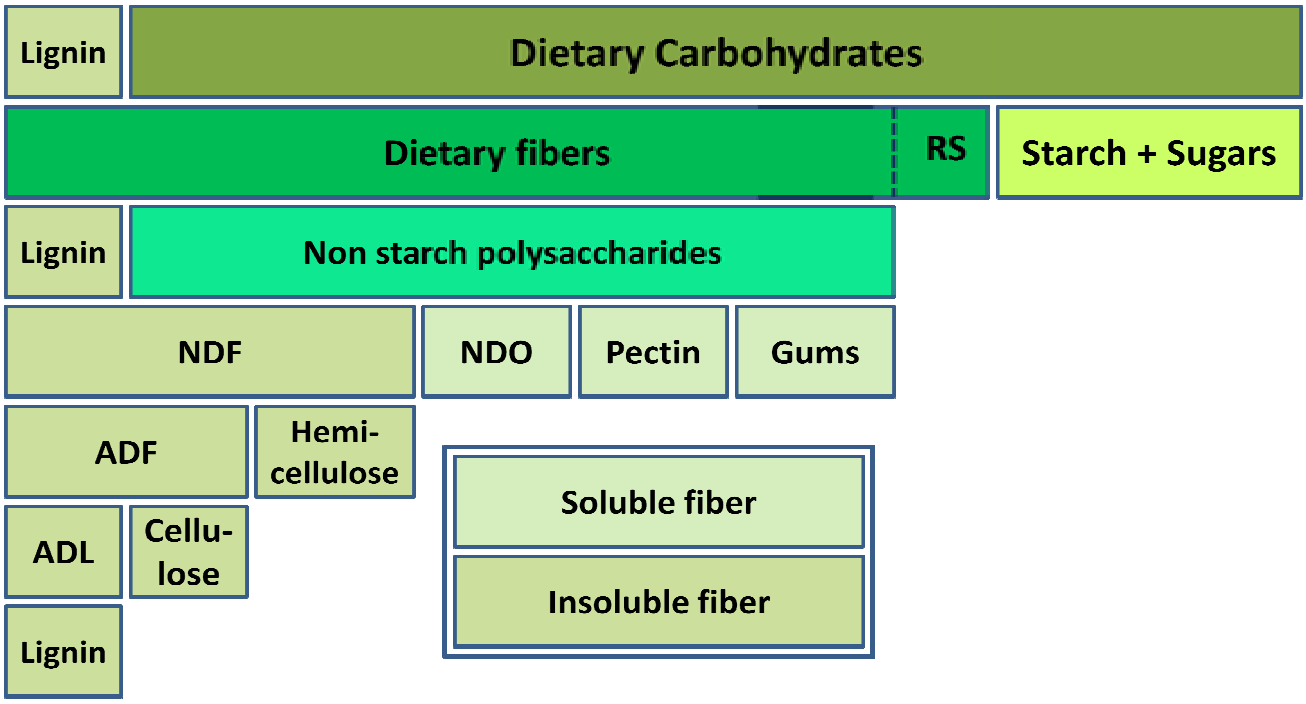
2.3.1.4. The role of dietary fiber on gastrointestinal physiology and microbiota.

Dietary fiber (DF) was first defined in the context of human medicine by Trowell *et al*. (1976) as the "the sum of polysaccharides and lignin that are not digested by endogenous secretions of the digestive tract of man". This definition is also commonly used for all non-ruminant animal species, including the pig. DF covers a wide range of carbohydrates known as non-starch polysaccharides (NSP) which include pectins, cellulose, hemicelluloses, β-glucans and fructans. Oligosaccharides and resistant starch are also considered in the DF fraction. (Montagne, *et al*., 2003; Bindelle *et al*., 2008). Figure 2.3 present a schematic representation of the dietary carbohydrates and where it can also see that the NSP can be divided in soluble and insoluble fiber, according to the carbohydrate composition that confer a solubility in water or not and influence the fermentation rate in the GIT.

Dietary fiber intake provides many health benefits in humans (Anderson *et al*., 2009), but its inclusion on pig diets may have a negative impact on the animal performance, depending on its level of inclusion, because it may reduce the protein and energy digestibility (Noblet *et al*., 2001), decrease feed intake (Wenk, 2001), likely due to gastric signals caused by elongation of the stomach wall (Langhans, 1999); and stimulation effects on the GIT motility (Wilfart *et al*., 2007).

It has been recognized generally as “anti-nutritive” for young animals due to these negative influences on digestion. These anti-nutritive effects appear to be more important in chickens than in pigs, where it seems to be more important in piglets than in growing and finishing pigs (Fernandez *et al*., 2000). But recently the interest of the inclusion of DF on piglet diet has increased, likely influenced by the search of alternatives to the use of growth promoters antibiotics (Stein and Kil, 2006). In this way, several investigators conducted *in vivo* experiments (McDonald *et al*., 2001; Awati *et al*., 2006; Hedemann *et al*., 2006; Mateos *et al*., 2007; Hermes *et al*., 2009, 2010; Molist *et al*., 2009, 2010a,b) and assessed the influence of DF on the adaptation of the piglet after weaning. In general terms, these authors report that soluble fiber increases intestinal transit time, delays gastric emptying, delays glucose absorption, increases pancreatic secretion, and slows absorption, whereas insoluble fiber decreases transit time, enhances water holding capacity and assists fecal bulking in non-ruminant animals.

Figure 2.3. Schematic representation of the carbohydrates composition (adapted from Gerrits and Verstengen, 2006).



ADF: acid-detergent fiber; ADL: acid-detergent lignin; NDF: neutral-detergent fiber; NDO: non-digestible oligosaccharides; RS: resistant starch.

A major impact of fibrous ingredients on the digestive process is in the bacterial fermentation; leading to the production of VFA, favoring the growth of acid lactic bacteria and finally stimulating the colonization resistance (Williams *et al*., 2001). Fermentable fiber in the large intestine of non-ruminant animals may also to decrease protein fermentation and the production of potentially toxic substances. In young animals, the reduction of protein fermentation could be a mitigate factor in the etiology of post-weaning diarrhea (Awati *et al*., 2006; Bikker *et al*., 2006; Piva *et al*., 2006; Kim *et al*., 2008; Hermes *et al*., 2009; 2010; Molist 2010). In addition, as DF interacts, both with the mucosa and the microflora, it may have an important role in the control of "gut health" (Montagne *et al*., 2003; de Lange *et al*., 2010), contributing to the adaptation of digestive function in the large intestine (Wellock *et al*., 2007).

But not all DF ingredients promote positive effects on gut health and animal performance. For example, PWC in pigs might be by some fiber fractions, as it has been described for the NSP from guar gum (McDonald *et al*., 1999) or pearl barley (McDonald *et al*. 2001b, Hopwood *et al*., 2004) and also the carboxymethylcellulose (McDonald *et al*. 2001a; Montagne *et al*., 2004), likely due to a higher viscosity of intestinal contents that might provide a favorable luminal environment for the establishment and growth of bacteria, especially *E. coli.*

Some of the sources of DF which have been proposed to have beneficial impact in the nutrition of young pigs can be divided according to its composition and the positive results found in the literature:

a) Soluble fibers (rapidly fermented carbohydrates, usually fermented in the small intestine) such as: sugar beet pulp; pectin, dietary gums, and the non-digestible oligosaccharides such as raffinose, stachyose, oligofructose or inulin (Lizardo *et al*., 1997; Konstantinov *et al*., 2003: Pierce *et al*., 2005; Awati *et al*., 2006; Pierce *et al*., 2006; Hedemann *et al*., 2006; Wellock *et al*., 2007; Weber *et al*., 2008; Lynch *et al*., 2009).

b) Insoluble fiber (slowly fermented carbohydrates, usually reach the large intestine intact): husks from cereals; brans from wheat or oat; hulls from soybean, rice, barley or oat; distillers dried

grains with solubles (Hedemann *et al*., 2006; Hogberg and Lindberg, 2006; Mateos *et al*., 2006; Carneiro *et al*., 2007; Mateos *et al*., 2007; Wellock *et al*., 2007; Kim *et al*., 2008; Weber *et al*., 2008; Molist *et al*., 2010a,b, 2011);

c) Both: combination of soluble and insoluble fiber (Bikker *et al*., 2006; Hermes *et al*., 2009, 2010; Molist *et al*., 2009);

d) Resistant starch: from potato starch (Martinez-Puig *et al*., 2003; Hedemann and Bach Knudsen, 2007; Martinez-Puig *et al*., 2007).

This intriguing field of study still needs to be better investigated in order to understand the mechanisms through which DF influences the GIT function, microbiota, immunity and how it interacts with other components of the high complexity weaner diets (de Lange *et al*., 2010).

2.3.2. Feed additives:

Due to the fact that the use of antibiotics as growth promoters has been banned in Europe Union and that an expansion of this policy to other countries can now may be expected, intensive research has focused on the development of alternative strategies with the aim of maintaining animal health and performance (see the most recent reviews by Gallois *et al*., 2009; Lallès *et al*., 2009; Oliver *et al*, 2009; de Lange *et al*., 2010, Vondruskova *et al*., 2010; Doyle and Erickson, 2011). Among them, de Lange *et al*. (2010) proposed to divide them according to its expected mode of action in: a) immune response enhancers; b) antimicrobial agents; c) establishment stimulants of a beneficial gut microbiota; and d) stimulants of digestive function. But a lot of them can act in more than one way and this is not a definitive division list, especially because the numbers of commercially available products that combine different feed additives (aiming for a synergic effect) are growing.

Various “natural” materials, many of which are commercially available, have been investigated as efficient alternatives to antibiotic growth promoters. At present, probiotics, prebiotics, organic acids, enzymes, phytobiotics, clay adsorbents and others are under investigation.

These materials can exert beneficial effects on the microbiota composition and consequently affect animal health and growth performance and it will be present further.

2.3.2.1. Medication: Antibiotic growth promoters.

Antibiotics are compounds that suppress or inhibit the growth of microorganisms. This class of compounds includes the antibiotics, naturally occurring substances produced by yeasts, molds, and other microorganisms; and the chemotherapeutics, chemically synthesized substances (Cromwell, 2002). They are added to feed at low (subtherapeutic) levels for growth and reproductive promotion, improvement of feed utilization, and reduction of mortality and morbidity (NRC, 1998).

The efficacy of antimicrobials in improving the rate and efficiency of growth in pigs is well documented. A summary of 1,194 experiments involving 32,555 pigs indicated that antimicrobials improved growth rate by 16.4% in weanling pigs (7 to 25 kg body weight) and improvements in efficiency of feed utilization by 6.9%. Responses in pig growth to the feeding of antimicrobials are greater under field conditions than in controlled experiments at research stations (Cromwell, 2002). A summary of 67 field trials with young pigs indicated that the feeding of antimicrobials reduced mortality by one-half (4.3 versus 2.0 percent), with even greater reductions in mortality when disease levels were high (15.6 versus 3.1 percent) (NRC, 1998).

Subtherapeutic levels of antibiotics have been shown to reduce the incidence or severity of swine diseases, mainly the enteric-related. It has been proposed that antibiotics alter the normal, non-pathogenic flora of the gut and these changes have a beneficial effect on digestive processes and the utilization of nutrients in feed. It has been estimated that as much as 6% of the energy in a pig's diet may be lost due to microbial fermentation in the stomach and small intestine. Intestinal bacteria also inactivate pancreatic digestive enzymes and metabolize dietary protein with the production of the toxic ammonia and biogenic amines. Antibiotics inhibit these activities and increase the digestibility of dietary protein. Antibiotics also appear to prevent irritation of the

intestinal lining and may enhance uptake of nutrients from the intestine by thinning of the mucosal layer. Thus the effects of subtherapeutic antibiotics may extend beyond digestion (Doyle, 2001).

On the other hand, the increased use of antibiotics has given rise to a fear of the development of resistant pathogenic bacterial strains (Wegener *et al*., 1998; Kyriakis *et al*., 1999; Budino *et al*., 2005) and residual contamination of the food chain with antibiotics (Chen *et al*., 2005; Dibner and Richards, 2005; Roselli *et al*., 2005). This has led to the adoption of safety measures and a gradual withdrawal of antibiotic promoters from pig diets. In 2006, the use of antibiotics as growth promoters was forbidden in the EU and the search for alternative additives is currently being investigated (Lallès *et al*., 2009).

2.3.2.2. Zinc oxide (ZnO).

Zinc is a component of many metalloenzymes, including DNA and RNA synthetases and transferases, many digestive enzymes, and is associated with insulin secretion. As such, it plays a crucial role in lipid, protein and carbohydrate metabolism in the pig (Pluske *et al*., 2002, Ou *et al*., 2007).

The feed industry are currently using high doses of zinc oxide (2400 – 3000 ppm), compared to the maintenance recommended doses (100 ppm), claiming an alternative to antibiotics, since it was strongly reported that may reduce the incidence of post weaning diarrhea and mortalities, as well as improve growth of newly weaned piglets (Poulsen, 1981; Hahn and Baker, 1993; Smith *et al*., 1997; Carlson *et al*. 1999; Hill *et al*., 2000; Mavromichalis *et al*. 2000, Amezcua *et al*., 2002). This activity could be explained by an antibacterial effect (Holm and Poulsen, 1996), but the complete mechanism of action of zinc is still unclear (Jacela *et al*., 2010a).

Zinc seems to play a role in stabilizing cell membranes and modifying membrane functions (Bray and Bettger, 1990), as demonstrate that high levels of Zn in a weaner diet increased tight junction protein expression and hence decreases intestinal permeability (Zhang and Guo, 2009). The

authors speculated that decreasing intestinal permeability due to dietary Zn supplementation may have prevented translocation of pathogenic bacteria through the intestinal barrier.

Recently, Slade *et al*. (2011) reported positive effects with the supplementation with high dosis of ZnO (3100 ppm) on diets of weaned piglets challenged with ETEC K88. The ZnO supplementation generated positive parameters on the intestinal morphology, favorable changes in lactic acid bacteria-to coliform ratio that were also associated with increased rates of feed intake and growth, suggesting benefits not only on the suppression of ETEC proliferation, but also altered development of small intestine mucosa and associated microbiota. These results contradict those found by Hojberg *et al*. (2005) and Molist *et al*. (2011) which observed a reduction in the lactobacilli population with the inclusion of high doses of ZnO, despite that Molist *et al*. (2011) also observed a decreased in coliform counts compared to a diet with basal levels of ZnO. It seems that the ZnO effect on microbiota could be unspecific and may act as a general antimicrobial.

Despite the ambiguity related to the exact mechanism(s) of action of ZnO, this terapeutic approach is well accepted by the swine industry because it is a cost effective nutritional tool and is a powerful anti-inflammatory agent and the gut often suffers a non-specific inflammatory response after weaning (Lallès *et al*., 2007).

However, the high level of Zn is mostly excreted in the faeces resulting in an environmental concern. In Europe high levels of ZnO can now only be used under veterinary prescription, and alternatives such as organic sources (Case and Carlson, 2002) or technological processing (Singh *et al*., 2009) could be useful in the future.

2.3.2.3. Acidifiers

The digestive system of the early-weaned pig is not sufficiently developed to handle the transition from diets based on milk proteins (liquid) to those based on cereal, and animal by-products (solid). Weaned piglets are physiologically immature and may not produce enough hydrochloric acid (HCl) to keep stomach pH at an optimum of approximately 3.5. At this pH,

digestion of proteins and populations of beneficial bacteria (lactobacilli) are maximized and harmful bacteria are inhibited (Maxwell and Carter, 2001). Organic acids (see Table 2.3) are widely distributed in nature as normal constituents of plants or animal tissue. They are also formed through microbial fermentation of carbohydrates predominantly in the large intestine of pigs (Partanen and Mroz, 1999).

It has been suggested that the benefits of including organic acids and (or) their salts are related to the antimicrobial properties of their cations and anions (Partanen, 2001). As a consequence there is an improvement in gastro-intestinal health, resulting in enhanced growth performance by increasing voluntary food intake and improving feed efficiency (Giesting *et al*., 1991; Overland *et al*., 2000), and by reducing the incidence and severity of diarrhea of the animals (Tsiloyiannis *et al*., 2001; Owusu-Asiedu *et al*., 2003). Organic acids also have been used to preserve high-moisture grains and as mold inhibitors in feeds (NRC, 1998) and to control the in-feed contamination of Salmonella spp. (Creus *et al*., 2007).

Although efficacy of acidifiers has been demonstrated in numerous studies, responses have been inconsistent between different types of acidifiers. Ravindran and Kornegay (1993) published a review of studies summarizing the effects of numerous acidifiers on pig performance. They suggested that variables that influence more the efficacy of acidifiers include type of diet, age of pigs, type and dosage of acidifier and existing environmental conditions. The observed differences in performance responses to dietary organic acids may also be due to differences in dietary buffering capacity. The buffering capacity varies substantially between different feedstuffs (Blank, 1999). The acid-buffering capacity is lowest in cereals and cereal by-products, intermediate or high in protein feedstuffs and very high in mineral sources, except in dicalcium and monosodium phosphates (Mroz *et al*., 2000).

Table 2.3. Some physicochemical properties of most common organic acids and their salts, used in pig diets (from Mroz, 2005).

Name	Physical form	Mol.wt/GE (MJ/kg)	Dissociation constant (pK_a)	Corrosiveness rate	Odour
1.Formic	Liquid	46.03/5.7	3.75	++(+)	Pungent
2.Acetic	Liquid	60.05/14.6	4.76	+++	Pungent
3.Propionic	Oily liquid	74.08/20.6	4.88	++	Pungent
4.Butyric	Oily liquid	88.12/24.8	4.82	+	Rancid
5.Lactic	Liquid	90.08/15.1	3.86	(+)	Sour milk
6.Sorbic	Solid	112.1/27.8	4.76	(+)	Mildly acrid
7.Fumaric	Solid	116.1/11.5	3.02/4.38	0 to (+)	Odourless
8.Malic	Solid/Liquid	134.1/10.0	3.46/5.10	(+)	Apple
9.Citric	Solid	192.1/10.2	3.1/4.8/6.4	0 to ++	Odourless
10.Ca-formate	Solid	130.1/11.0		0	Neutral
11.Ca-lactate	Solid	308.3/30.0		0	Neutral
12.Ca-propionate	Solid	84.1/40.0		0	Neutral
13.K-diformate	Solid	130.0/11.4		0	Neutral
14.Ca-butyrate	Solid	214.0/48.0		0	Rancid
15.Mg-citrate	Solid	214.4/10.0		0	Neutral
16.Na-lactate	Solid	112.1/15.0		0	Neutral

Salts of organic acids, such as formates and diformates, are not as corrosive and can be used to significantly improve growth rate and feed conversion in weanling pigs (Overland *et al*., 2000; Paulicks *et al*., 2000). Sodium butyrate (SB) has also well-known beneficial effects on colonic mucosa, butyrate and butyrate precursors have been the focus of special attention (Hamer *et al*., 2008). It is more frequently used in pig feeding due to its less strong odour than the acid. It is presumed that the effect inside the gut is not different, because SB dissociates. In experiments

conducted by Manzanilla *et al*. (2006) and Castillo *et al*. (2006), SB was included in the starter diet at 3 g/kg and fed for 14 days to piglets weaned at 18–22 days of age. SB supplementation improved feed gain ratio in week 2 post-weaning and over the 14-day post-weaning period. SB was detected only in the stomach, suggesting quick gastric and (or) duodenal absorption or catabolism (Gálfi and Bakori, 1990; Manzanilla *et al*., 2006). SB increased gastric DM percentage (Manzanilla *et al*., 2006) and also induced large changes in both the biodiversity of the microbial ecosystem and species composition of the bacterial community in the jejunum (Castillo *et al*., 2006). Total microbial activity in digesta was lower in the caecum and distal colon when SB was present in the diet. Manzanilla *et al*. (2006) suggested that SB supplementation may have contributed to stabilise the gastrointestinal ecosystem while depressing amilolytic bacteria, thus improving the health status of the pigs and the efficiency of the use of nutrients for growth.

Another strategy to extend the effectiveness of acid supplements along the GIT and reduce corrosion damage to housing materials is the use of a slow-release form of acid. It consists of organic acids microencapsulated with fatty acids and mono - and diglycerides mixed to form microgranules (Piva *et al*., 2007, Doyle, 2001). This microencapsulation approach can also reduce the negative impact observed with the free forms of organic acids supplementation, where it reduced the number of HCl-secreting parietal cells and increased the somatostatin secreting cells, both leading to the reduction on gastric HCl secretion (Bosi *et al*., 2006).

Its generally accepted that the growth promoting effects of acids are most prominent in the first few weeks after weaning, when the gastro-intestinal tract of the piglet is not fully developed and is most vulnerable to infection (Mroz, 2005).

2.3.2.4. Phytogenic feed additives.

Herbs have been in use in human nutrition for thousands of years due to renowned antiseptic qualities (Cowan, 1999). Numerous plants, their extracts or other natural substances possess anti-bacterial activity. Research has focused on natural components with antimicrobial activity as this

was thought to be one of the modes of actions of antibiotic growth promoters (Lallès *et al*., 2009). However, many other different effects have been reported, such as: 1) changes in immune function (Koh *et al*., 1998; Boyaka *et al*., 2001); 2) enzyme stimulation (Platel and Srivasan, 1996); 3) antiparasitic (Force *et al*., 2000); 4) antifungal (Mahmound, 1994); 5) antiviral (Aruoma *et al*., 1996; Benencia and Courrèges, 2000; Garcia *et al*., 2003); 6) antitoxigenic activity (Azumi *et al*., 1997); 7) antioxidant activity (Aruoma *et al*., 1996; Dorman *et al*., 2000; Teissedre and Waterhouse, 2000). Their inclusion in the diet has also been shown to stimulate appetite by improving palatability (Close, 2000; Doyle, 2001). Given this wide range of effects, plant extracts must be considered one of the main candidates to study, concerning not only pig weaning but also other problems of animal production (Kamel, 2001).

In this way, essential oils are preferably used in the production of feed phytogenics additives, because they contain the active principles of plants (Windisch *et al*., 2008). But, the production of essential oils is extremely difficult to control, since the concentration, quality and composition of the active principles are highly influenced by ecological factors and climatic conditions (soil, nutrients, water, light and temperature); as well as by the plant cultivation, processing and isolation methods (Máthé, 2009). Probably for this reason there is a multiplicity of controversial results obtained in different scientific studies examining the effect of these substances in animal nutrition. Accordingly, it is necessary to select effective plant extracts, standardize them and investigate a potential synergistic benefit deriving from their combination (Budzinski *et al*., 2000; Oetting *et al*., 2006) or other potentially toxic effects (Di Pasqua *et al*., 2007).

Studies on the bioavailability and pharmacokinetics of various plant extracts, major compounds involved in the antimicrobial activity of essential oils, show that they are rapidly absorbed and metabolized (Kohlert *et al*., 2000). Rapid absorption limits the luminal availability of these compounds for antimicrobial activity and could explain why different results have been obtained. Their effect on the microbiota could be improved by the use of microencapsulation technology, which would allow a sustained release along the gastrointestinal tract and thereby

increase luminal availability in the ileum and colon (Meunier *et al*., 2006). Furthermore, this technology should be used to avoid detrimental effects like irritant or bad taste effects (Piva *et al*., 2007). Various encapsulation technologies are now emerging like the rotary fluidized bed technologies which can perform pelletization and coating in a single machine (Meunier *et al*., 2007).

In Table 2.4 are presented a summary of some experimental results carried out recently with the inclusion of phytogenic feed additives in pig diets. From these results and a review published by Windisch *et al*. (2008) when it compared with antibiotic growth promoters and organic acids, the phytogenic substances currently used in practice similarly seem to modulate relevant gastrointestinal variables, such as microbial colony counts, fermentation products (including undesirable or toxic substances), digestibility of nutrients, gut tissue morphology, and reactions of the gut-associated lymphatic system.

Furthermore, some isolated observations seem to support the claimed enhancements of digestive enzyme activity and absorption capacity through phytogenic compounds. In addition, phytogenic products may stimulate intestinal mucus production, which may further contribute to relief from pathogen pressure through inhibition of adherence to the mucosa. Unfortunately, respective experimental results are available only from commercial products containing blends of phytogenic substances. Therefore, there is still a need for a systematic approach to explain the efficacy and mode of action for each of type and dose of active compound, as well as its possible interactions with other feed ingredients. Nevertheless, the current experience in feeding such compounds to swine and poultry seems to justify the assumption that phytogenic feed additives may have the potential to promote production performance and productivity, and thus be added to the set of nonantibiotic growth promoters, such as organic acids and probiotics (Windisch *et al*., 2008).

Table 2.4. Plant extracts in pig nutrition (adapted from Vondruskova *et al*., 2010)

Plant	The effect observed	Reference
Oregano, cinnamon and Mexican pepper	Decreased ileum total microbial mass, increased lactobacilli:enterobacteria ratio	Manzanilla *et al*. (2004)
Sanguinarine (alkaloids of *Macleaya cordata*)	Increased body weight gain and feed conversion by growing pigs	Borovan (2004)
Cinnamon, thyme, oregano	Inhibited pathogenic *E. coli* in piglet intestine	Namkung *et al*. (2004)
Thyme, clove, oregano, eugenol and carvacrol	Improved pig performance	Oetting *et al*. (2006)
Clove, oregano	Growth performance of pigs close to pigs fed antimicrobials	Costa *et al*. (2007)
Specific blend of herbal extract	Increase ADG, decrease feed conversion ratio in finishing pigs	Liu *et al*. (2008)
Aged garlic extract, allicin	Improved body weight, morphological properties of intestine villi and non-specific defense mechanisms of piglets	Tatara *et al*. (2008)
Camellia sinensis	Decrease of clostridia and enterococci counts in the faeces of piglets	Zanchi *et al*. (2008)
Carvacrol microencapsulated in Ca-alginate microcapsules	*In vitro* antimicrobial activity against enterotoxigenic *E. coli* K88	Wang *et al*., (2009)
Plant polyphenols (from cooca beans and plant tannins)	Decreased *in vitro* the adhesion and toxin binding of porcine enterotoxigenic *E. coli*	Verhelst *et al*., (2010)

2.3.2.5. Microbiota manipulation strategies: Pro, pre and symbiotics.

2.3.2.5.1. Probiotics:

Although there are several definitions of a probiotic, they generally refer to a live culture of microorganisms that exerts a beneficial effect on the host by improving the indigenous microbial balance (Collins and Gibson, 1999)

The likely mechanisms of action of probiotics are based on: competition between them and pathogenic microorganisms for binding sites in the intestinal mucosa (Bomba *et al*., 2002; Harvey *et al*, 2005; Gaggìa *et al*., 2010); nutrient availability (Bomba *et al*., 2002); and total inhibition of pathogen growth by production of organic acids and antibiotic-like compounds, called bacteriocins (MantereAlhonen, 1995; Zimmermann *et al*., 2001; Bomba *et al*., 2002; Marinho *et al*., 2007; Mazmanian *et al*., 2008).

Probiotics also influence the digestive process in the body by increasing the activity of microbial probiotic enzymes and the digestibility of food (Roselli *et al*., 2005). They stimulate the immune system and the regeneration of intestinal mucosa (Gaggìa *et al*., 2010). They can elicit an increase in immunoglobulin a production (Perdigon *et al*., 1995) and stimulate macrophages and natural killers cells (Chiang *et al*., 2000; Matsuzaki and Chin, 2000). They can also regulate anti- and pro-inflammatory cytokine production (Lessard and Brisson, 1987; Shu *et al*., 2001; Roselli *et al*., 2005).

Several different organisms have been fed to humans and animals (Hammes and Hertel, 2006) as a source of probiotics in the diet. But the most prevalent probiotics genera used include *Lactobacillus* spp., *Bacillus* spp., *Enterococcus* spp.; *Bifidobacterium* spp., the yeast *Saccharomyces cerevisiae*, and combinations of these organisms (Turner *et al*., 2001; Gaggìa *et al*., 2010). Moreover, even an avirulent *E. coli* strain was recently proposed to be used in weaning pigs to inhibit the infection by ETEC K88 (Setia *et al*., 2009).

Addition of probiotics to swine diets was reported to improve growth rate, feed conversion, and survival in weanling, growing, and finishing pigs. For example, improvements in growth

performance have also been observed in young pigs in response to supplementation of the diet with strains of *Lactobacillus acidophilus* (Pollmann *et al*., 1980); *Bacillus cereus* (Zani *et al*., 1998), *Bacillus subtilis* (Guo *et al*., 2006), viable spores of *Bacillus licheniformis* or *Bacillus cereus* var. *toyoi* (Kyriakis *et al*., 1999) or with live yeast (van Heugten *et al*, 2001). On the other hand, no improvement in growth performance of weanling pigs were observed neither when live yeast was added to a corn-soybean meal diet (Kornegay *et al*., 1995) nor with the inclusion of *Lactobacillus spp*., *Enterococus faecium* and *Bacillus cereus* var. *toyoi* to experimentally and/or naturally infected pigs (De Cupere *et al*., 1992; Johansen *et al*., 1996; Friendship *et al*., 2002).

The inconsistent response of weanling pigs to microbial supplementation of the diet most likely depends on whether colonization of organisms in the intestine occurs (Maxwell and Carter, 2001) and the probiotic strain used (Reid and Friendship, 2002). To fully understand how probiotics enhance gut health and improve nutrient utilization, further studies are needed to adequately characterize the mode of action by which probiotics alter the gastrointestinal environment in swine (Lallès *et al*., 2007).

2.3.2.5.2 Prebiotics:

Prebiotics are defined as ‘nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and (or) activity of one or a limited number of bacteria in the colon, and hence improve host health’ (Gibson & Roberfroid, 1995). They have been referred to as the bifidus factor, because they support the growth and/or activities of probiotic microorganisms in the gastrointestinal tract (Gibson, 2004; Rayes *et al*., 2009).

Gaggìa *et al*. (2010) pointed out the factors for a dietary substrate to be classed as a prebiotic. The investigators cited that at least three criteria are required: 1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine; 2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria; and 3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host.

So, the prebiotic positive effects on GIT are the same to those described above for DF. In fact most identified prebiotics are carbohydrates and oligosaccharides presented in fibers, but the most promising are the nondigestible oligosaccharides (Gaggìa *et al*., 2010). Its supplementation is strongly related to the ability of increasing GIT concentrations of short-chain fatty acids produced by bifidobacteria and lactic acid bacteria and which it can serve as a dominant energy source for enterocytes (Houdijk *et al*., 2002; Hamer *et al*., 2008).

The most common sources currently used as prebiotics in pig nutrition are: galactoligosaccharides (from trans-galactosilation of lactose); fructoligosaccharides (from partial enzymatic hydrolysis of inulin and transfructosylation of saccharose); lactulose (from alkali isomeration) and inulin from chicory root (Vondruskova *et al*., 2010).

From the literature, dietary supplementation with inulin (present in a number of raw materials of vegetable origin such as onion, garlic, asparagus, banana and chicory root) has a positive effect on VFA production, sufficient height of intestinal villi, stimulation of natural microflora and improvement of efficiency parameters (Crittenden and Playne, 1996).

Lactulose is another prebiotic widely used in the prevention and treatment of enteric disorders in animals and humans (Vondruskova *et al*., 2010). It cannot be hydrolyzed by the β-galactosidase of the digestive tract and hence, it cannot be absorbed from the small intestine. It passes down to the large intestine where resident microflora consumes it and produce lactic and/or acetic acid (Gibson, 2004; Bohacenko *et al*., 2007). Consequently, it stimulates the growth and/or activity of indigenous intestinal microflora, especially of the genera *Bifidobacterium* and *Lactobacillus,* and reduces the activity of proteolytic bacteria. Pig diets are usually supplemented with one per cent lactulose (Kamphues *et al*., 2007).

Although mannanoligosaccharides (MOS. from mannose enzymatic synthesis or extracted from cell wall of *Saccharomyces cerevisae*) have been used in the same manner as the prebiotics listed above, they do not selectively enrich for beneficial bacterial populations. Investigation on the mode of action of mannanoligosaccharide pointed out that these compounds are able to bind to

mannose-specific lectin of gram-negative pathogens that express Type- 1 fimbriae such as *Salmonella* (Spring *et al*., 2000; Price *et al*., 2010) and *E. coli*, (Thomas *et al*., 2004; Baurhoo *et al*., 2007; Kiarie *et al*., 2011) resulting in their excretion from the intestine. Some authors have reported better growth performance and digestibility rates for weanling pigs fed mannanoligosaccharides than for control pigs (Le Mieux *et al*., 2003; Rozeboom *et al*., 2005, Shim *et al*., 2005). However, these results are not consistently observed (White *et al*., 2002; Burkey *et al*., 2004). Kiarie *et al*., (2011) also reported that the MOS supplementation can positively influence the GIT functionality and decrease the adhesion of ETEC to the intestinal tract of weaned piglets challenged by an ETEC K88.

There is still a wide open field of study to understand the mechanisms that govern the dynamic interplay between diet, intestinal microbiota and host nutrition. Considering the new frontiers that the analysis of bacterial genomes and metagenomes it has been opened, it will allow the rational production of a desired prebiotic molecule with specific functional properties (Candela *et al*., 2010; Lane *et al*., 2010).

2.3.2.5.3. Synbiotics

A synbiotic is defined as "a mixture of probiotics and prebiotics that has beneficial effect on the host by improving the survival and persistence of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria" (Gibson and Roberfroid, 1995).

Some studies have confirmed a significant synergistic growth-stimulating effect (Kumprecht and Zobac, 1998; Shim *et al*., 2005; Modesto *et al*., 2009), reducing mortality and increasing the counts of species that are components of natural microflora (Nemcova *et al*., 1999; Frece *et al*., 2009). Furthermore, it was found that synbiotic preparations increase VFA production (Shim *et al*., 2007; Bird *et al*., 2009) and reduce environmental contamination by fecal noxious gas emission (Lee *et al*., 2009).

Protective effects have been attributed to different combinations. The positive contribution of maltodextrins and *L. paracasei* (Bomba *et al*., 2002, 2006) or *E. faecium* (Kumprecht and Zobac, 1998) on increasing the efficiency of piglets, reducing pathogenic *E. coli* growth in the digestive tract and their adhesion to intestinal mucosa have often been observed. Improved performance efficiency was also observed after the feeding of animals with biological preparations containing *L. fermentum*, *L. brevis*, *L. salivarius* or *E. faecium* with lactulose or lactitol (Bomba *et al*., 2002; Piva *et al*., 2005). Feeding a mixture of fructooligosaccharides and *L. paracasei* has been demonstrated to have a stimulating effect on the growth of natural intestinal microorganisms, to decrease the numbers of undesirable microflora including coliforms, *Clostridium* and *Enterobacteriaceae* and to improve the morphology of intestinal villi (Spencer *et al*., 1997; Nemcova *et al*., 1999; Bomba *et al*., 2002; Shim *et al*., 2005). Moreover, a higher effectiveness of synbiotics was shown when they were given to animals during the preweaning period (Shim *et al*., 2005). In the experiments performed by Bird *et al*. (2009), proliferation of *Bifidobacteria* in the intestinal tract occurred as a consequence of diet supplementation with *B. animalis* subsp. *lactis* and/or *B. choerinum* with fructooligosaccharides.

Potential advantages of using probiotics, prebiotics and/or its combination from a health and growth-promotion standpoint include partial replacement of antibiotic growth promoters. However, studies showing more consistent results are needed to justify its use as additives to pig diets. For all the claimed beneficial effects and studies conducted, a consensus has yet to be reached by the scientific community that prebiotics and probiotics consistently provide benefits in commercial settings. Moreover, their addition in the diet entails additional cost and thus must be evaluated thoroughly (Jacela *et al*., 2010).

2.3.2.6. Phage therapy

Bacteriophages (phages) are viruses that are excellent agents for killing pathogenic bacteria because they are not toxic to the animal host and they multiply in the bacterial host, leading to an

increase in titer of the phage as they destroy the bacterial that cause infection in humans and animals (Hanlon, 2007; O'Flaherty *et al*., 2009). Smith and Huggins (1982) started the investigation using phages and demonstrated its effectiveness to control septicemic *E. coli* infections and then applied this approach to prevent and treat an ETEC experimental disease in neonatal pigs, calves and lambs (Smith and Huggins, 1983; Smith *et al*., 1987 a, b). These studies firmly established the potential for phage therapy against ETEC infections in newborn pigs, but there have been no studies of phage therapy against ETEC K88 in weaned piglets (Fairbrother *et al*., 2005).

The most significant barrier to the deployment of phages against PWC in pigs is likely the requirements for licensing of phage products. The concern is that phages might become involved in transferring virulence and/or antimicrobial drug resistance genes in the intestine. It will therefore be necessary to demonstrate that phages used for prophylaxis and/or therapy lacks this capability (Fairbrother *et al*., 2005). Brüssow (2005) reviewed the use of phage therapy to treat *E. coli* infections and pointed out the applications of this technology for food sanitation and treatment of diarrhea, but also cited the industrial phage production and the steps to safely evaluate the effects of the phage therapy and successfully used for *E. coli* diarrhea (Denou *et al*., 2009).

2.3.2.7. Clay minerals

Another possibility for PWC prevention is the use of natural and modified clay minerals, which are naturally extracted from clays (bentonite, zeolite, kaolin etc.) and are a mixture of various minerals differing in chemical composition. The layers can be interconnected by a system of hydrogen bonds or a group of cations, and this space is termed an interlayer (Vondruskova *et al*., 2010). A common feature of clay minerals is a high sorption capacity determined by their stratified structure. Within the layers of these clays, substitution of other metal ions for silicon or aluminium can occur resulting in a net negative charge on the surface of the clay platelet. This charge imbalance is offset by hydrated cations, the predominant ones being Na^{+} and Ca_2^{+}. These interlaminar cations can be exchanged with other metal cations. In aqueous solutions, water is

intercalated into the interlaminar space of clay, leading to an expansion of the minerals (Ma and Guo, 2008).

Due to their sorption capacity and absence of primary toxicity, clay minerals are regarded as a simple and effective tool for chemical prevention of a series of toxic materials, not only in the environment, but also in the living bodies (Lemke *et al*., 2001; Phillips *et al*., 2002; Trckova *et al*., 2004). Multiple studies have confirmed the decontaminating properties of clay minerals. They can bind: aflatoxins (Phillips *et al*., 2002); plant metabolites (Dominy *et al*., 2004); and heavy metals (Hassen *et al*., 2003).

Kaolin-based medication is used for the therapy of diarrhea and digestive disorders in human medicine (Narkeviciute *et al*., 2002). There is also an abundance of published data which indicate that the dietary use of clay minerals reduces the incidence and decreases the severity and the duration of diarrhea in pigs (Ramu *et al*., 1997; Narkeviciute *et al*., 2002; Dominy *et al*., 2004; Papaioannou *et al*., 2004). Many studies have also documented significant improvements in body weight gain and feed conversion after supplementation of a diet with clay minerals (Papaioannou *et al*., 2004, 2005; Chen *et al*., 2005; Alexopoulos *et al*., 2007; Trckova *et al*., 2009).

The mechanisms by which the clay mineral reduce diarrhea could be related with several factor, as its ability to retard the rate of digestive passage through the intestines and to absorb water in more compact and better shaped faeces (Vondruskova *et al*., 2010); the ability to bind feed antigens or antinutritional components which can cause intestinal hypersensitivity, reduce the digestive enzyme activity and which can both result in a predisposition to infectious enteritis (Papaioannou, *et al*., 2004, 2005; Ma and Guo, 2008); the reduction of the number of pathogenic microorganisms and the depression of the activity of bacterial enzymes in the small intestinal digesta (Trckova *et al*., 2009); and the prevention of irritation and damage of mucosa impoving its morphological characteristics (Xia *et al*. 2004, 2005; Trckova *et al*., 2009).

However, the most direct effect in the gut environment is the likely ability to bind to potentially pathogenic bacteria as enterotoxigenin *E. coli* strains in weaned piglets (Hu and Xia,

2004; Trckova *et al*. 2009) and its enterotoxins (Ramu *et al*., 1997). Due to these consistent effects, clays are currently being study as a good alternative feed additive and it was also report that modifications of clay minerals by the addition of Cu2+ can improve their antibacterial activity (Xia *et al*. 2004; 2005), which is then a result of two mechanisms: one is electrostatic attraction which promotes the adherence of *E. coli* to the surface of clay minerals and the other is the slow release of Cu2+, which can kill bacteria (Xia *et al*., 2004).

The question of how much clay minerals reduce the occurrence and severity of PWC in piglets due to their adsorption qualities is still under discussion, but the supplementation of a diet with one to three per cent of clay-based adsorbents is generally recommended (Vondruskova *et al*., 2010).

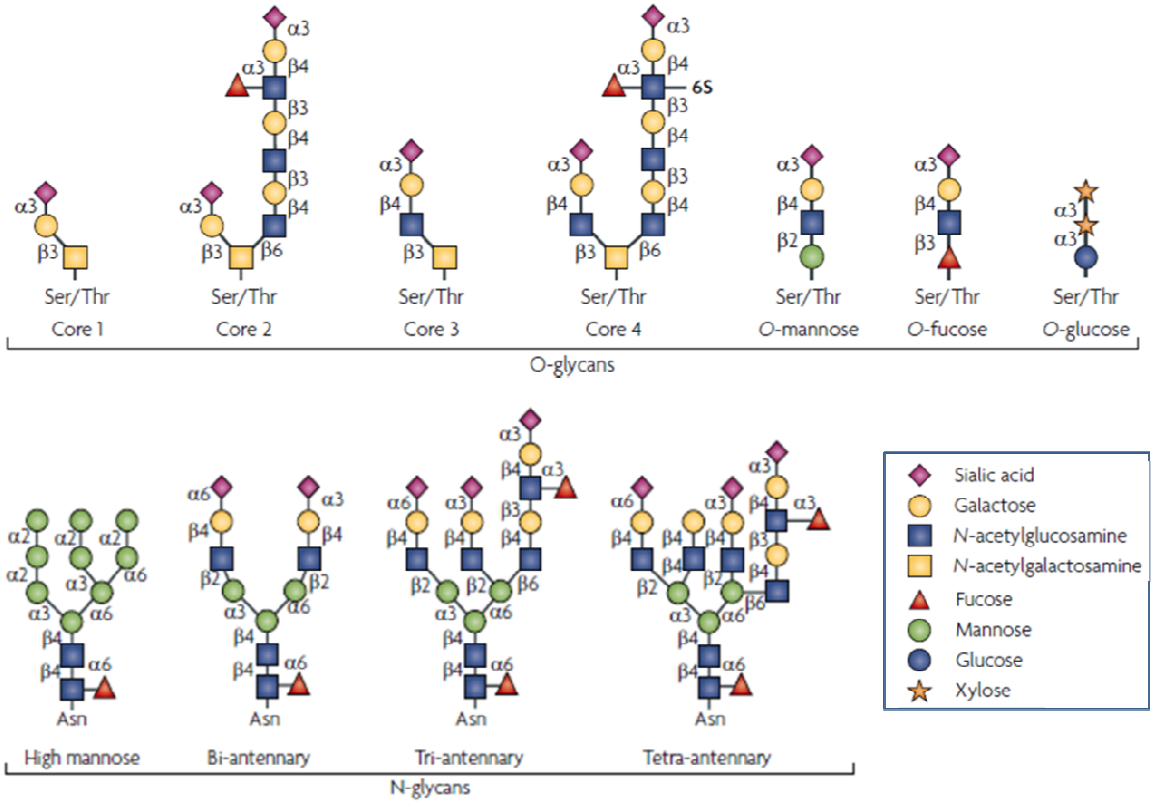
2.3.3. Interference with the adhesion process as a strategy.

The first and essential step in the infection process for most pathogens is to overcome the physical, chemical and immunological barriers of the host, and adhere to the target cells. But, this adhesion process does not occur by chance. Most pathogenic bacteria developed a recognition system using specific adhesin molecules, which recognize the affinitive receptor at the surface of the target cell and bind to it in order to find the conditions to survive and multiply. That specificity characteristic could explain why some *Salmonella enterica* strains are able to adhere and cause gastrointestinal infections while some uropathogenic *E coli* strains are not, but it can cause a severe urinary infection when reach this tissue (Shoaf-Sweeney and Hutkins, 2009).

Most of these receptors are of high complexity. They are compound by an arrangement through a natural enzymatic process that produces glycosidic linkages of saccharides to other saccharides, proteins and lipids (See Fig. 2.4), known as “glycosylation” (Marth and Grewal, 2008). The glycosylation characteristic of a receptor present multiple biological effects and encompasses ligands for proteinaceous structures known as “lectins” that are present in unicellular and multicelullar organisms such as bacteria, plants and animals (Sharon, 2009).

In this way, the characterization of these receptors and the affinitive lectins are of great importance to design or identify molecules able to interfere in the adhesion process and break the pathogenesis of the infectious disease, improving the health of the animals. The different feed manipulation strategies that can overlap the interactions between the intestinal receptors and pathogenic bacteria are further discussed.

Figure 2.4. Schematic representation of the composition of *O*- and *N*-glycosylation, where it can be observed how the sugars and proteins are arranged (from Marth and Grewal, 2008).



2.3.3.1. Proteases and F-4 receptors.

It was described that some exogenous and endogenous proteases may lower the activity of intestinal receptors. One of this proteases extensively studied is a mixture of proteolytic enzyme derived from pineapple and called bromelain. It has been reported that it has the capability of remove certain cell surface molecules that affect lymphocyte migration and activation (Hale, 2004) and could present an anti-inflammatory effect in the *in vitro* infection by *Salmonella enterica*

serovar Typhimurium (Mynott *et al*., 2002). Due to this anti-inflamatory effect, it was suggested to be used as a novel therapy for inflammatory bowel disease (Onken *et al*., 2007).

More specifically in the case of ETEC K88 infection, *in vitro* and *in vivo* studies showed that bromelain, administered orally to pigs, and reduced binding of ETEC K88 to the brush border in a dose-dependent manner (Mynott *et al*., 1996). Chandler and Mynott (1998) also reported that treatment with enteric-coated bromelain reduced the incidence of diarrhea following challenge with ETEC K88. In other studies however, bromelain only temporally inhibited the F4 receptor activity for approximately 30h, suggesting the necessity of a continuous treatment (Fairbrother *et al*., 2005). In any case the efficacy in a clinical situation remains to be demonstrated (Fairbrother and Gyles, 2006).

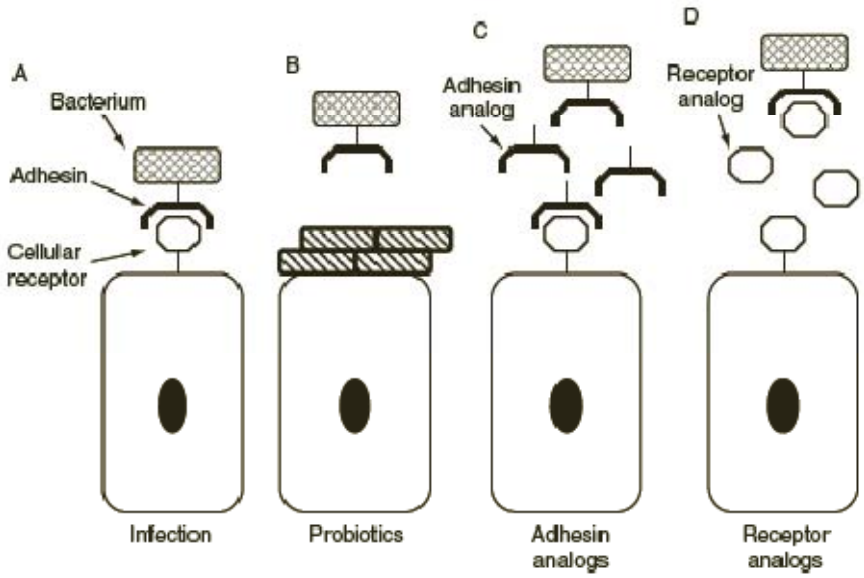
2.3.3.2. Blockage of bacterial fimbriae and intestinal receptors.

Figure 5 illustrate the hypothetical schematic adherence process (Fig. 2.5. A) and the likely anti-adhesives strategies (Fig 2.5. B-C). Probiotics, despite there is several mechanisms to inhibit enteric pathogens (already described in the previous section); it also may occupy the surface of the intestinal epithelium avoiding the attachment of pathogens to its receptors (Fig. 2.5.B). There are some other products, which we could call adhesin analogs, that may show anti-adhesive properties (Fig. 2.5.C), based in the fact that in a soluble state it may bind to their intended receptor, thereby blocking pathogen adherence (Shoaf-Sweeney and Hutkins, 2009). This strategy has not been practical to be used because they are almost always macromolecules, not readily available, must be used in high concentrations, and due to their innate nature may be toxic or immunogenic (Ofek *et al*., 2003). However, the most promising and well-studied anti-adhesive mechanisms are the inhibition of bacterial adherence via receptor analogs (Fig. 2.5. D), which it will be further presented.

Inhibition of bacterial adherence via receptor analogs is the most well-studied of the anti-adhesive mechanisms. 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. Soluble oligosaccharides that resemble or mimic host oligosaccharide receptors interrupt the adherence process by acting as receptor analogs or decoys. More precisely, rather than binding to host cells, pathogens bind to the soluble oligosaccharide decoys and are displaced from the intestinal tract preventing infection initiation and subsequent host tropism (Shoaf-Sweeney and Hutkins, 2009).

Figure 2.5. Schematic illustration of adherence (A) and anti-adhesive agents: probiotics (B), adhesin analogs (C), and receptor analogs (D). From Shoaf-Sweeney and Hutkins, 2009.



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, vegetables, fibrous ingredients, berries, and other foods (Lane *et al*., 2010). Moreover, there is now considerable evidence demonstrating that soluble oligosaccharides, specific for an adhesin, can competitively inhibit pathogens binding to target intestinal cells (Hyland *et al*., 2006a, 2008; Humphries *et al*., 2010) and the synthesis on gold nanoparticles seems to increase its inhibitory effect (Hyland *et al*., 2006b; Zhao *et al*., 2010).

The disruption or inhibition of pathogen attachment to host cells via anti-adherence agents has attracted considerable attention for several reasons. First, this approach is considered gentler

and ecologically sound compared to alternative approaches, such as using chemotherapy or antibiotic treatments (Shoaf-Sweeney and Hutkins, 2009). Some of the candidate anti-adhesive agents are even found naturally in foods (Sharon, 2009). In addition, although some resistance to anti-adhesive agents may possibly occur, dissemination of bacterial strains that are resistant to anti-adhesives will likely occur at a significantly lower rate compared to antibiotic-resistant strains (Ofek *et al*., 2003).

2.3.3.2.1. Plant compounds.

The potential of certain vegetal compounds to inhibit the adherence of microorganisms, specifically *E. coli*, to the intestinal epithelium has been reported dating back to the 1960s and showed that addition of insoluble fiber sources such as the husks from cereals could reduce the excretion of hemolytic *E. coli* and the incidence of PWC. For example, Smith & Halls (1968) found that barley hulls prevented disease in weaned piglets inoculated with *E. coli*. However, likely due to the negative connotation as "anti-nutritive" feed ingredient, the fibrous feedstuffs were not used rather than to control appetite in sows. But, more recently, a great interest has been paid specially to its capability to ferment, promote the stabilization of a healthy intestinal microbiota (Konstatinov *et al*., 2004) and control enteric bacterial disease in weaned piglets (Pluske *et al*., 2002). Different reports have described that increasing the fermentable carbohydrate content, through the inclusion of wheat bran, sugar beet pulp, native starch (Bikker *et al*., 2006; Carneiro *et al*., 2007; Hermes *et al*., 2009), or inulin (Wellock *et al*., 2008) reduced counts of coliform bacteria. Mateos *et al*. (2006) and Kim *et al*. (2008) also described that the inclusion of moderate levels of dietary fiber, such as oat hulls, reduced the incidence of diarrhea in diets based on cooked maize or processed (cooked or extruded) rice. However these *in vivo* studies do not elucidate the role of dietary fiber to improve the animal heath. One of the likely mechanisms involved could be the inhibition of the adherence of potentially pathogenic bacteria to the intestinal tract.

The *in vitro* assessment of the interactions between the vegetal compounds and pathogenic bacteria was possible with a miniaturized adhesion test (Becker *et al*., 2007), where it was successfully employed to evaluate the ETEC K88 adhesion to different naturally occurring polysaccharides present in some plants (Becker and Galletti, 2008). Using this model, our group identify the high affinity of ETEC K88 to a wheat bran (WB) extract (Molist *et al*., 2011), among other fibrous feedstuffs. This finding was also useful to explain the good results observed *in vivo* (Molist *et al*., 2010) with a 4% inclusion of WB in weaning diets and the reduction on the adhesion of *E. coli* to the ileal mucosa after an ETEC K88 challenge in piglets.

Other feedstuffs that could potentially inhibit or diminish the adhesion of bacteria in the intestinal tract are the mannan-rich plants, such as oil plam and carob seeds, with more than 50% of mannans that could specially act as binding sites for type-1 fimbriae bacteria, attracted by mannose carbohydrate reported (Becker and Galletti, 2008), whereas for other type of adhesins expressions, fucosylated, galactosylated, lactosyalated, and sialylated saccharides have been reported (Shoaf-Sweeney and Hutkins, 2009). For that reason a significant attention was paid for the anti-adhesive capability of milk oligosaccharides (especially for those rich in sialic acid and fucose) that will be further discussed.

2.3.3.2.2. Milk oligosaccharides

Milk contains a wide variety of glycoproteins that are involved in many biological activities, such as: antimicrobial activity, prebiotic effect and in the regulation of the immune system (Lonnerdal, 2003). Regarding the anti-adhesive capability, the initial evidence that such substances might exist was based on the long-standing observation that breast-fed infants appeared to suffer from fewer diarrheal diseases than formula-fed infants (Newburg, 2005). This apparent reduction in infection by diarrheal pathogens has been attributed to several components in human breast milk, including lactoferrin, casein peptides, and human milk oligosaccharides (Coppa *et al*., 2006;

Rhoades *et al*., 2005; Lane *et al*., 2010). These oligosaccharides can be found in nonconjugated (free) or conjugated form (glycolipids, glycoproteins).

The potential of milk-derived compounds to inhibit the adherence of microorganisms, specifically *E. coli*, to the intestinal epithelium has been studied *in vitro* by various authors. Schwertmann *et al*. (1999) described the potential of different milk glycoproteins to block the fimbriae of *E. coli*; Grange *et al*. (2002) described different proteins and glycoesfingolipids from pig serum; and Rhoades *et al*. (2005) described a milk peptide capable of reducing the numbers of *E. coli* in human colon adenocarcinoma epithelial cells cultured *in vitro*. Some reports suggest that milk contains glycoconjugates that have structural homology to the glycan moieties of intestinal mucosal cell surface and may act as competitive inhibitors of pathogen binding to their glycoconjugate receptors. Examples include oligosaccharides containing α1,2 linked fucosylated oligosaccharides (Newburg *et al*., 2004) or glycoproteins containing sialic compounds (Schwertmann *et al*., 1999).

In this way, the casein glycomacropeptide (CGMP), a glycoprotein originating from the C-terminal portion of κ-casein during cheese manufacture, was extensively characterized for its multiple health effects (Thomä-Worringer *et al*., 2006). The CGMP is entirely free of the aromatic amino acids, which has permitted its use on human phenylketonuria diets (LaClair *et al*., 2009). Furthermore, threonine constitutes 18% of the total amino acid content and a large portion is glycosylated resulting in a sialic acid content of around 4.2% (Nakano *et al*., 2007).

Several reviews described the biological activities of CGMP (Brody, 2000; Lonnerdal, 2003; Thomä-Worringer *et al*., 2006; Krissansen, 2007), and probably one of the most studied effects has been its interaction with the microbiota through the activity of carbohydrate moieties of the molecule. Some authors have reported that CGMP are able to bind the cholera toxin of *Vibrio cholera* (Kawasaki *et al*., 1992) and can promote the *in vitro* growth of *Bifidobacteria* (Idota *et al*., 1994) and *Lactococcus* species (Bouhallab *et al*., 1993), but inhibiting the growth of *Bacillus subtilis*, *Salmonella enterica* serovars Typhimurium and Enteriditis in Luria-Bertani medium

(Wong *et al*., 2006). Moreover, others studies have shown that CGMP inhibits the adhesion of pathogenic *E. coli* to the mucosal surface (Newburg, 1997, Rhoades *et al*., 2005) or its growth *in vitro* (Malkoski *et al*., 2001).

However, the activity of CGMP *in vivo*, especially in the distal segments of the gastrointestinal tract, has not been well explored. Peptides derived from CGMP have been detected in the intestinal lumen and blood after ingestion of milk products in humans (Chabance *et al*., 1998; Ledoux *et al*., 1999) and animals (Fosset *et al*., 2002), which suggests that, some CGMP fragments may resist the protein digestion, probably due to its *O*-glycosylation (Boutrou *et al*., 2008) and reach distal segment of the gastrointestinal tract.

2.3.3.2.3. Byproducts of fermentation industry.

The food industry use yeasts (mainly *Saccharomyces cerevisiae*) to ferment anaerobically organic compounds and generate alcohol, fermented foods and/or improve the conservation of food. The “residue” of this process is a product rich in microorganisms and cell walls from which they can be extracted fractions containing different kind of oligosaccharides, such as glucans and mannans. The structure of the mannan component resembles that of the surface glycoproteins containing mannose present on the mucosal surface of the intestine. The mannans act as high-affinity ligands for the mannose-specific type-1 fimbriae of pathogenic bacteria such as *Escherichia coli* (Tiralongo and Moran, 2010) and Salmonellae (Borowsky *et al*., 2009). In theory, pathogenic bacteria that normally adhere to mannans on the mucosal surface of the intestine may instead bind to the mannan component of MOS (Miguel *et al*., 2004; Kiarie *et al*., 2011). Because these pathogens do not attach to the mucosal surface of the intestine, they are flushed from the intestinal tract.

An *in vitro* agglutination experiment demonstrated that five of seven strains of *E coli* and seven of ten strains of *Salmonella enterica* serovar Typhimurium and Enteritidis were agglutinated by mannan oligosaccharide (Spring *et al*., 2000) Elimination of pathogens would presumably

enhance the health and growth of the young pigs. Castillo *et al*., (2008) reported that dietary MOS inclusion reduced the jejunal numbers of enterobacteria of early-weaned piglets and White *et al*. (2002) observed a reduction on the fecal coliform counts after an ETEC K88 challenge in piglets. Becker and Galletti (2008) presented a wide study comparing different feed ingredients rich in mannans, where it was measured the *in vitro* adhesion to several enteropathogens such as *Salmonella enterica* and *E. coli* K88 and concluded that the yeast MOS source showed a pronounced inhibitory effect on ETEC K88 adhesion, compared to other sources.

Aspergillus oryzae, a fungus used to ferment several traditional Japanese food products, possess galactomannans-proteins (Goto, 2007), O-glycans with monosyl residues (Nakajima and Ichishima, 1994) and fucose-specific lectins (Matsumura *et al*., 2007) which could act as binding sites for the enteropathogenic bacteria due to its mannose moieties. To our knowledge, there is not relevant information about the use of *Aspergillus oryzae* fermentation extract in pig nutrition. Despite that this products have been widely used in ruminant species (Caton *et al*., 1993; Varel and Kreikemeier, 1994; Zerby *et al*., 2011) aiming a positive impact on ruminal fermentation and microbiota; and avian species (Torres-Rodriguez *et al*., 2005; Tellez *et al*., 2010), with positive impact on the intestinal development and growth. But there is not enough information published describing the effects of the inclusion of this extract in the pig nutrition.

2.3.3.2.4. Bacterial exopolysaccharides.

Several lactic acid bacteria produce exopolysaccharides (EPS) that are secreted into the growth media or remain tightly attached to the cell surface. These high-molecular-weight sugar polymers have found a major application in the manufacture of fermented dairy products, specially low-fat yoghurt and cheese, providing thickening and gelling properties at low concentrations (Rodriguez-Carvajal *et al*., 2008).

However, adhesion studies with EPS are limited and only recently (Wang *et al*., 2010), it was report a study using EPS from lactic acid bacteria and porcine ETEC strains in a

hemagglutination assay to evaluate the adhesion of ETEC to porcine erythrocytes. The investigators reported that EPS can interfere with ETEC adhesion and therefore have the potential to benefit the swine industry. Moreover they suggest that antiadherence ability of EPS are due to a certain degree of structural specificity and further studies on the structure/function relationships among EPSs, ETEC adhesins, and host cell receptors are needed to gain a deeper understanding of the antiadhesive properties of bacterial EPS.

2.3.3.3. The immunomodulatory effect of anti-adhesive feedstuffs.

Presumably, the most direct effect of blocking the adhesion of pathogens to the intestinal tract it is the reduction of the innate immune response (earlier presented on section 2.1). However, it was described that certain bioactive feedstuffs can also stimulate the host defenses and are able to modulate the immune functions leading to the activation of phagocitic cells and/or the inflammation signs (Gallois *et al*., 2009).

The diet composition is important for the development of the immune system at the level of both organs and cells. Nutrients can influence host defense during the acute phase of the immune response, as this requires immediate changes, involving cell activation, proliferation and differentiation (Klasing, 2007). The most promising approach is reached by the addition of specific ingredients (such as fermentable carbohydrates) to the diet which will have potentially beneficial effects on the composition and activity of the GIT microbiota (Gil and Rueda, 2002).

Bauer *et al*. (2006) gather the scientific evidences about the influence of DF on the intestinal immune function. Although the mechanisms by which explain the immunomodulating effects of fermentable carbohydrates are still largely unknown, it seems that fiber act in two different ways, that may be contradictory. The first one is the positive stimulus to the growth of acid lactic bacteria, which directly activate the function of phagocitic cells (through the contact of colonic microbiota with GALT) and the production of immunoglobulins (specially IgA). But on the other hand, fermentable carbohydrates can increase the production of VFA, specially butyric acid which has an

anti-inflammatory effect (Segain *et al*., 2000); and it can influence positively the mucin production that prevents the adherence and translocation of bacteria across the epithelium (Looijer-van Langen and Dieleman, 2009). Also it has been reported that DF can directly up-regulate the expression of proinflammatoty cytokines in weaned piglets (Pié *et al*., 2007).

Glucans and mannans are already proposed as potential immunomodulatory agents for prophylaxis and therapy of infections in human (Masihi, 2000) and pigs (Sohn *et al*., 2000; Kogan and Kocher, 2007). However, its influence on immunity is not always reliable, as well as their effects on piglet performances. In piglets challenged with enteric pathogens (*E. coli* K88, *S. enterica*), health benefits of dietary mannans are not consistent (Gallois *et al*., 2009). For example, β-glucans have been shown to have anti-inflammatory properties (Li *et al*., 2005; 2006) and have been shown to modulate the acute phase response, whose regulation is known to be orchestrated by pro-inflammatory cytokines like IL-1 β, IL-6 or TNF-α (Niewold, 2007).

But, contradictorily, other investigators (Gantner *et al*., 2003; Eicher *et al*., 2006) reported that it may be involved in the binding to specific receptors on monocytes/macrophages and granulocytes triggering a cascade of immunological events. The same contradictory results were found with mannans, on one hand some authors found a decreased on blood neutrophils:lymphocytes ratio (Davis *et al*., 2004a), a reduction on lymphocyte proliferation (Davis *et al*., 2004b) and the recruitment of IEL into the small intestine lamina propia (Lizardo *et al*., 2008), suggesting that mannan supplementation could alleviate the inflammatory response associated with the stress of weaning. On the other hand other investigators reported that mannans can enhance the blood lymphocyte proliferation (Jensen *et al*., 2007) and the acute-phase protein response (Burkey *et al*., 2004). These conflicted results may be explained by their ability to adhere to a wide range of pathogenic bacteria (Kogan and Kocher, 2007) and to modulate immune functions (Sohn *et al*., 2000) or by its high glycosilation complexity that could create variable results (Marth and Grewal, 2008).

The inhibition of adhesion it could be also the mechanism by which other dietary ingredients may regulate the intestinal immune response. It was observed, for example, that milk, egg-yolk, honey, fungal, bacterial and/or plant oligosaccharides can inhibit the adhesion of pathogenic bacteria and it may be implicated in the reduction of intestinal inflammation (Daddaoua *et al*., 2005; Sharon, 2009; Tiralongo and Moran, 2009; Lane *et al*., 2010; Ghazarian *et al*., 2011; Mills *et al*., 2011).

The presented review allow us to understand that intestinal environment is a complex ecosystem where the cross interactions that occur between the nutrients, microbiota and the host cells determine how our body creates defenses to protect and overcome a dietary or microbial challenge. Recently, the discovery of new technologies such as the lectin arrays, proteo- and glyco-genomics (Sharon, 2009), as well as the massive sequencing of bacterial genomes and metagenomes (Candela *et al*., 2010) are allowing the identification and characterization of lectin-like interactions in cell recognition and adhesion from natural sources (Chow *et al*., 2010; Lane *et al*., 2010; Tiralongo and Moran, 2010; Ghazarian *et al*., 2011). These techniques will also improve our capability to select food and/or develop synthesized compounds which will be inexpensive, safe, interfere positively in immunity (Marth and Grewal, 2008; Chow *et al*., 2010) and promote health (Candela *et al*., 2010; Laparra and Sanz, 2010; Szarc vel Szic *et al*., 2010) in humans and animals.

2.4. Literature cited

Aarestrup FM, Seyfarth AMH, Emborg K, *et al*. (2001) Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* **45**, 2054-2059.

Akkermans ADL, Konstantinov SR, Zhu WYF, *et al*. (2003) Postnatal development of the intestinal microbiota of the pig. *Proceedings of the 9th International Symposium on Digestive Physiology in Pigs* [RO Ball, editor], 1st ed., pp 49-56. University of Alberta, Banf, Alberta, Canada.

Alexander TJL, Thornton K, Boon G, *et al*. (1980) Medicated early weaning to obtain pigs free from pathogens endemic in the herd of origin. *Vet Rec* **106**, 114-119.

Alexopoulos C, Papaioannou DS, Fortomaris P, *et al*. (2007) Experimental study on the effect of in-feed administration of a clinoptilolite-rich tuff on certain biochemical and hematological parameters of growing and fattening pigs. *Livest Sci* **111**, 230-241.

Anderson JW, Baird P, Davis Jr. RH, *et al.* (2009) Health benefits of dietary fiber. *Nutr Rev* **67**, 188-205.

Andersson M (2010) On the immune response in porcine enteric diseases, with special reference to swine dysentery, proliferative enteropathy and PMWS. PhD thesis. Swedish University, Uppsala, Sweden.

Arce C, Ramírez-Boo M, Lucena C, *et al.* (2010) Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from *Salmonella* Typhimurium. *Comp Immunol Microbiol Infect Dis* **33**, 161-174.

Bach Knudsen KE and Jensen BB (1991) Effect of source and level of dietary fibre on microbial fermentation in the large intestine. *Proceedings of the 5th International Symposium on Digestive Physiology*. [MW Verstegen, J Huisman and den LA Hartog, editors], pp 389-393. Waggeningen Academic Publishers, Waggeningen, The Netherlands.

Bailey M, Haverson K, Inman C, *et al*. (2005) The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effect or function. *Proc Nutr Soc* **64**, 451-457.

Bauer E, Williams BA, Verstegen MWA, *et al*. (2006) Fermentable carbohydrates: Potential dietary modulators of intestinal physiology, microbiology and immunity in pigs. In: *Biology of Growing Animals*, pp.33-63 [R Mosenthin, editor]. Elsevier, The Netherlands.

Baurhoo B, Letellier A, Zhao X, *et al.* (2007) Cecal populations of Lactobacilli and Bifidobacteria and *Escherichia coli* populations after *in vivo Escherichia coli* challenge in birds fed diets with purified lignin or mannanoligosaccharides. *Poult Sci* **86**, 2509-2516.

Barszcz M and Skomial J (2011) The development of the small intestine of piglets - chosen aspects. *J Anim Feed Sci* **20**, 3-15.

Becker PM, Galletti S, Hil PJR, *et al.* (2007) Validation of growth as measurand for bacterial adhesion to food and feed ingredients. *J Appl Microbiol* **103**, 2686-2696.

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

Bikker P, Dirkzwager A, Fledderus J, *et al*. (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J Anim Sci* **84**, 3337-3345.

Bird A, Vuaran M, Crittenden R, *et al*. (2009) Comparative effects of a high-amylose starch and a fructooligosaccharide on fecal *Bifidobacteria* numbers and short-chain fatty acids in pigs fed *Bifidobacterium animalis*. *Dig Dis Sci* **54**, 947-954.

Blank R, Mosenthin R, Sauer WC, *et al*. (1999) Effect of fumaric acid and dietary buffering capacity on ileal and fecal amino acid digestibilities in early-weaned pigs. *J Anim Sci* **77**, 2974-2984.

Bomba A, Nemcová R, Gancarcíková S, *et al*. (2002) Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fructo-oligosaccharides and polyunsaturated fatty acids. *Br J Nutr* **88**, S95-S99.

Bomba A, Jonecova Z, Koskova J, *et al*. (2006) The improvement of probiotics efficacy by synergistically acting components of natural origin: a review. *Biologia* **61**, 729-734.

Borovan L (2004) Plant alkaloids enhance performance of animals and improve the utilizability of amino acid. *Krmivarstvi* **6**, 36-37.

Borowsky L, Corção G, Cardoso M (2009) Mannanoligosaccharide agglutination by *Salmonella enterica* strains isolated from carrier pigs. *Braz J Microbiol* **40**, 458-464.

Bosi P, Casini L, Finamore A, *et al*. (2004) Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic *Escherichia coli* K88. *J Anim Sci* **82**, 1764-1772.

Bosi P, Mazzoni M, De Filippi S, *et al*. (2006) A continuous dietary supply of free calcium formate negatively affects the parietal cell population and gastric RNA expression for H+/K+-ATPase in weaning pigs. *J Nutr* **136**, 1229-1235.

Boudry G, Lallès JP, Malbert CH, *et al*. (2002) Diet-related adaptation of the small intestine at weaning in pigs is functional rather than structural. *J Pediatr Gastroenterol Nutr* **34**, 180-187.

Bouhallab S, Favrot C, Maubois JL (1993) Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactococcus lactis* subsp *lactis*. *Lait* **73**, 73-77.

Boutrou R, Jardin J, Blais A, *et al.* (2008) Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J Agric Food Chem* **56**, 8166-8173.

Boyaka PN, Marinaro M, Jackson RJ, *et al*. (2001) Oral QS-21 requires early IL-4 help for induction of mucosal and systemic immunity. *J Immunol* **166**, 2283-2290.

Bray TM and Bettger WJ (1990) The physiological role of zinc as an antioxidant. *Free Radic Biol Med* **3**, 281-291

Brody EP (2000) Biological activities of bovine glycomacropeptide. *Br J Nutr* **84**(SupplementS1), 39-46.

Bruininx EM, Binnendijk GP, van der Peet-Schwering CP, *et al*. (2002) Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs. *J Anim Sci* **80**, 1413-1418.

Bruininx EM, van der Peet-Schwering CM, Schrama JW, *et al*. (2001) The IVOG feeding station: A tool for monitoring the individual feed intake of group-housed weanling pigs. *J Anim Physiol Anim Nutr* **85**, 81-87.

Brussow H (2005) Phage therapy: the *Escherichia coli* experience. *Microbiol* **151**, 2133-2140.

BSAS, British Society of Animal Science (2003) *Nutrient Requirement Standards for Pigs.* Penicuik, Midlothian, UK: British Society of Animal Science.

Budiño FEL, Thomaz MC, Kronka RN, *et al*. (2005) Effect of probiotic and prebiotic inclusion in weaned piglet diets on structure and ultra-structure of small intestine. *Braz Archiv Biol Technol* **48**, 921-929.

Budzinski JW, Foster BC, Vandenhoek S, *et al*. (2000) An *in vitro* evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. *Phytomedicine* **7**, 273-282.

Burkey TE, Dritz SS, Nietfeld JC, *et al.* (2004) Effect of dietary mannanoligosaccharide and sodium chlorate on the growth performance, acute-phase response, and bacterial shedding of weaned pigs challenged with *Salmonella enterica* serotype Typhimurium. *J Anim Sci* **82**, 397-404.

Callaway TR, Morrow JL, Edrington TS, *et al*. (2006) Social stress increases fecal shedding of *Salmonella* Typhimurium by early weaned piglets. *Curr Issues in Intest Microbiol* **7**, 65-71.

Candela M, Maccaferri S, Turroni S, *et al.* (2010) Functional intestinal microbiome, new frontiers in prebiotic design. *Int J Food Microbiol* **140**, 93-101.

Carlson MS, Hill GM, Link JE (1999) Early- and traditionally weaned nursery pigs benefit from phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. *J Anim Sci* **77**, 1199-1207.

Carneiro M, Lordelo M, Cunha LF, *et al*. (2007) Microbial activity in the gut of piglets: II. Effect of fibre source and enzyme supplementation. *Livest Sci* **108**, 262-265.

Castillo M (2006) Development of gut microbiota in pig: modulation of bacterial communities by different feeding strategies. PhD thesis. Universitat Autònoma de Barcelona, Spain.

Castillo M, Martín-Orúe SM, Manzanilla EG, *et al*. (2006a) Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet Microbiol* **114**, 165-170.

Castillo M, Martin-Orue SM, Roca M, *et al*. (2006b) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. J Anim Sci 84, 2725-2734.

Castillo M, Skene G, Roca M, *et al*. (2007) Application of 16S rRNA gene-targetted fluorescence in situ hybridization and restriction fragment length polymorphism to study porcine microbiota along the gastrointestinal tract in response to different sources of dietary fibre. *FEMS Microbiol Ecol* **59**, 138-146.

Castillo M, Martin-Orue SM, Taylor-Pickard JA, *et al*. (2008) Use of mannanoligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: Effects on microbiota and gut function. *J Anim Sci* **86**, 94-101.

Caton JS, Erickson DO, Carey DA, *et al.* (1993) Influence of *Aspergillus oryzae* fermentation extract on forage intake, site of digestion, in situ degradability, and duodenal amino acid flow in steers grazing cool-season pasture. *J Anim Sci* **71**, 779-787.

Cera KR, Mahan DC, Cross RF, *et al*. (1988) Effect of age, weaning and postweaning diet on small intestinal growth and jejunal morphology in young swine. *J Anim Sci* **66**, 574-584.

Chabance B, Marteau P, Rambaud JC, *et al.* (1998) Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **80**, 155-165.

Chandler DS and Mynott TL (1998) Bromelain protects piglets from diarrhoea caused by oral challenge with K88 positive enterotoxigenic *Escherichia coli*. *Gut* **43**, 196-202.

Chen X, Gao S, Jiao X *et al.* (2004) Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. *Vet Microbiol* **103**, 13-20.

Chen J, Kwon J, Min BJ, *et al*. (2005) The effects of dietary biotite V supplementation as an alternative substance to antibiotics in growing pigs. *Asian Autral J Anim* **18**, 1642-1645.

Chiang BL, Sheih YH, Wang LH, *et al*. (2000) Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *Eur J Clin Nutr* **54**, 849-855.

Chow J, Lee SM, Shen Y, *et al.* (2010) Host–Bacterial symbiosis in health and disease. In: *Advances in Immunology*, pp.243-274 [Sidonia Fagarasan and Andrea Cerutti, editor]. Academic Press.

Close WH (2000) Producing pigs without antibiotic growth promoters. In: *Proceedings of the 2000 Banff Pork Seminar*, pp.47-56. University of Alberta, Edmonton, Canada.

Coates M, Fuller R, Harrison G, *et al*. (1963) A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. *Br J Nutr* **17**, 141-150.

Coddens A, Verdonck F, Tiels P, *et al.* (2007) The age-dependent expression of the F18+ *E. coli* receptor on porcine gut epithelial cells is positively correlated with the presence of histo-blood group antigens. *Vet Microbiol* **122**, 332-341.

Coffey RD & Cromwell GL (1995) The impact of environment and antimicrobial agents on the growth response of early-weaned pigs to spray-dried porcine plasma. *J Anim Sci* **73**, 2532-2539.

Conway PL, Welin A, Cohen PS (1990) Presence of K88-specific receptors in porcine ileal mucus is age dependent. *Infect Immun* **58**, 3178-3182.

Conway PL (1997) Development of intestinal microbiota. Gastrointestinal microbes and host interactions. *Gastrointestinal Microbiology*. [RI Mackie, BA White and RE Isaacson, editors], pp. 3-39. Chapman and Hall, London, UK.

Coppa GV, Zampini L, Galeazzi T, *et al.* (2006) Human Milk Oligosaccharides Inhibit the Adhesion to Caco-2 Cells of Diarrheal Pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. *Pediatr Res* **59**, 377-382.

Corring T, Aumaitre A and Durand G (1978) Development of digestive enzymes in the piglet from birth to 8 weeks. *Ann Nutr Metab* **22**, 231-243.

Costa LB, Tse MLP, Miyada VS (2007) Herbal extracts as alternatives to antimicrobial growth promoters for weanling pigs. *Rev Bras Zootec* **36**, 589-595.

Cowan MM (1999) Plant Products as Antimicrobial Agents. *Clin Microbiol Rev* **12**, 564-582.

Creus E (2007) Mesures diIntervenció per al control de *Salmonella* en la cadena de producció porcina. PhD thesis. Barcelona, Spain: Universitat Autònoma de Barcelona.

Creus E, Pérez JF, Peralta B, *et al.* (2007) Effect of Acidified Feed on the Prevalence of Salmonella in Market-age Pigs. *Zoonoses and Public Health* **54**, 314-319.

Cromwell GL (2002) Why and how antibiotics are used in swine production. *Anim Biotechnol* **13**, 7-27.

Cutler SA, Lonergan SM, Cornick N, *et al.* (2007) Dietary inclusion of Colicin E1 is effective in preventing postweaning diarrhea caused by F18-positive *Escherichia coli* in pigs. *Antimicrob Agents Chemother* **51**, 3830-3835.

Daddaoua A, Puerta V, Zarzuelo A, *et al.* (2005) Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J Nutr* **135**, 1164-1170.

Davies P, Funk J and Morrow WEM (1997) Fecal shedding of *Salmonella* by a cohort of finishing pigs in North Carolina. *J Swine Health Prod* **7**, 231-234.

Davis ME, Brown DC, Maxwell CV, *et al.* (2004a) Effect of phosphorylated mannans and pharmacological additions of zinc oxide on growth and immunocompetence of weanling pigs. *J Anim Sci* **82**, 581-587.

Davis ME, Maxwell CV, Erf GF, *et al.* (2004b) Dietary supplementation with phosphorylated mannans improves growth response and modulates immune function of weanling pigs. *J Anim Sci* **82**, 1882-1891.

de Haan Ld and Hirst TR (2004) Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms - review. *Mol Membr Biol* **21**, 77-92.

Denou E, Bruttin A, Barretto C, *et al.* (2009) T4 phages against *Escherichia coli* diarrhea: potential and problems. *Virology* **388**, 21-30.

Dibner J and Richards J (2005) Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* **84**, 634-643.

Di Pasqua R, Betts G, Hoskins N, *et al.* (2007) Membrane Toxicity of Antimicrobial Compounds from Essential Oils. *J Agric Food Chem* **55**, 4863-4870.

Do T, Stephens C, Townsend K, *et al.* (2005) Rapid identification of virulence genes in enterotoxigenic *Escherichia coli* isolates associated with diarrhoea in Queensland piggeries. *Aust Vet J* **83**, 293-299.

Dominy NJ, Davoust E, Minekus M (2004) Adaptive function of soil consumption: an in vitro study modeling the human stomach and small intestine. *J Exp Biol* **207**, 319-324.

Dorman HJD & Deans SG (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol* **88**, 308-316.

Doyle MP & Erickson MC (2011) Opportunities for mitigating pathogen contamination during on-farm food production. *Int J Food Microbiol* **In Press, Corrected Proof**.

Dreyfus LA, Harville B, Howard DE, *et al.* (1993) Calcium influx mediated by the *Escherichia coli* heat-stable enterotoxin B (STb). *Proc Natl Acad Sci USA* **90**, 3202-3206.

Eicher SD, McKee CA, Carroll JA, *et al.* (2006) Supplemental vitamin C and yeast cell wall β-glucan as growth enhancers in newborn pigs and as immunomodulators after an endotoxin challenge after weaning. *J Anim Sci* **84**, 2352-2360.

Ermer PM, Miller PS, Lewis AJ (1994) Diet preference and meal patterns of weanling pigs offered diets containing either spray-dried porcine plasma or dried skim milk. *J Anim Sci* **72**, 1548-1554.

Erume J, Berberov EM, Kachman SD, *et al.* (2008) Comparison of heat-labile enterotoxin and heat-stable enterotoxin-b expression to the virulence of F4ac enterotoxigenic *Escherichia coli* in young pigs. *Infect Immun* **76**, 3141-3149.

Ewing WN and Cole DJA (1994) *The living gut*. 1st ed. The Midlands Book. Dungannom, N. Ireland,

Falk PG, Hooper LV, Midtvedt T, *et al*. (1998) Creating and maintaining the gastrointestinal ecosystem: What we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* **62**, 1157-1170.

Fairbrother JM, Nadeau É, Gyles CL (2005) *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* **6**, 17-39.

Fairbrother JM and Gyles CL (2006) *Escherichia coli* infections. In: *Diseases of Swine*, pp.639-674 [Straw BE, Zimmerman JJ, D'Allaire S *et al.*, editors]. Ames, Iowa, USA: Blackwell Publishing Professional.

Force M, Sparks WS, Ronzio RA (2000) Inhibition of enteric parasites by emulsified oil of oregano *in vivo*. *Phytother Res* **14**, 213-214.

Fosset S, Fromentin G, Gietzen DW, *et al.* (2002) Peptide fragments released from Phe-caseinomacropeptide in vivo in the rat. *Peptides* **23**, 1773-1781.

Frece J, Kos B, Svetec IK, *et al*. (2009) Synbiotic effect of Lactobacillus helveticus M92 and prebiotics on the intestinal microflora and immune system of mice. *J Dairy Res* **76**, 98-104.

Francis DH (2002) Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. *J Swine Health Prod* **10**, 171-175.

Francis DH, Grange PA, Zeman DH, *et al.* (1998) Expression of mucin-type glycoprotein K88 receptors strongly correlates with piglet susceptibility to K88+ enterotoxigenic *Escherichia coli*, but adhesion of this bacterium to brush borders does not. *Infect Immun* **66**, 4050-4055.

Franklin MA, Mathew AG, Vickers JR, *et al*. (2002) Characterization of microbial populations and volatile fatty acid concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 vs 24 days of age. *J Anim Sci* **80**, 2904-2910.

Friesen KG, Goodband RD, Nelssen JL, *et al*. (1993) The effect of pre- and postweaning exposure to soybean meal on growth performance and on the immune response in the early-weaned pig. *J Anim Sci* **71**, 2089-2098.

Frydendahl K (2002) Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. *Vet Microbiol* **85**, 169-182.

Frydendahl K, Jensen TK, Andersen JS, *et al.* (2003) Association between the porcine *Escherichia coli* F18 receptor genotype and phenotype and susceptibility to colonisation and postweaning diarrhoea caused by *E. coli* O138:F18. *Vet Microbiol* **93**, 39-51.

Funk JA, Davies PR, Nichols MA (2001) Longitudinal study of *Salmonella enterica* in growing pigs reared in multiple-site swine production systems. *Vet Microbiol* **83**, 45-60.

Gaggìa F, Mattarelli P, Biavati B (2010) Probiotics and prebiotics in animal feeding for safe food production. *Int J Food Microbiol* **141**, S15-S28.

Gálfi P & Bokori J (1990) Feeding trial in pigs with a diet containing sodium n-butyrate. *Acta Vet Hung* **38**, 3-17.

Gallois M, Rothkötter HJ, Bailey M, *et al*. (2009) Natural alternatives to in-feed antibiotics in pig production: can immunomodulators play a role? *Animal* **3**, 1644-1661.

Gantner BN, Simmons RM, Canavera SJ, *et al.* (2003) Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. *J Experim Med* **197**, 1107-1117.

Garcia CC, Talarico L, Almeida N, *et al*. (2003) Virucidal activity of essential oils from aromatic plants of San Luis, Argentina. *Phytother Res* **17**, 1073-1075.

Geary TM and Brooks PH (1998) The effect of weaning weight and age on the post-weaning growth performance of piglets fed fermented liquid diets. *Pig Journal* **42**, 10-23.

Geens MM and Niewold TA (2010) Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli*, *Escherichia coli*, and *E. coli* lipopolysaccharide. *Comp Funct Genom* **2010**, 1-11.

Ghazarian H, Idoni B, Oppenheimer SB (2011) A glycobiology review: Carbohydrates, lectins and implications in cancer therapeutics. *Acta Histochem* **113**, 236-247.

Giesting DW, Roos MA, Easter RA (1991) Evaluation of the effect of fumaric acid and sodium bicarbonate addition on performance of starter pigs fed diets of different types. *J Anim Sci* **69**, 2489-2496.

Gil A & Rueda R (2002) Interaction of early diet and the development of the immune system. *Nutr Res Rev* **15**, 263-292.

Goto M (2007) Protein O-glycosylation in fungi: diverse structures and multiple functions. *Biosci Biotechnol Biochem* **71**, 1415-1427.

Grange PA, Erickson AK, Anderson TJ, *et al.* (1998) Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of *Escherichia coli*. *Infect Immun* **66**, 1613-1621.

Grange PA, Mouricout MA, Levery SB, *et al.* (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**, 2336-2343.

Griffith RW, Schwartz KJ, Meyerholz DK (2006) Salmonella. In: *Diseases of Swine*. [BE Straw, JJ Zimmerman, S D'Allaire and DJ Taylor, editors], 9th ed., chapter 45, pp. 739-754. Blackwell Publishing Professional, Ames, Iowa, USA.

Guo X, Li D, Lu W, *et al*. (2006) Screening of Bacillus strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of *Bacillus subtilis* MA139 in pigs. *Antonie Van Leeuw J Microb* **90**, 139-146; 146.

Hahn JD and Baker DH (1993) Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. *J Anim Sci* **71**, 3020-3024.

Hale LP (2004) Proteolytic activity and immunogenicity of oral bromelain within the gastrointestinal tract of mice. *Int Immunopharmacol* **4**, 255-264.

Hamer HM, Jonkers D, Venema K *et al*. (2008) Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* **27**, 104-119.

Hammes W and Hertel C (2006) The genera *lactobacillus* and *carnobacterium*. In: *The Prokaryotes*, pp.320-403; 403 [Stackebrandt E, editor]. Springer New York.

Hampson DJ, Pluske JR, Pethick DW (2001) Dietary manipulation of enteric disease. *Pig News and Inform* **22**, 21-28.

Hanlon GW (2007) Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents* **30**, 118-128.

Harvey RB, Anderson RC, Genovese KJ, *et al*. (2005) Use of competitive exclusion to control enterotoxigenic strains of *Escherichia coli* in weaned pigs. *J Anim Sci* **83**, E44-47.

Hassen A, Jamoussi F, Saidi N, *et al*. (2003) Microbial and copper adsorption by smectitic clay-an experimental study. *Environ Technol* **24**, 1117-1127.

Hedemann MS, Eskildsen M, Laerke HN, *et al.* (2006) Intestinal morphology and enzymatic activity in newly weaned pigs fed contrasting fiber concentrations and fiber properties. *J Anim Sci* **84**, 1375-1386

Heo J, Kim J, Hansen CF, *et al.* (2008) Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. *Arch Anim Nutr* **62**, 343-358.

Heo JM, Kim JC, Hansen CF, *et al.* (2009) Feeding a diet with a lower protein content reduces indices of protein fermentation and the incidence of post-weaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of *Escherichia coli*. *J Anim Sci* **87**, 2833-2843.

Heo JM, Kim JC, Hansen CF, *et al.* (2010) Effects of dietary protein level and zinc oxide supplementation on the incidence of post-weaning diarrhoea in weaner pigs challenged with an enterotoxigenic strain of *Escherichia coli*. *Livestock Science* **133**, 210-213.

Hermes RG, Molist F, Ywazaki M, *et al*. (2009) Effect of dietary level of protein and fiber on the productive performance and health status of piglets. *J Anim Sci* **87**, 3569-3577.

Hermes RG, Molist F, Ywazaki M, *et al*. (2010) Effects of type of cereal and fibre level on growth and parameters of the gastrointestinal tract in young pigs. *Livest Sci* **133**, 225-228.

Hill GM, Cromwell GL, Crenshaw TD, *et al*. (2000) Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). *J Anim Sci* **78**, 1010-1016.

Hill JE, Hemmingsen SM, Goldade BG, *et al*. (2005) Comparison of ileum microflora of pigs fed corn-, wheat-, or barley-based diets by chaperonin-60 sequencing and quantitative PCR. *Appl Environ Microbiol* **71**, 867-875.

Hooper LV, Wong MH, Thelin A, *et al*. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. *Science* **291**, 881-884.

Hoblet KH, Kohler EM, Saif LJ, *et al.* (1986) Study of porcine postweaning diarrhea involving K88(-) hemolytic *Escherichia coli*. *Am J Vet Res* **47**, 1910-1912.

Hopwood DE and Hampson DJ (2003) Interactions between the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immediate post-weaning period. *Weaning the Pig. Concepts and Consequences*, 1st ed., pp. 199-218 [JR Pluske, J Le Dividich and MWA Verstegen, editors]. Wageningen Academic Publishers, Wageningen, The Netherlands.

Hopwood DE, Pethick DW, Pluske JR, *et al*. (2004) Addition of pearl barley to a rice-based diet for newly weaned piglets increases the viscosity of the intestinal contents, reduces starch digestibility and exacerbates post-weaning colibacillosis. *Br J Nutr* **92**, 419-427.

Houdijk JGM, Hartemink R, Verstegen MWA, *et al.* (2002) Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. *Archiv Anim Nutr* **56**, 297-307.

Houdijk JGM, Campbell FM, Fortomaris PD, *et al.* (2007) Effects of sub-clinical post-weaning colibacillosis and dietary protein on acute phase proteins in weaner pigs. *Livestock Science* **108**, 182-185.

Hu C & Xia M (2006) Adsorption and antibacterial effect of copper-exchanged montmorillonite on *Escherichia coli* K88. *Appl Clay Sci* **31**, 180-184.

Humphries RM, Griener TP, Vogt SL *et al.* (2010) N-acetyllactosamine-induced retraction of bundle-forming pili regulates virulence-associated gene expression in enteropathogenic *Escherichia coli*. *Mol Microbiol* **76**, 1111-1126.

Hyland RM, Griener TP, Mulvey GL, *et al.* (2006) Basis for N-acetyllactosamine-mediated inhibition of enteropathogenic *Escherichia coli* localized adherence. *J Med Microbiol* **55**, 669-675.

Hyland R, Beck P, Mulvey G, *et al.* (2006) N-acetyllactosamine conjugated to gold nanoparticles inhibits enteropathogenic *Escherichia coli* colonization of the epithelium in human intestinal biopsy specimens. *Infect Immun* **74**, 5419-5421.

Hyland RM, Sun J, Griener TP, *et al.* (2008) The bundlin pilin protein of enteropathogenic *Escherichia coli* is an N-acetyllactosamine-specific lectin. *Cell Microbiol* **10**, 177-187.

Idota T, Kawakami H, Nakajima I (1994) Growth-promoting effects of N-Acetylneuraminic acid-containing substances on Bifidobacteria. *Biosci Biotechnol Biochem* **58**, 1720-1722.

Inoue R, Tsukahara T, Nakanishi N, *et al*. (2005) Development of the intestinal microbiota in the piglet. *J Gen Appl Microbiol* **51**, 257-265.

Jacela JY, DeRouchey JM, Tokach MD (2010a) Feed additives for swine: Fact sheets – high dietary levels of copper and zinc for young pigs, and phytase. *J Swine Health Prod* **18**, 87-91.

Jacela JY, DeRouchey JM, Tokach MD (2010b) Feed additives for swine: Fact sheets – prebiotics and probiotics, and phytogenics. *J Swine Health Prod* **18**, 132-136.

Jensen BB and Jorgensen H (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60**, 1897-1904.

Jensen GM, Frydendahl K, Svendsen O, *et al.* (2006) Experimental infection with *Escherichia coli* O149:F4ac in weaned piglets. *Veterinary Microbiology* **115**, 243-249.

Jiang R, Chang X, Stoll B, *et al*. (2000) Dietary plasma protein reduces small intestinal growth and lamina propria cell density in early weaned pigs. *J Nutr* **130**, 21-26.

Jin LZ, Baidoo SK, Marquardt RR, *et al*. (1998) *In vitro* inhibition of adhesion of enterotoxigenic *Escherichia coli* K88 to piglet intestinal mucus by egg-yolk antibodies. *FEMS Immunol Med Microbiol* **21**, 313-321.

Kaeffer B, Bottreau E, Velge P, *et al.* (1993) Epithelioid and fibroblastic cell lines derived from the ileum of an adult histocompatible miniature boar (d/d haplotype) and immortalized by SV40 plasmid. *Eur J Cell Biol* **62**, 152-162.

Kamel C (2001) Tracing modes of action and roles of plant extracts in non-ruminants. In: *Recent Advances in Animal Nutrition*, pp.135-150 [Garnsworthy PC & Wiseman J, editors]. Nottingham: Nottingham University Press.

Kamphues J, Tabeling R, Stuke O, *et al.* (2007) Investigations on potential dietetic effects of lactulose in pigs. *Livest Sci* **109**, 93-95.

Katouli M, Lund A, Wallgren P, *et al*. (1997) Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. *J Appl Microbiol* **83**, 147-154.

Kawasaki Y, Isoda H, Tanimoto M, *et al.* (1992) Inhibition by lactoferrin and κ-Casein glycomacropeptide of binding of cholera toxin to its receptor. *Biosci Biotechnol Biochem* **56**, 195-198.

Kelly D, Smyth JA and McCracken KJ (1991) Digestive development of the early-weaned pig. *Br J Nutr* **65**, 169-180.

Kenworthy R and Crabb WE (1963) The intestinal flora of young pigs, with reference to early weaning, *Escherichia coli* and scours. *J Comp Pathol* **73**, 215-228.

Kiarie E, Bhandari S, Scott M, *et al.* (2011) Growth performance and gastrointestinal microbial ecology responses of piglets receiving *Saccharomyces cerevisiae* fermentation products after an oral challenge with *Escherichia coli* (K88). *J Anim Sci* **89**, 1062-1078.

Kim JC, Mullan BP, Hampson DJ, *et al*. (2008) Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract. *Br J Nutr* **99**, 1217-1225.

King DE, Asem EK and Adeola O (2000) Ontogenetic development of intestinal digestive functions in white pekin ducks. *J Nutr* **130**, 57-62.

Klasing KC (2007) Nutrition and the immune system. *Br Poult Sci* **48**, 525-- 537.

Kogan G and Kocher A (2007) Role of yeast cell wall polysaccharides in pig nutrition and health protection. *Livest Sci* **109**, 161-165.

Koh SY, George S, Brözel V, *et al.* (2008) Porcine intestinal epithelial cell lines as a new *in vitro* model for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli*. *Vet Microbiol* **130**, 191-197.

Konstantinov SR, Favier CF, Zhu WY, *et al.* (2004) Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. *Anim Res* **53**, 317-324.

Konstantinov SR, Awati AA, Williams BA, *et al*. (2006) Post-natal development of the porcine microbiota composition and activities. *Environ Microbiol* **8**, 1191-1199.

Koh WS, Yoon SY, Kwon BM, *et al*. (1998) Cinnamaldehyde inhibits lymphocyte proliferation and modulates T-cell differentiation. *Int J Immunopharmacol* **20**, 643-660.

Kohlert C, van Rensen I, Marz R, *et al*. (2000) Bioavailability and pharmacokinetics of natural volatile terpenes in animals and humans. *Planta Med* **66**, 495-505.

Konstantinov SR, Zhu YH, Williams BA, *et al*. (2003) Effect of fermentable carbohydrates on piglet faecal bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *FEMS Microbiol Ecol* **43**, 225-235.

Krissansen GW (2007) Emerging health properties of whey proteins and their clinical implications. *J Am Coll Nutr* **26**, 713S-723.

Kyriakis SC, Tsiloyiannis VK, Vlemmas J, *et al*. (1999) The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. *Res Vet Sci* **67**, 223-228.

LaClair CE, Ney DM, MacLeod EL, *et al.* (2009) Purification and use of glycomacropeptide for nutritional management of phenylketonuria. *J Food Sci* **74**, E199-E206.

Lallès, JP, Boudry G, Favier CF, *et al*. (2004) Gut function and dysfunction in young pigs: physiology. *Anim Res* **53**, 301-316.

Lallès JP, Bosi P, Smidt H, *et al*. (2007) Weaning — A challenge to gut physiologists. *Livest Sci* **108**, 82-93.

Lallès JP, Bosi P, Janczyk P, *et al*. (2009) Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. *Animal* **3**, 1625-1643.

Lane JA, Mehra RK, Carrington SD, *et al.* (2010) The food glycome: A source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol* **142**, 1-13.

Lange CFM, Pluske J, Gong J, *et al.* (2010) Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livestock Science* **134**, 124-134.

Laparra JM and Sanz Y (2010) Interactions of gut microbiota with functional food components and nutraceuticals. *Pharmacological Research* **61**, 219-225.

Ledoux N, Mahé S, Dubarry M, *et al.* (1999) Intraluminal immunoreactive caseinomacropeptide after milk protein ingestion in humans. *Nahrung/Food* **43**, 196-200.

Le Dividich J and Sève B (2000) Effects of underfeeding during the weaning period on growth, metabolism, and hormonal adjustments in the piglet. *Domest Anim Endocrin* **19**, 63-74.

Lee SJ, Shin NH, Ok JU, *et al*. (2009) Effects of dietary synbiotics from anaerobic microflora on growth performance, noxious gas emission and fecal pathogenic bacteria population in weaning pigs. *Asian Austral J Anim* **22**, 1202-1208.

Lemke SL, Mayura K, Reeves WR, *et al*. (2001) Investigation of organophilic montmorillonite clay inclusion in zearalenone-contaminated diets using the mouse uterine weight bioassay. *J Toxicol Environ Health* **62**, 243-258.

Li J, Xing J, Li D, *et al.* (2005) Effects of β-glucan extracted from *Saccharomyces cerevisiae* on humoral and cellular immunity in weaned piglets. *Arch Anim Nutr* **59**, 303-312.

Li J, Li DF, Xing JJ, *et al.* (2006) Effects of β -glucan extracted from *Saccharomyces cerevisiae* on growth performance, and immunological and somatotropic responses of pigs challenged with *Escherichia coli* lipopolysaccharide. *J Anim Sci* **84**, 2374-2381.

Lin B, Gong J, Wang Q, *et al.* (2011) *In-vitro* assessment of the effects of dietary fibers on microbial fermentation and communities from large intestinal digesta of pigs. *Food Hydroc* **25**, 180-188.

Liu P, Piao XS, Kim SW, *et al*. (2008) Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of *Escherichia coli* and Lactobacillus in weaning pigs. *J Anim Sci* **86**, 2609-2618.

Lizardo R, Nofrarias M, Guinvarch J, *et al*. (2008) Influence de l'incorporation de levures Saccharomyces cerevisiae ou de leurs parois dans l'aliment sur la digestion et les performances zootechniques des porcelets en post-sevrage. *40èmes Journées de la Recherche Porcine, Paris, France* , 183-190.

Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr ; Am J Clin Nutr* **77**, 1537S-1543.

Looijer-van Langen M and Dieleman LA (2009) Prebiotics in chronic intestinal inflammation. *Inflamm Bowel Dis* **15**, 454-462.

Lotz M, Ménard S, Hornef M (2007) Innate immune recognition on the intestinal mucosa. *Int J Med Microbiol* **297**, 379-392.

Mackie RI, Sghir A and Gaskins HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. *Am J Clin Nutr* **69**, 1035-1045.

Madec F, Bridoux N, Bounaix S, *et al.* (2000) Experimental models of porcine post-weaning colibacillosis and their relationship to post-weaning diarrhoea and digestive disorders as encountered in the field. *Vet Microbiol* **72**, 295-310.

Mahmoud AL (1994) Antifungal action and antiaflatoxigenic properties of some essential oil constituents. *Lett Appl Microbiol* **19**, 110-113.

Makkink CA, Negulescu GP, Qin G, *et al*. (1994) Effect of dietary protein source on feed intake, growth, pancreatic enzyme activities and jejunal morphology in newly-weaned piglets. *Br J Nutr* **72**, 353-368.

Malkoski M, Dashper SG, O'Brien-Simpson NM, *et al.* (2001) Kappacin, a Novel Antibacterial Peptide from Bovine Milk. *Antimicrob Agents Chemother ; Antimicrob Agents Chemother* **45**, 2309-2315.

Mantere-Alhonen S (1995) Propionibacteria used as probiotics - A review. *Lait* **75**, 447-452.

Manzanilla EG, Perez JF, Martin M, *et al*. (2004) Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *J Anim Sci* **82**, 3210-3218.

Manzanilla EG, Nofrarias M, Anguita M, *et al*. (2006) Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. *J Anim Sci* **84**, 2743-2751.

Mariani V, Palermo S, Fiorentini S, *et al.* (2009) Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. *Vet Immunol Immunopathol* **131**, 278-284.

Marth JD and Grewal PK (2008) Mammalian glycosylation in immunity. *Nat Rev Immunol* **8**, 874-887.

Martinez-Puig D, Manzanilla EG, Morales J, *et al*. (2007) Dietary nucleotide supplementation reduces occurrence of diarrhoea in early weaned pigs. *Livest Sci* **108**, 276-279.

Marinho MC, Lordelo MM, Cunha LF, *et al*. (2007) Microbial activity in the gut of piglets: I. Effect of prebiotic and probiotic supplementation. *Livest Sci* **108**, 236-239.

Masihi KN (2000) Immunomodulatory agents for prophylaxis and therapy of infections. *Int J Antimicrob Agents* **14**, 181-191.

Mateos GG, Martin M, Latorre MA, *et al*. (2006) Inclusion of oat hull in diets for young pigs based on cooked maize or cooked rice. *Anim Sci* **82**, 57-63.

Mateos GG, López E, Latorre MA, *et al*. (2007) The effect of inclusion of oat hulls in piglet diets based on raw or cooked rice and maize. *Anim Feed Sci Technol* **135**, 100-112.

Máthé Á (2009) Essential oils - biochemistry, production and utilization. In: *Phytogenics in Animal Nutrition. Natural Concepts to Optimize Gut Health and Performance*, pp.1-18 [Steiner T, editor]. Nottingham, United Kingdom: Nottingham University Press.

Matsumura K, Higashida K, Ishida H, *et al.* (2007) Carbohydrate binding specificity of a fucose-specific lectin from *Aspergillus oryzae*. *J Biol Chem* **282**, 15700-15708.

Matsuzaki T and Chin J (2000) Modulating immune responses with probiotic bacteria. *Immunol Cell Biol* **78**, 67-73.

Maxwell CV and Carter SD (2001) Feeding the weaned pig. In: *Swine Nutrition*, pp.691-715 [Lewis, Austin J. and Lee Southern, L., editor]. Boca Raton, Florida, USA: CRC Press LLC.

Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* **453**, 620-625.

Mezoff AG, Jensen NJ, Cohen MB (1991) Mechanisms of increased susceptibility of immature and weaned pigs to *Escherichia coli* heat-stable enterotoxin. *Pediatr Res* **29**, 424-428.

McCracken BA, Gaskins HR, Ruwe-Kaiser PJ (1995) Diet-dependent and diet-independent metabolic responses underlie growth stasis of pigs at weaning. *J Nutr* **125**, 2838-2845.

McCracken BA, Spurlock ME, Roos MA, *et al.* (1999) Weaning anorexia may contribute to local inflammation in the piglet small intestine. *J Nutr* **129**, 613-619.

McDonald DE, Pethick DW, Pluske JR, *et al*. (1999) Adverse effects of soluble non-starch polysaccharide (guar gum) on piglet growth and experimental colibacillosis immediately after weaning. *Res Vet Sci* **67**, 245-250.

McDonald DE, Pethick DW, Mullan BP, *et al*. (2001a) Increasing viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulates proliferation of enterotoxigenic *Escherichia coli* in newly-weaned pigs. *Br J Nutr* **86**, 487-498.

McDonald DE, Pethick DW, Mullan BP, *et al*. (2001b) Soluble non-starch polysaccharides from pearl barley exacerbate experimental post-weaning colibacillosis. In: *Proceedings of the 8th Symposium on Digestive Physiology in Pigs*, pp.280-292 [Lindberg JE & Ogle B, editors]. Wallingford, UK: CABI Publishing.

Meunier JP, Cardot JM, Manzanilla EG, *et al*. (2007) Use of spray cooling technology for development of micro-encapsulated capsicum oleoresin for the growing pig as an alternative to in-feed antibiotics : Study of release using in vitro models. *J Anim Sci* **85**, 2699-2710

Miguel JC, Rodriguez-Zas S, Pettigrew JE (2004) Efficacy of a mannan oligosaccharide (Bio-Mos®) for improving nursery pig performance. *J Swine Health Prod* **12**, 296-307.

Mills S, Ross RP, Hill C, *et al.* (2011) Milk intelligence: Mining milk for bioactive substances associated with human health. *Int Dairy J* **21**, 377-401.

Modesto M, D'Aimmo MR, Stefanini I, *et al.* (2009) A novel strategy to select Bifidobacterium strains and prebiotics as natural growth promoters in newly weaned pigs. *Livest Sci* **122**, 248-258.

Molist F, de Segura AG, Gasa J, *et al*. (2009) Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. *Anim Feed Sci Technol* **149**, 346-353.

Molist F, Gómez de Segura, Pérez JF, *et al*. (2010) Effect of wheat bran on the health and performance of weaned pigs challenged with *Escherichia coli* K88+. *Livest Sci* **133**, 214-217.

Molist F, Hermes RG, Gómez de Segura A, *et al*. (2011) Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *Br J Nut* 1-10.

Montagne L, Cavaney FS, Hampson DJ, *et al*. (2004) Effect of diet composition on postweaning colibacillosis in piglets. *J Anim Sci* **82**, 2364-2374.

Moradpour Z, Sepehrizadeh Z, Rahbarizadeh F, *et al*. (2009) Genetically engineered phage harbouring the lethal catabolite gene activator protein gene with an inducer-independent promoter for biocontrol of *Escherichia coli*. *FEMS Microbiol Lett* **296**, 67-71.

Mroz Z, Jongbloed AW, Partanen KH, *et al*. (2000) The effects of calcium benzoate in diets with or without organic acids on dietary buffering capacity, apparent digestibility, retention of nutrients, and manure characteristics in swine. *J Anim Sci* **78**, 2622-2632.

Mroz Z (2005) Organic acids as potential alternatives to antibiotic growth promoters for pigs. In: *Proceedings of Banff Pork Seminar 2005. Advances in Pork Production*, pp.169-182 Edmonton, Alberta, Canada.

Mynott TL, Luke RK, Chandler DS (1996) Oral administration of protease inhibits enterotoxigenic *Escherichia coli* receptor activity in piglet small intestine. *Gut* **38**, 28-32.

Mynott TL, Crossett B, Prathalingam SR (2002) Proteolytic inhibition of *Salmonella enterica* serovar Typhimurium-induced activation of the mitogen-activated protein kinases ERK and JNK in cultured human intestinal cells. *Infect Immun* **70**, 86-95.

Nabuurs MJA, Van Zijderveld FG, De Leeuw PW (1993) Clinical and microbiological field studies in the Netherlands of diarrhoea in pigs at weaning. *Res Vet Sci* **55**, 70-77.

Nagy B and Fekete PZ (1999) Enterotoxigenic *Escherichia coli* in farm animals. *Vet Res* **30**, 259-284.

Nagy B and Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *International Journal of Medical Microbiology* **295**, 443-454.

Namkung H, Li M, Gong J, *et al*. (2004) Impact of feeding blends of organic acids and herbal extracts on growth performance, gut microbiota and digestive function in newly weaned pigs. *Canad J Anim Sci* **84**, 697-704.

Narkeviciute I, Rudzeviciene O, Leviniene G, *et al*. (2002) Management of Lithuanian children's acute diarrhoea with Gastrolit solution and dioctahedral smectite. *Eur J Gastroenterol Hepatol* **14**, 419-424.

Nessmith WB, Nelssen JL, Tokach MD, *et al*. (1997) Effects of substituting deproteinized whey and crystalline lactose for dried whey on weanling pig performance. *J Anim Sci* **75**, 3222-3228.

Newburg DS (1997) Do the Binding Properties of Oligosaccharides in Milk Protect Human Infants from Gastrointestinal Bacteria? *J Nutr* **127**, 980S-984S

Newburg DS, Ruiz-Palacios GM, Altaye M, *et al.* (2004) Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiol* **14**, 253-263.

Newburg DS (2005) Innate Immunity and Human Milk. *The Journal of Nutrition* **135**, 1308-1312.

Niewold TA (2007) The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial Growth Promoters, the Real Mode of Action? A Hypothesis. *Poult Sci* **86**, 605-609.

Nollet H, Deprez P, Van Driessche E, *et al*. (1999) Protection of just weaned pigs against infection with F18+ *Escherichia coli* by non-immune plasma powder. *Vet Microbiol* **65**, 37-45.

NRC (1998) Nutrient Requirements of Swine. 10th ed. Washington, DC, USA Nat Academic Press.

Nyachoti CM, Omogbenigun FO, Rademacher M, *et al.* (2006) Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *J Anim Sci* **84**, 125-134.

Oetting LL, Utiyama CE, Giani PA, *et al*. (2006) Effects of herbal extracts and antimicrobials on apparent digestibility, performance, organs morphometry and intestinal histology of weanling pigs. *Rev Brasil Zootec* **35**, 1389-1397.

Ofek I, Hasty DL, Sharon N (2003) Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immunology & Medical Microbiology* **38**, 181-191.

O'Flaherty S, Ross RP, Coffey A (2009) Bacteriophage and their lysins for elimination of infectious bacteria. *FEMS Microbiol Rev* **33**, 801-819.

Oliver SP, Patel DA, Callaway TR, *et al.* (2009) ASAS Centennial Paper: Developments and future outlook for preharvest food safety. *J Anim Sci* **87**, 419-437.

Onken JE, Greer PK, Calingaert B, *et al.* (2008) Bromelain treatment decreases secretion of pro-inflammatory cytokines and chemokines by colon biopsies in vitro. *Clin Immunol* **126**, 345-352.

Osek J, Gallien P, Truszczyñski M, *et al.* (1999) The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. *Comp Immunol Microbiol Infect Dis* **22**, 163-174.

Ou D, Li D, Cao Y, *et al*. (2007) Dietary supplementation with zinc oxide decreases expression of the stem cell factor in the small intestine of weanling pigs. *J Nutr Biochem* **18**, 820-826.

Owusu-Asiedu A, Nyachoti CM, Marquardt RR (2003) Response of early-weaned pigs to an enterotoxigenic *Escherichia coli* (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic. *J Anim Sci* **81**, 1790-1798.

Pabst R, Geist M, Rothkotter HJ (1988) Postnatal development and lymphocyte production of jejunal and ileal peyer's patches in normal and gnotobiotic pigs. *Immunology* **64**, 539-544.

Papaioannou DS, Kyriakis CS, Alexopoulos C, *et al*. (2004) A field study on the effect of the dietary use of a clinoptilolite-rich tuff, alone or in combination with certain antimicrobials, on the health status and performance of weaned, growing and finishing pigs. *Res Vet Sci* **76**, 19-29.

Papaioannou D, Katsoulos PD, Panousis N, *et al*. (2005) The role of natural and synthetic zeolites as feed additives on the prevention and/or the treatment of certain farm animal diseases: A review. *Microp Mesop Mat* **84**, 161-170.

Partanen KH and Mroz Z (1999) Organic acids for performance in pig diets. *Nutr Res Rev* **12**, 117-145.

Partanen KH (2001) Organic acids - their efficacy and modes of action in pigs. In: *Gut Enviroment of Pigs*, pp.201-217 [Piva A, Bach Knudsen KE, Lindberg JE, editors]. Nottingham, UK: Nottingham Universtiy Press.

Pearson JP & Brownlee IA (2005) Structure and function of mucosal surfaces. In: Colonization *of Mucosal Surfaces*. pp.3-16 [Nataro JP, Cohen PS, Mobley HLT *et al*., editors]. Washington, D.C., USA: ASM Press.

Pepper L, Cho Y, Boder ET, *et al*. (2008) A decade of yeast surface display technology: Where are we now? *Comb Chem High Throughput Screen* **11**, 127-134.

Perdigon G, Alvarez S, Rachid M, *et al*. (1995) Immune System Stimulation by Probiotics. *J Dairy Sci* **78**, 1597-1606.

Paulicks BR, Roth FX, Kirchgessner M (2000) Effects of potassium diformate (FormiR LHS) in combination with different grains and energy densities in the feed on growth performance of weaned piglets. *J Anim Physiol Anim Nutr* **84**, 102-111.

Phillips TD, Lemke SL, Grant PG (2002) Characterization of clay-based enterosorbents for the prevention of aflatoxicosis. In: *Advances in Experimental Medicine and Biology* pp.157-171. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Pierce KM, Callan JJ, McCarthy P, *et al*. (2007) The interaction between lactose level and crude protein concentration on piglet post-weaning performance, nitrogen metabolism, selected faecal microbial populations and faecal volatile fatty acid concentrations. *Anim Feed Sci Technol* **132**, 267-282.

Pierce KM, Sweeney T, Brophy PO, *et al*. (2006) The effect of lactose and inulin on intestinal morphology, selected microbial populations and volatile fatty acid concentrations in the gastro-intestinal tract of the weanling pig. *Anim Sci* **82**, 311-318.

Piva A, Panciroli A, Meola E, *et al*. (1996) Lactitol enhances short-chain fatty acid and gas production by swine cecal microflora to a greater extent when fermenting low rather than high fiber diets. *J Nutr* **126**, 280-289.

Piva A, Casadei G, Gatta PP, *et al*. (2005) Effect of lactitol, lactic acid bacteria, or their combinations (synbiotic) on intestinal proteolysis in vitro, and on feed efficiency in weaned pigs. *Can J Anim Sci* **85**, 345-353.

Piva A, Galvano F, Biagi G, *et al.* (2006) Chapter 1 intestinal fermentation: Dietary and microbial interactions. In: *Biology of Growing Animals*, pp.3-31 [R. Mosenthin, editor]. Elsevier.

Piva A, Pizzamiglio V, Morlacchini M, *et al*. (2007) Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. *J Anim Sci* **85**, 486-493.

Platel K & Srinivasan K (1996) Influence of dietary spices or their active principles on digestive enzymes of small intestinal mucosa in rats. Int J Food Sci Nutr 47(1), 55-59.

Pluske JR, Siba PM, Pethick DW, *et al*. (1996) The incidence of swine dysentery in pigs can be reduced by feeding diets that limit the amount of fermentable substrate entering the large intestine. *J Nutr* **126**, 2920-2933.

Pluske JR, Hampson DJ and Williams IH (1997) Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest Produc Sci* **51**, 215-236.

Pluske JR, Durmic Z, Pethick DW, *et al*. (1998) Confirmation of the role of rapidly fermentable carbohydrates in the expression of swine dysentery in pigs after experimental infection. *J Nutr* **128**, 1737-1744.

Pluske JR, Pethick DW, Hopwood DE, *et al*. (2002) Nutritional influences on some major enteric bacterial diseases of pigs. *Nut Res Rev* **15**, 333-371.

Pluske JR, Durmic Z, Payne HG, *et al*. (2007) Microbial diversity in the large intestine of pigs born and reared in different environments. *Livest Sci* **108**, 113-116.

Pollmann DS, Danielson DM, Peo ER (1980) Effect of *Lactobacillus acidophilus* on starter pigs fed a diet supplemented with lactose. *J Anim Sci* **51**, 638-644.

Ramu J, Clark K, Woode GN, *et al*. (1997) Adsorption of cholera and heat-labile *Escherichia coli* enterotoxins by various adsorbents: An *in vitro* study. *J Food Protect* **60**, 358-362.

Ravindran V and Kornegay ET (1993) Acidification of weaner pig diets: A review. *J Sci Food Agric* **62**, 313-322.

Rhoades JR, Gibson GR, Formentin K, *et al.* (2005) Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. *J Dairy Sci* **88**, 3455-3459.

Rodríguez-Carvajal MA, Ignacio Sánchez J, Campelo AB, *et al.* (2008) Structure of the high-molecular weight exopolysaccharide isolated from *Lactobacillus pentosus* LPS26. *Carbohydr Res* **343**, 3066-3070.

Roediger WE and Moore A (1981) Effect of short-chain fatty acid on sodium absorption in isolated human colon perfused through the vascular bed. *Dig Dis Sci* **2**, 106.

Rolfe RD (1996) Colonization resistance. Gastrointestinal microbes and host interactions. *Gastrointestinal Microbiology*, 1st ed. [RI Mackie, BA White and RE Isaacson, editors]. Chapman and Hall, London, UK.

Rostagno MH, Hurd HS, McKean JD *et al*. (2003) Preslaughter holding environment in pork plants is highly contaminated with *Salmonella enterica*. *Appl Environ Microbiol* **69**, 4489-4494.

Roselli M, Finamore A, Maria SB, *et al*. (2005) Alternatives to in-feed antibiotics in pigs: Evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of *in vitro* and *in vivo* results. *Anim.Res* **54**, 203-218.

Roselli M, Britti MS, Le Huërou-Luron I, *et al.* (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic *Escherichia coli* K88 in pig intestinal cells. *Toxicol in Vitro* **21**, 224-229.

Rossi R, Pastorelli G, Cannata S, *et al.* (2010) Recent advances in the use of fatty acids as supplements in pig diets: A review. *Anim Feed Sci Technol* **162**, 1-11.

Saif, LJ and Sestak K (2006) Transmissible gastroenteritis and porcine respiratory coronavirus. *Diseases of Swine*, 9th ed chapter 30, 489-516. [BE Straw, JJ Zimmerman, S. D'Allaire, and D. J. Taylor, editors]. Blackwell Publishing Professional, Ames, Iowa, USA.

Sanson BF and Gleed PT (1981) The ingestion of sow's faeces by suckling piglets. *Br J Nutr* **46**, 451-456.

Sargeant HR, McDowall KJ, Miller HM, *et al.* (2010) Dietary zinc oxide affects the expression of genes associated with inflammation: Transcriptome analysis in piglets challenged with ETEC K88. *Vet Immunol Immunopathol* **137**, 120-129.

Schierack P, Nordhoff M, Pollmann M, *et al*. (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. *Histochem. Cell Biol* **125**, 293-305.

Schwertmann A, Shroten H, Hacker H, *et al.* (1999) S-Fimbriae from *Escherichia coli* bind to soluble glycoproteins from human milk. *J Pediat Gastroenterol Nutr* **28**, 257-263.

Segain J, de la Blétière DR, Bourreille A, *et al.* (2000) Butyrate inhibits inflammatory responses through NFκB inhibition: implications for Crohn's disease. *Gut* **47**, 397-403.

Setia A, Bhandari SK, House JD, *et al*. (2009) Development and *in vitro* evaluation of an *E. coli* probiotic able to inhibit the growth of pathogenic *E. coli* K88. *J Anim Sci* **87**, 2005-2012.

Sharon N (2009) Lectins. In: *Encyclopedia of Life Sciences*, pp- 1-11. Chichester, UK: John Wiley & Sons, Ltd.

Shibasaki S, Maeda H and Ueda M (2009) Molecular display technology using Yeast—Arming technology— *Anal Sci* **25**, 41-49.

Shim SB, Verdonk JMAJ, Pellikaan WF, *et al*. (2007) Differences in microbial activities of faeces from weaned and unweaned pigs in relation to *in vitro* fermentation of different sources of inulin-type oligofructose and pig feed ingredients. *Asian Austral J Anim* **20**, 1444-1452

Shim SB, Verstegen MWA, Kim H, *et al*. (2005) Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or synbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota. *Arch Anim Nutr* **59**, 419-427.

Shoaf‐Sweeney KD and Hutkins RW (2009) Adherence, Anti-adherence, and oligosaccharides: Preventing pathogens from sticking to the host. In: *Advances in Food and Nutrition Research*, pp.101-161 [Steve L. Taylor, editor]. Elsevier, Academic Press.

Shu Q, Qu F, Gill HS (2001) Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with Rotavirus and *Escherichia coli* infection in a piglet model. *J Pediatr Gastroenterol Nutr* **33**, 171-177.

Singh J, Im J, Whitten JE, *et al*. (2009) Encapsulation of zinc oxide nanorods and nanoparticles. *Langmuir* **25**, 9947-9953.

Slade RD, Kyriazakis I, Carroll SM, *et al*. (2011) Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic *Escherichia coli* O149 challenge. *Animal* pp. 1-9.

Smith HW and Halls S (1968) The production of oedema disease and diarrhoea in weaned pigs by the oral administration of *Escherichia coli*: factors that influence the course of the experimental disease. *J Med Microbiol* **1**, 45-59.

Smith JW, Tokach MD, Goodband RD, *et al*. (1997) Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. *J Anim Sci* **75**, 1861-1866.

Smith HW and Huggins MB (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J Gen Microbiol* **128**, 307-318.

Smith HW and Huggins MB (1983) Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *J Gen Microbiol* **129**, 2659-2675.

Smith HW, Huggins MB, Shaw KM (1987) Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *J Gen Microbiol* **133**, 1127-1135.

Snoeck V, Huyghebaert N, Cox E, *et al.* (2004) Gastrointestinal transit time of nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. *J Control Release* **94**, 143-153.

Sohn KS, Kim MK, Kim JD, *et al.* (2000) The role of immunostimulants in monogastric animal and fish - review. *Asian Austral J Anim Sci* **13**, 1178-1187.

Solà-Oriol D (2008) Quantitative evaluation of the palatability of feed ingredients in swine. PhD thesis. Barcelona, Spain: Universitat Autònoma de Barcelona.

Solà-Oriol D, Roura E, Torrallardona D (2007) Pig preference for cereal based diets, relationship with their digestibility and physical properties. *Livest Sci* **108**, 190-193.

Solà-Oriol D, Roura E, Torrallardona D (2009a) Feed preference in pigs: Effect of cereal sources at different inclusion rates. *J Anim Sci* **87**, 562-570.

Solà-Oriol D, Roura E, Torrallardona D (2009b) Use of double-choice feeding to quantify feed ingredient preferences in pigs. *Liv Sci* **123**, 129-137.

Songer JG and Uzal F (2005) Clostridial enteric infections in pigs. *J Vet Diagn Invest* **17**, 528-536.

Songer JG and Taylor DJ (2006) Clostridial Infections. *Diseases of Swine*, 9th ed., chapter 36, pp. 613-628. [BE Straw, JJ Zimmerman, S. D'Allaire and DJ Taylor, editors]. Blackwell Publishing Professional, Ames, Iowa, USA.

Spreeuwenberg MAM, Verdonk JMAJ, Gaskins HR, *et al*. (2001) Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. *J Nutr* **131**, 1520-1527.

Spring P, Wenk C, Dawson K, *et al.* (2000) The effects of dietary mannanoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of Salmonella-challenged broiler chicks. *Poult Sci* **79**, 205-211.

Stein HH and Kil DY (2006) Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, Part 2. *Anim Biotechnol* **17**, 217-231.

Steinbach G and Hartung M (1999) An attempt to estimate the share of human cases of salmonellosis attributable to *Salmonella* originating from swine. *Berl Munch Tierarztl Wochenschr* **112**, 296-300.

Stokes CR, Bailey M, Haverson K, *et al*. (2004) Postnatal development of intestinal immune system in piglets: Implications for the process of weaning. *Anim Res* **53**, 325-334.

Swords WE, Wu CC, Champlin FR (1993) Postnatal changes in selected bacterial groups of the pig colonic microflora. *Biol Neonate* **63**, 191-200.

Szarc vel Szic K, Ndlovu MN, Haegeman G, *et al.* (2010) Nature or nurture: Let food be your epigenetic medicine in chronic inflammatory disorders. *Biochem Pharmacol* **80**, 1816-1832.

Takeda K and Akira S (2005) Toll-like receptors in innate immunity. *Int Immunol* **17**, 1-14.

Tannock GW (1999) Modification of the normal microbiota by diet, stress, antimicrobial agents and probiotics. *Gastrointestinal Microbiology*. [RI Mackie and BA White, editors]. Chapman and Hall Microbiology Series, New York, USA.

Tatara MR, Sliwa E, Dudek K, *et al*., (2008) Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared in artificial sow. *Ann Agric Environ Med* **15**, 63–69.

Teissedre PL and Waterhouse AL (2000) Inhibition of oxidation of human low-density lipoproteins by phenolic substances in different essential oils varieties. *J Agric Food Chem* **48**, 3801-3805.

Tellez G, Nava GM, Vicente JL, *et al.* (2010) Evaluation of dietary *Aspergillus* meal on intestinal morphometry in turkey poults. *International Journal of Poultry Science* **9**, 875-878.

Thiagarajah JR and Verkman A (2003) CFTR pharmacology and its role in intestinal fluid secretion. *Curr Opin Pharmaco* **3**, 594-599.

Thomä-Worringer C, Sørensen J, López-Fandiño R (2006) Health effects and technological features of caseinomacropeptide. *Int Dairy J* **16**, 1324-1333.

Thomas WE, Nilsson LM, Forero M, *et al.* (2004) Shear-dependent ?stick-and-roll? adhesion of type 1 fimbriated *Escherichia coli*. *Mol Microbiol* **53**, 1545-1557.

Thomson JR (2006) Diseases of the digestive system. In: *Diseases of Swine*, pp.37-55 [Straw BE, Zimmerman JJ, D'Allaire S *et al.*, editors]. Ames, Iowa, USA: Blackwell Publishing Professional.

Tiralongo J and Moran AP (2010) Bacterial lectin-like interactions in cell recognition and adhesion. In: *Microbial Glycobiology*, pp.549-565 [Otto Holst, Patrick J. Brennan, Mark von Itzstein, editors]. San Diego: Academic Press.

Tokach MD, Dritz SS, Goodlad RA, *et al*. (2003) Nutritional requirements of the weaned pig. In: *Weaning the Pig: Concepts and Consequences*, pp.259-299 [Pluske JR, Le Dividich J, Verstegen MWA, editors]. Wageningen, The Netherlands: Wageningen Academic Publishers.

Torres-Rodriguez A, Sartor C, Higgins SE, *et al.* (2005) Effect of Aspergillus meal prebiotic (Fermacto) on performance of broiler chickens in the starter phase and fed low protein diets. *J Appl Poult Res* **14**, 665-669.

Trckova M, Vondruskova H, Zraly Z, *et al*. (2009) The effect of kaolin feeding on efficiency, health status and course of diarrhoeal infections caused by enterotoxigenic *Escherichia coli* strains in weaned piglets. *Vet Med-Czech* **54**, 47-63.

Trevisi P, Corrent E, Messori S, *et al.* (2010) Healthy newly weaned pigs require more tryptophan to maximize feed intake if they are susceptible to *Escherichia coli* K88. *Livest Sci* **134**, 236-238.

Tsiloyannis VK, Kyryakis SC, Vlemmas J, *et al*. (2001) The effect of organic acids on the control of porcine post-weaning diarrhoea. *Res Vet Sci* **70**, 287-293.

Turner JL, Dritz SS, Minton JE (2001) Review: Alternatives to conventional antimicrobials in swine diets. *The Professional Scientist* **17**, 217-226.

Ulgheri C, Paganini B, Rossi F (2010) Antisecretory factor as a potential health-promoting molecule in man and animals. *Nut Res Rev* **23**, 300-313.

van Beers-Schreurs, HMG, Nabuurs MJA, Vellenga L, *et al*. (1998) Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *J Nutr* **128**, 947-953.

van den Broeck W, Cox E, Goddeeris BM (1999) Receptor-dependent immune responses in pigs after oral immunization with F4 Fimbriae. *Infect Immun* **67**, 520-526.

van der Meulen, J, Koopmans SJ, Dekker RA, *et al*. (2010) Increasing weaning age of piglets from 4 to 7 weeks reduces stress, increases post-weaning feed intake but does not improve intestinal functionality. *Animal* **4**, 1653.

van der Wolf PJ, van Schie FW, Elbers ARW, *et al.* (2001) Administration of acidified drinking water to finishing pigs in order to prevent Salmonella infections. *Vet Q* **23**, 121-125.

van Dijk AJ, Everts H, Nabuurs MJA, *et al*. (2001) Growth performance of weanling pigs fed spray-dried animal plasma: a review. *Livest Prod Sci* **68**, 263-274.

van Heugten E, Funderburke DW, Dorton KL (2003) Growth performance, nutrient digestibility, and fecal microflora in weanling pigs fed live yeast. *J Anim Sci* **81**, 1004-1012.

Varel VH and Kreikemeier KK (1994) Influence of feeding *Aspergillus oryzae* fermentation extract (Amaferm) on in situ fiber degradation, ruminal fermentation, and microbial protein synthesis in nonlactating cows fed alfalfa or bromegrass hay. *J Anim Sci* **72**, 1814-1822.

Varel VH and Yen JT (1997) Microbial perspective on fiber utilization by swine. *J Anim Sci* **75**, 2715-2722.

Verhelst R, Schroyen M, Buys N, *et al.* (2010) The effects of plant polyphenols on enterotoxigenic *Escherichia coli* adhesion and toxin binding. *Livest Sci* **133**, 101-103.

Vicente B, Valencia DG, Perez-Serrano M, *et al.* (2008) The effects of feeding rice in substitution of corn and the degree of starch gelatinization of rice on the digestibility of dietary components and productive performance of young pigs. *J Anim Sci* **86**, 119-126.

Vidotto MC, Lima NCS, Fritzen JTT, *et al.* (2009) Frequency of virulence genes in *Escherichia coli* strains isolated from piglets with diarrhea in the North Parana State, Brazil. *Brazilian J Microbiol* **40**, 199-204.

Visek WJ (1984) Ammonia: Its effects on biological systems, metabolic hormones, and reproduction. *J Dairy Sci* **67**, 481-498.

Vondruskova H, Slamova R, Trckova MZ, *et al*. (2010) Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: a review. *Vet Med-Czech* **55**, 199-224.

Wales AD, Cook AJC, Davies RH (2011) Producing salmonella-free pigs: A review focusing on interventions at weaning. *Vet Rec* **168**, 267-276.

Wang Q, Gong J, Huang X, *et al.* (2009) In vitro evaluation of the activity of microencapsulated carvacrol against *Escherichia coli* with K88 pili. *J Appl Microbiol* **107**, 1781-1788.

Wang Y, Ganzle MG, Schwab C (2010) Exopolysaccharide synthesized by *Lactobacillus reuteri* decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes. *Appl Environ Microbiol* **76**, 4863-4866.

Wegener H, Aarestrup F, Jensen L, *et al*. (1999) Use of antimicrobial growth promoters in food animals and Enterococcus faecium resistance to therapeutic antimicrobial drugs in Europe. *Emerging Infect Dis* **5**, 329-335.

Wellock IJ, Fortomaris PD, Houdijk JGM, *et al.* (2008) The consequences of non-starch polysaccharide solubility and inclusion level on the health and performance of weaned pigs challenged with enterotoxigenic *Escherichia coli*. *Br J Nutr.* **99**, 520-530.

Westwater C, Kasman LM, Schofield DA, *et al*. (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial infections. *Antimicrob Agents Chemother* **47**, 1301-1307.

White LA, Newman MC, Cromwell GL, *et al.* (2002) Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. *J Anim Sci* **80**, 2619-2628.

Williams BA, Verstegen MWA, Tamminga S (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutr Res Rev* **14**, 207-227.

Windisch W, Schedle K, Plitzner C, *et al*. (2008) Use of phytogenic products as feed additives for swine and poultry. *J Anim Sci* **86**, E140-148.

Winfield MD and Groisman EA (2003) Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl Environ Microbiol* **69**, 3687-3694.

Wong PYY, Nakamura S, Kitts DD (2006) Functional and biological activities of casein glycomacropeptide as influenced by lipophilization with medium and long chain fatty acid. *Food Chem* **97**, 310-317.

Wostmann BS (1996) Germfree and gnotobiotic animal models: background and applications. . Boca Raton, Florida, USA, CRC Press.

Xia MS, Hu CH, Xu ZR, *et al*. (2004) Effects of copper-bearing montmorillonite (Cu-MMT) on *Escherichia coli* and diarrhea on weanling pigs. *Asian Austral J Anim* **17**, 1712-1716

Xia MS, Hu CH, Xu ZR (2005) Effects of copper bearing montmorillonite on the growth performance, intestinal microflora and morphology of weanling pigs. *Anim Feed Sci Technol* **118**, 307-317.

Yen JT (2001) Anatomy of the digestive system and nutritional physiology. *Swine Nutrition*, 2nd ed., pp. 31-63 [AJ Lewis and LL Southern, editors]. CRC Press LLC ed. CRC Press LLC, Boca Raton, Florida, USA.

Yuan L, Stevenson GW, Saif LJ (2006) Rotavirus and reovirus. *Diseases of Swine*, 9th ed. chapter 26: pp. 435-454 [BE Straw, JJ Zimmerman, S D'Allaire and DJ Taylor, editors] Blackwell Publishing Professional, Ames, Iowa, USA.

Zanchi R, Canzi E, Molteni L, *et al*. (2008) Effect of *Camellia sinensis* whole plant extract on piglet intestinal ecosystem. *Ann Microbiol* **58**, 147-152; 152.

Zani JL, da Cruz FW, dos Santos AF, *et al*. (1998) Effect of probiotic CenBiot on the control of diarrhoea and feed efficiency in pigs. *J Appl Microbiol* **84**, 68-71.

Zerby HN, Bard JL, Loerch SC, *et al.* (2011) Effects of diet and *Aspergillus oryzae* extract or *Saccharomyces cervisiae* on growth and carcass characteristics of lambs and steers fed to meet requirements of natural markets. *J Anim Sci* **In press**.

Zhang W, Zhao M, Ruesch L, *et al.* (2007) Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Vet Microbiol* **123**, 145-152.

Zhang B and Guo Y (2009) Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *Br J Nutr* **102**, 687-693

Zhao Y, Tian Y, Cui Y, *et al.* (2010) Small molecule-capped gold nanoparticles as potent antibacterial agents that target gram-negative bacteria. *J Am Chem Soc* **132**, 12349-12356.

Zimmermann B, Bauer E, Mosenthin R (2001) Pro- and prebiotics in pig nutrition – potential modulators of gut health? *J Anim Feed Sci* **10**, 47-56.

Chapter 3

Experimental objectives

During the last years, our research group has been working in several projects funded by the Spanish government: AGL2005-07438-C02-01/GAN (2004 - 2007); AGL2007-60851/GAN (2007-2010); and AGL2009-07328/GAN (2009 – 2012) with the aim of evaluating different feeding strategies to improve the health of young pigs around weaning. This topic has received a special interest after the ban of antibiotics as growth promoters in the animal industry. These projects have allowed the collaboration of our group (Grup de Nutrició Animal del Departament de Ciència Animal i dels Aliments) with different outstanding research centers in Finland, United Kingdom Germany, Italy, Canada, USA, Japan and New Zealand.

The present experimental work has been developed in the frame of all these projects to attend the following main experimental objectives:

1. To assess what are the likely effects of incorporating fibrous ingredients in the diet of early weaned piglets in order to promote the gut maturation and the establishment of a healthy intestinal microbiota.

2. To evaluate, *in vitro* and *in vivo*, some of the mechanisms that has been proposed to explain the dietary effects of some ingredients on the microbiota, such as the assessment of a likely interference on the adhesion of fimbriated bacteria to porcine intestinal epithelium.

3. To evaluate the mucosal immune response in cell culture *in vitro* models after an enteropathogen challenge and to elucidate the role of anti-adherence feedstuffs in the immune modulation.

4. To determine the likely role of some of these strategies to prevent the development of post-weaning colibacillosis in piglets, promoting the better adaptation from weaning to fattening of the animals.

In order to accomplish these objectives, five experiments were designed and carried out as presented in chapters 4 to 8.

In the experiment 1 (Hermes *et al*., 2009), the interaction effects of two levels of crude protein and two levels of fiber supplementation were evaluated in a 2x2 factorial design using 96

early weaned piglets. The main objective of this study was to clarify if early weaned piglets may get a benefit in the animal performance from an increased level of fiber in the diet and its impact on the intestinal microbiota and function. Moreover it aimed to find if the DF supplementation response depends on a likely interaction with the level of protein in the diet.

Experiment 2 (Hermes *et al*., 2010) had a similar objective, but evaluating the results of incorporating fibrous ingredients as dependent on the main cereal used in the basal diet. We planned to measure the animal performance parameters, fermentation and the effects on the intestinal microbiota as an index of intestinal health.

From these two *in vivo* experiments, we decided to move to *in vitro* studies and focus our interest on the mechanisms likely interacting between the diet and the intestinal microbiota. In particular, we focused on the likely role of some compounds to block the intestinal attachment of some pathogens to the intestinal epithelium.

Experiment 3 was planned to perform an *in vitro* screening of feedstuffs which could adhere to the K88 ETEC, and in that way inhibit or block its attachment to the intestinal tract of piglets. The adhesion study was planned to be performed with more than 30 natural sources of feedstuffs, being the best ones chosen for further studies of likely blockage of the intestinal ETEC adhesion.

Experiment 4 was designed to elucidate, *in vitro*, the role of 5 chosen anti-adhesive feedstuffs on the pathological process of entero-pathogenic bacteria and the influence on the innate immune response of the GIT. To reach these objectives it was used a culture of a porcine intestinal cell line (IPEC-J2), which represents a good *in vitro* model to study the molecular aspects regarding the interaction between diets, intestinal pathogens and the epithelial cells.

Finally, experiment 5 was designed to confirm, *in vivo,* the effect in early weaned piglets of one of these products having the highest adherence and blocking activity *in vitro*: the casein glycomacropeptide extracted from bovine milk. In this study the animals were proposed to be challenged with an ETEC K88 to monitor the response on microbiology, immunology, digestion and animal performance of piglets fed on diets containing or not the casein glycomacropeptide.

Chapter 4

Effect of dietary level of protein and fiber on the productive performance and health status of piglets. *J Anim Sci*, 2009, **87**: 3569-3577.

4.1 Introduction

Feed specifications for the newly weaned pigs are still a controversial area in the pig industry. High standard requirements for AA (standardized ileal digestible Lys, 12.0 to 14.0 g/kg diet) and energy (10.4 MJ NE/kg diet, BSAS, 2003) are defined for the piglets. However, diets containing a high level of protein may disturb the intestinal microbiota as a consequence of protein fermentation (Bertschinger *et al*., 1979). Some authors have reported that reducing dietary CP with AA supplementation reduced the ammonia and SCFA in the small intestine (Nyachoti *et al*., 2006; Htoo *et al*., 2007; Pierce *et al*., 2007), but also reduced the pig performance, probably as a consequence of the lack of some essential nutrients or AA imbalance (Nyachoti *et al*., 2006). Alternatively, some authors have proposed to reduce protein fermentation in the small intestine by the inclusion of fermentable carbohydrates in the diet (Awati *et al*., 2006). Increasing the fermentable carbohydrate content, through the inclusion of wheat bran, sugar beet pulp, native starch (Bikker *et al*., 2006; Carneiro *et al*., 2007) or inulin (Wellock *et al*., 2008) has been proved to stimulate lactic and butyric acid production in the small and large intestine, and to reduce counts of coliform bacteria. Mateos *et al*. (2006) and Kim *et al*. (2007) also described that the inclusion of moderate levels of fiber such as oat hulls reduced the incidence of diarrhea in diets based on cooked maize or processed (cooked or extruded) rice.

However, there are discrepancies in the literature about the effect of fiber on the productive performance of the young piglets. Some authors found no significant changes or even observed a decrease in the growth performance of piglets when fermentable carbohydrates were supplemented into low CP diets (16%; Bikker *et al*., 2006; 16 to 18.5%, Pierce *et al*., 2007) or diets with a high content of NSP (greater than 10% NDF; Freire *et al*., 1998). On the other hand, others have reported a significant increase in the ADG and ADFI when insoluble, non-viscous carbohydrates or lactose were supplemented to high CP diets (21%, Pierce *et al*., 2007) or diets with a low fiber content (rice based diets, 3.43% of NDF; Mateos *et al*., 2006). The reasons for these discrepancies are not clear

but they appear to depend on the basal diets composition (CP and NDF content) and the ability of the supplementary fiber to enhance the intestinal function or prevent harmful products released from protein fermentation.

The objective of the present work was to confirm the potential interaction between the level of dietary CP and NDF on the growth performance and health status of early weaned piglets, with the aim of clarify discrepancies between studies and understand the physiological mechanisms involved.

4.2. Material and Methods

4.2.1. Animals and Housing

This experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval from the Animal Protocol Review Committee of this institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

A total of 96 piglets [(Large White x Landrace) x Pietrain] were acquired from the experimental facilities of IRTA Mas de Bove (Reus, Spain). Animals were weaned at 21d of age and fed for 14 d a weaning diet based on rice (60%), sweet milk whey (13.7%), soybean protein (10.9 %) and potato protein (10%). On d 14 after weaning (35 d of age), piglets were transported to the Universitat Autònoma de Barcelona facilities, weighed (9.1 ± 0.60 kg BW) and placed in 32 pens (3 animals/pen). The pens were allotted to 4 treatments (Table 4.1) in a 2×2 factorial design that included two levels of CP content (16 vs. 20% CP) and two levels of DF (LF vs. HF, 5.3 and 7.15 % of NDF as-fed basis, respectively). The HF content was obtained by including 40 g/kg of wheat bran, the coarse outer covering of the wheat kernel, and 20 g/kg of sugar beet pulp. Diets were isoenergetic and isonitrogenous and formulated to satisfy the nutrient requirement standards for pigs (BSAS, 2003). Each pen ($3m^2$) had a feeder and a water nipple to ensure *ad libitum* feeding

and free water access. The experiment was conducted at the summer season with an average temperature in the rooms of 30ºC (± 2ºC).

4.2.2. Experimental Procedures

Animals received the experimental diets over 3 wk. Individual BW and pen feed consumption was registered weekly. Animals were checked daily to evaluate the health status. Consistency of the feces samples was evaluated and assigned a ranking between: 1 – liquid, 2 – soft, 3 – normal, 4 – hard feces on the first and the last day of the experiment. Piglets with clinical signs of diarrhea (watery feces) and simultaneous dehydration, fever, growth check and apathy were daily treated (during a 3 d period) with 1mL (i.m. injection) per animal of marbofloxacin (Marbocyl® 2%, Vétoquinol S.A., Lure Cedex, France). The number of antibiotic injections administered in each pen and mortality as a result of diarrhea was also recorded. At the beginning and on the last day of experiment, a sample of feces was taken from the rectum of one piglet from each pen for immediate conventional microbiological analyses. A total of 32 samples (8 per treatment) were obtained from piglets non-treated previously with antibiotics. A subsample of feces was stored at -20ºC for further molecular microbiological analyses.

On the final day of the experiment, the animal with the median weight of each pen was blood sampled and euthanized with an intravenous injection of sodium pentobarbital (200 mg/kg BW). Animals were bled, and the abdomen was immediately opened to tie, remove and weigh the whole gastrointestinal tract and the liver. The stomach, small intestine and the large intestine were separated by double ties, weighed (including digesta), and sampled. Samples of proximal colon digesta were collected, and maintained at -20ºC for further short-chain fatty acids (SCFA), ammonia and physicochemical characterization. A section of 4 cm from the proximal colon was removed, opened longitudinally and fixed by immersion in 10% (vol/vol) buffered formalin for histological study.

Table 4.1. Ingredients (g/kg) and analyzed chemical composition of the experimental diets.

	Diets[a]			
CP (%)	16		20	
Fiber content	Low	High	Low	High
Ingredient , g/kg of feed				
Rice	442.9	383.5	361.7	302.5
Barley	200.0	200.0	200.0	200.0
Wheat bran	-	40.0	-	40.0
Sugar beet pulp	-	20.0	-	20.0
Sweet whey	147.2	147.2	147.2	147.2
Potato protein	40.0	40.0	83.6	83.6
Soybean meal, 44%CP	101.6	93.1	146.1	137.4
Animal fat	28.4	36.8	30.3	38.8
Calcium carbonate	6.3	6.1	6.2	6.0
Dicalcium phosphate	12.8	12.1	12.1	11.5
Sodium chloride	2.1	2.0	2.0	2.0
L-Lys·HCl	6.8	6.8	2.4	2.6
DL-Met	2.9	3.0	1.6	1.7
L-Thr	2.7	2.7	0.6	0.7
L-Trp	1.3	1.3	0.8	0.8
Choline chloride, 50%	1.2	1.2	1.2	1.2
Vitamins and minerals[b]	4.0	4.0	4.0	4.0
Calculated content				
NE, MJ/kg	10.32	10.32	10.32	10.32
Starch	42.1	38.8	36.3	33.1
Crude fiber	1.9	2.6	2.1	2.7
Lysine, %	1.4	1.4	1.4	1.4
Methionine	0.56	0.57	0.53	0.53
Threonine	0.92	0.92	0.96	0.97
Tryptophan	0.31	0.32	0.33	0.33
Isoleucine	0.69	0.69	0.95	0.94
Valine	0.78	0.78	1.05	1.05
Analyzed composition				
Moisture, %	10.62	10.66	10.45	10.07
Ash, %	5.41	5.55	5.53	5.95
GE (MJ/Kg DM)	17.05	16.97	18.13	17.83
Ether Extract, %	4.59	4.75	4.28	5.15
CP, %	15.44	15.43	19.36	19.43
NDF, %	5.15	6.89	5.38	7.40
ADF, %	2.09	2.70	2.12	2.94

[a] Experimental diets: 16LF: low protein, low fiber; 16HF: low protein, high fiber; 20LF: high protein, low fiber; 20HF: high protein, high fiber. [b] Supplied per kilogram of feed: 5,000 IU of vitamin A, 2,000 IU of vitamin D3, 15.0 mg of vitamin E, 1.3 mg of vitamin B_1, 3.5 mg of vitamin B_2, 1.5 mg of vitamin B_6, 0.025 mg of vitamin B_{12}, 10.0 mg of calcium pantothenate, 15.0 mg of niacin, 0.1 mg of biotin, 0.6 mg of folic acid, 2.0 mg of vitamin K3, 80.0 mg of Fe, 6.0 mg of Cu, 0,75 mg of Co, 150 mg of Zn, 60 mg of Mn, 0.75 mg of I, 0.10 mg of Se and 150 mg of Etoxiquín as antioxidant (Capsoquin®, ITPSA, Barcelona, Spain).

4.2.3. Analytical Procedures

Chemical analyses of the diets were performed according to the Association of Official Analytical Chemists (AOAC, 1995) standard procedures. The viscosity of proximal colon digesta was measured in a Brookfield DV-E viscometer (Brookfield Engineering Laboratories, Inc, Stoughton, MA, USA) at a shear rate of 11 s^{-1}.

For bacterial counts, fresh digesta samples were suspended (10% wt/vol) in a buffered peptone solution and subsequently homogenized. Thereafter, 10-fold dilutions were made with a buffered peptone physiological salt solution (CM509, Oxoid Ltd., Basingstoke, Hampshire, UK; containing peptone 10.0 g/L, sodium chloride 5.0 g/L, di-sodium phosphate 3.5 g/L, and potassium dihydrogen phosphate 1.5 g/L). For the enumeration of enterococci, 100 µL of each dilution was pipetted as a drop onto Chromocult® Enterococci-Agar (Merck KGaA, Darmstadt, Germany; dissolving 33g of agar/L of distilled water). The plates were incubated for 24 h at 37°C. The red colonies with a diameter of 0.5 to 2 mm were counted. For the enumeration of *E. coli* and total coliforms, 1 mL of solution was pipetted onto an *E. coli*/coliform count plate (3M Petrifilm, Europe Laboratoires 3M Santé, Cergy-Pontoise, France) with Violet Red Bile gel and an indicator of glucuronidase activity. The plates were incubated for 48 h at 37°C, and all blue *E. coli* colonies and total coliform colonies were counted following the manufacturer's instructions. The DNA from feces was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK) and enterobacteria and lactobacilli were quantified by real time PCR using SyBR Green dye (Castillo *et al.*, 2005). The lactobacilli:enterobacteria ratio was calculated by subtracting log 16S rDNA gene lactobacilli copies/g of feces minus log 16S rDNA gene enterobacteria copies/g of feces (as-is basis).

Short-chain fatty acids and lactic acid were determined by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatization, as described by Jensen and Jorgensen (1994).

Ammonia was determined with the aid of a gas sensitive electrode (Crison ISE- 9665, Crison Instruments, S.A., Barcelona, Spain) combined with a digital voltmeter (Crison GLP 22). Three grams

of digesta were diluted (1:2) with 0.16 M NaOH, and after homogenization, samples were centrifuged for 10 min (1500 x g). The ammonia released was measured in the supernatant as different voltage in mV (Adapted from Diebold *et al*., 2004).

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphological measurements were performed with a light microscope (BHS, Olympus, Spain) (Nofrarías *et al*., 2006). Goblet cells and the number of intraepithelial lymphocytes (IEL) were measured in ten well-oriented crypts using a linear ocular micrometer (Olympus, Ref 209-35040, Microplanet, Barcelona, Spain). These variables were expressed per 100 µm. Differences between the nuclei of enterocytes, mitotic figures, goblet cells, and lymphocytes were clearly distinguishable at 400 x magnification. All the morphological measurements were made by the same person, who was blind to the treatments. Pig Major Acute-phase Protein (Pig-MAP) concentration was determined by a sandwich type ELISA (PigMAP kit ELISA, PigCHAMP Pro Europa S.A., Segovia, Spain), according to the manufacturer's instructions.

4.2.4. Statistical Analyses

Data on productive performance, organ measurements, microbial counts, SCFA, digesta viscosity and intestinal morphology were subjected to factorial analysis, with the initial BW as the covariation factor (whenever significant), using the GLM procedure of SAS (1999). For all data analyzed, the pen was used as the experimental unit. The alpha level used for the determination of significance for all the analysis was 0.05, data is presented as least square means and standard error of the mean.

4.3. Results

4.3.1. Animal Performance and Health Status

In Table 4.2, the BW, ADFI, ADG and G:F are presented. No significant differences were observed in the ADFI between treatments. Changes in the level of protein did not result in effects on animal performance. On the other hand, supplementation of fiber increased ($P \leq 0.01$) the overall ADG and the final BW of the animals. Furthermore, the supplementation of fiber increased the overall feed efficiency ($P \leq 0.001$).

In Table 4.3, the diarrhea incidence and antibiotic interventions during the 3 wk experimental period and the fecal score and serum concentration of Pig-MAP on d 35 after weaning are presented. A significant interaction ($P \leq 0.05$) was observed between protein and fiber levels for fecal score and antibiotic interventions. Supplementing the 16% CP diet with fiber reduced the fecal score and increased the antibiotic interventions whereas the opposite was observed in pigs fed the 20% CP diet. Furthermore, pigs fed on the 16% CP diet showed an increase ($P \leq 0.05$) in the serum concentration of Pig-MAP compared to 20% CP diet.

4.3.2. Gut Development and Morphology of the Intestinal Wall

Table 4.4 shows the results of the relative full intestinal tract weight and proximal colon morphology measurements at 35 d post-weaning. Changes in the protein and fiber level promoted shifts in the relative weight of the full intestines. A high protein level increased ($P \leq 0.05$) the relative weight of the small and large intestine, whereas a higher fiber level increased ($P \leq 0.05$) the relative weight of the large intestine. No differences between treatments were observed in relative liver weight (mean of treatments: 3.2% of BW, data not presented). A higher level of protein increased ($P \leq 0.05$) the number of goblet cells and reduced ($P \leq 0.05$) the intraepithelial lymphocytes.

Table 4.2. Effect of dietary crude protein (CP) and fiber (DF) on growth performance of piglets from 3 to 5 wk post weaning.

		Diets							
	CP (%)	16		20			P-values		
	Fiber content[1]	Low	High	Low	High	SEM	CP	FB	CP*FB
BW (kg)	Initial	8.97	9.07	9.18	9.20	0.60	0.415	0.785	0.836
	Final	16.89	18.01	16.7	18.45	1.04	0.659	0.001	0.465
ADFI, g/d	week 1	598	568	570	596	79	0.994	0.949	0.317
	week 2	875	774	765	833	215	0.739	0.827	0.281
	week 3	1,032	967	890	1,121	252	0.948	0.361	0.108
	Overall	835	769	742	849	158	0.908	0.709	0.136
ADG, g/d	week 1	350	362	338	371	56	0.928	0.272	0.616
	week 2	382	417	374	439	86	0.876	0.131	0.673
	week 3	453	563	444	594	81	0.700	0.001	0.494
	Overall	395	447	385	466	44	0.773	0.001	0.378
G:F	Overall	0.50	0.60	0.54	0.57	0.07	0.858	0.001	0.237

[1] Low fiber, 5.3% NDF and high fiber, 7.15% NDF; as-fed basis

Table 4.3. Effects of protein and dietary fiber on the diarrhea incidence and antibiotic interventions in piglets from wk 3 to 5 after weaning, and the fecal score and the serum Pig-Major Acute Phase Protein (Pig-MAP) concentration on d 35 after weaning.

	Diets							
CP (%)	16		20			P-values		
Fiber content[1]	Low	High	Low	High	SEM	CP	FB	CP*FB
Diarrhea incidence[2]	1.5	2.6	1.8	1.1	1.4	0.222	0.622	0.092
Fecal score[3]	3.16[ab]	2.87[b]	2.96[ab]	3.29[a]	0.74	0.490	0.890	0.041
Antibiotic interventions[4]	2.25[ab]	6.38[a]	2.75[ab]	1.88[b]	2.81	0.054	0.114	0.018
Pig-MAP[5], mg/mL	1.09	1.25	0.93	0.84	0.31	0.018	0.776	0.293

[a,b] Different superscripts in the same row denotes significant difference ($P \leq 0.05$).
[1] Low fiber, 5.3% NDF and high fiber, 7.15% NDF; as-fed basis
[2] Mean values of animals having shown diarrhea per pen at the overall experimental period.
[3] Fecal Score: 1 – liquid; 2 – soft; 3 – normal; 4 – hard. At the last experimental day.
[4] Mean values of injections of 1ml/IM of Marbofloxacin (Marbocyl® 2%, Vétoquinol S.A., Lure Cedex, France) per pen at the overall experimental period.
[5] At the last experimental day.

Table 4.4. Effects of protein and dietary fiber on gastrointestinal % of BW and the proximal colon morphology of piglets on d 35 after weaning.

	Diets							
CP (%)	16		20			P-values		
Fiber content[1]	Low	High	Low	High	SEM	CP	FB	CP*FB
Intestinal weight (with content)								
Small intestine, % of BW	6.2	6.8	7.6	7.8	1.10	0.004	0.346	0.158
Large intestine, % of BW	3.8	4.5	4.3	5.4	0.90	0.048	0.018	0.486
Morphology								
Goblet cells/100 μm	4.3	4.9	5.1	5.6	0.35	0.043	0.130	0.772
IEL, cells/100 μm^2	1.8	1.7	1.2	1.5	0.17	0.020	0.423	0.252

[1] Low fiber, 5.3% NDF and high fiber, 7.15% NDF; as-fed basis
[2] IEL = intraepithelial lymphocytes.

4.3.3. Physicochemical Characteristics of Digesta and Fermentation

In Table 4.5 the viscosity, ammonia concentration and SCFA (concentration and profile) of the proximal colon digesta are presented. A significant interaction ($P \leq 0.05$) between protein and fiber levels was observed on the viscosity values of proximal colon digesta. Supplementation of fiber increased the viscosity of the colonic digesta in pigs fed on the 16% CP diet and decreased it with the 20% CP diet. Supplementation of fiber in the 16% CP diet tended also to increase the ammonia concentration of the proximal colon digesta, but decreased it when it was supplemented in the 20% CP diet (interaction, P = 0.073).

High levels of protein (P = 0.066) or fiber ($P \leq 0.05$) increased the concentration of the total amount of SCFA in the colonic digesta. Protein level had no effect on the SCFA profile, but the fiber supplementation decreased ($P \leq 0.05$) the proportion of propionic acid. An interaction was observed in the isoacids (sum of isobutyric and isovaleric acids) proportion, where the fiber supplementation decreased ($P \leq 0.05$) their concentrations in the 20% CP diets, but increased their concentrations in the 16% CP diets.

Table 4.6 presents the results of standard microbiological testing for plate counts of enterococci and coliforms, and molecular microbiology using Real Time PCR for counts of 16S rDNA gene copies of enterobacteria and lactobacilli on the last day of the experiment. On the first day of sampling no significant differences were observed between treatments (data not shown). On d 35 after weaning, the increase in the level of protein did not promote changes on the counts of bacteria in feces. On the other hand, the increase in the level of fiber reduced ($P \leq 0.05$) the coliform counts measured by conventional microbiology and enterobacteria measured by molecular microbiology. A significant increase was observed in the lactobacilli:enterobacteria ratio after fiber supplementation.

Table 4.5. Effect of protein and dietary fiber on the physicochemical characterization of colon digesta and the amount and profile of short-chain fatty acid (SCFA) in the proximal colon digesta of piglets on d 35 after weaning.

	Diets							
CP (%)	16		20			P-values		
Fiber content[1]	Low	High	Low	High	SEM	CP	FB	CP*FB
Viscosity (Log of cP, SR11)	2.8^{ab}	3.1^{a}	3.1^{ab}	2.6^{b}	0.44	0.515	0.559	0.033
Ammonia (mM/kg of FM)	8.4	10.8	9.2	8.2	2.52	0.365	0.479	0.073
SCFA, mmol/L	126	147	146	155	20.2	0.066	0.048	0.376
Profile, %SCFA								
Acetic	51.2	55.5	53.0	63.4	4.47	0.936	0.153	0.235
Propionic	30.8	27.6	31.7	26.5	4.29	0.947	0.011	0.531
Butyric	12.3	11.7	10.5	14.2	3.49	0.806	0.223	0.095
Isoacids[2]	0.7^{b}	1.1^{a}	0.9^{ab}	0.6^{b}	0.31	0.28	0.653	0.010
Lactic	0.1	0.3	0.2	0.4	0.513	0.452	0.333	0.877

[a,b] Different superscripts in the same row denotes significant difference ($p \leq 0.05$).

[1] Low fiber, 5.3% NDF and high fiber, 7.15% NDF; as-fed basis

[2] Sum of isobutyric and isovaleric acids.

Table 4.6. Effects of protein and dietary fiber on the counts of bacteria measured by conventional microbiological analyses (enterococci and coliforms) and by Real-Time PCR (qPCR, enterobacteria and lactobacilli) in the feces of piglets on d 35 after weaning.

	Diets							
CP(%)	16		20			P-values		
Fiber content[1]	Low	High	Low	High	SEM	CP	FB	CP*FB
Plate count, Log of CFU/g								
Enterococci	5.3	6.0	5.3	5.3	0.92	0.183	0.222	0.256
Coliforms	7.5	7.1	8.3	7.0	1.20	0.254	0.005	0.122
qPCR, log 16S rDNA gene copies/g								
Enterobacteria	10.7	10.4	11.1	10.3	1.13	0.623	0.061	0.406
Lactobacilli	11.6	11.7	11.7	11.8	0.36	0.391	0.463	0.917
Ratio lacto:entero[2]	0.91	1.28	0.61	1.46	1.131	0.828	0.036	0.388

[1] Low fiber, 5.3% NDF and high fiber, 7.15% NDF; as-fed basis

[2] log 16S rDNA gene lactobacilli copies/g of feces minus log 16S rDNA gene enterobacteria copies/g of feces (as-is basis).

4.4. Discussion

4.4.1. Influence of the Level of CP on the Productive Performance

Previous reports have described the possibility of reducing CP level in piglet feeds in association with an adequate AA supplementation without affecting gain and protein deposition (Le Bellego and Noblet, 2002). These authors described a reduction in the N excretion and an increase in the feed intake when CP was reduced from 22.4% to 20.4%. However, the practical application of this strategy to lower levels of dietary protein may be limited by the availability of essential AA in relation to the requirements of the piglets (Figueroa *et al*., 2002). In a recent study, Nyachoti *et al*. (2006) observed a significant reduction on the ADG and ADFI of piglets when received diets containing 19% or less CP. The authors suggested that Ile, Val or some other essential AA might have limited piglet performance. In the present study pigs fed on the 16% CP diet showed similar performance than those pigs fed on the 20% CP diet, but showed a lower intestinal weight and a lower number of mucus-containing goblet cells. These results are similar to those presented by Le Bellego and Noblet, (2002) who described that protein restriction (22.4 vs. 16.9%) in association with an adequate AA supplementation did not affect the level of performance of the piglets, but decreased the contribution of the internal organs (including the GIT) to the empty BW. As stated previously, diets used in the present study were formulated to satisfy requirement standards for pigs between 10 to 30 kg (BSAS, 2003) on a NE, Lys, Met, Thr and Trp basis, but no corrections were performed for other essential amino acids, such as Ile or Val (Table 1). The nutritional state appears to be a key factor in regulating the GIT development, the mucous layer and the gut barrier integrity. Some AA, such as Leu, Pro and Gln, which were at lower contents in the 16%CP diet, are recognized as essential substrates for the intestinal anabolic and catabolic processes (Wu, 1998; Buddington *et al*., 2001); and for the development of an adequate intestinal enzymatic activity (Zhang *et al*., 1998; Adeola and King, 2006).

4.4.2. Influence of the Level of Fiber on the Productive Performance

Results from the study indicate that the supplementation of the piglet diet with WB (40 g/kg) and SBP (20 g/kg) improved the productive performance and the feed efficiency of piglets from wk 3 to 5 after weaning. Differences in the BW at 5 wk after weaning ranged from 16.8 to 18.2 kg with the LF and HF diets, respectively. However, it should be pointed out that the increased weight gain with the HF diets was also due in part to the increased weight of the internal organs, possibly by a higher weight of the gut contents. Differences for the full small and large intestine weight averaged from 1.99 to 2.43 kg in piglets fed the LF and HF 20%CP diets respectively, which account for approximately 30 to 40% of the BW differences.

In the literature, variable results are reported on the influence of DF in the daily gain and feed efficiency of piglets. It is generally accepted that fiber in the growing pig diets may reduce the voluntary intake and the fat and energy digestibility. Thus, in some studies, supplementation of the diets with NSP has been associated with a decrease in the daily weight gain of growing animals, even if formulations were calculated to provide a similar calculated net energy supply. Wellock *et al*. (2008) reported a decrease in the weight gain of piglets (wk 1 and 2 after weaning) fed on diets when non-starch polysaccharides (NSP) content was increased from 9.4 to 17.7% of NSP with high amounts (15%) of inulin and highly purified cellulose. Similar results were observed by Freire *et al*. (1998) when piglets were fed on diets supplemented with 15% of wheat bran (from 10 to 15% of NDF).

In contrast, other studies support the idea of no adverse effects on the weight gain or feed efficiency when the piglets are fed on moderate amounts of non-viscous fibrous sources. Longland *et al*. (1994) and Gill *et al*. (2000) reported no significant differences in the weight gain and the apparent digestibility of N and GE when 4 to 8 wk old piglets were fed a cereal based diet containing 0 or 15 to 18.5% of sugar-beet pulp (NSP from 13.8 to 21.8%, NDF 12.1 to 16.5%). Any negative effect was restricted to the first week after weaning, when high fiber diets tended to restrict voluntary intake and significantly reduced daily gain. Mateos *et al*. (2006) showed an interaction

between the type of cereal and a moderate amount of oat hulls inclusion (2 to 4%) on their effect on the feed intake and BW gain of piglets from wk 1 to 5 after weaning. Additional fiber increased performance in piglets given rice but reduced it in piglets given maize. In both cases, oat hulls supplementation increased the apparent total tract digestibility coefficients of OM, CP and energy. The authors suggested that young pigs may have a minimal requirement for fiber of 6% NDF for 6 to 12 kg piglets, that was not reached in the rice diets (3.43% of NDF). The BSAS (2003) nutrient requirement standard for pigs suggests a NDF content of 7 to 13% for weaned pigs of 10 to 30 kg live weight. In our study, the increase on the NDF content from 5.3 to 7.15% was associated with an enhanced growth performance of the animals, and confirms the fact that pigs may have a minimal requirement for fiber. Diets with low dietary fiber content and mean particle size, below requirements for a healthy development of the gastrointestinal tract of young pigs, may constrain the voluntary intake and the efficient utilization of nutrients.

4.4.3. Influence of the Level of CP and Fiber on the Intestinal Health

Our experiment showed an interaction between the protein and fiber level in the incidence of diarrhea and antibiotic interventions. The incorporation of fiber increased diarrhea in pigs fed on the 16% CP diet, but reduced it in pigs fed on the 20% CP diet. Intriguingly, this interaction was not observed on the productive performance or the microbial counts in the colonic digesta, which it may be likely explained by the higher antibiotic interventions applied to piglets fed on the HF 16%CP diet or by changes in other bacteria species not accounted for in our microbial study. Similar results on the interaction between CP concentration and fermentable carbohydrates have been reported by other authors in the productive performance or the intestinal microbiota. Bikker *et al*. (2006) reported that a HF diet (19.5 vs. 13.2% NSP) reduced growth performance of pigs from 4 to 8 wk of age, when fed on a low CP diet (15.2%) but not on a high CP diet (21.6%). In the same way, Pierce *et al*. (2007) showed that increasing concentrations of lactose in the postweaning diet, from 3 to 6 wk of age, allowed significant increases in the ADFI and ADG of piglets fed on a 21% CP diet but

reduced it when supplemented to a 16% CP diet. The authors also reported significant increases with the lactose supplementation in the lactobacilli and *Bifidobacterium* concentration in feces, and significant decreases in the *E. coli* counts, especially in the high protein diet.

Protein fermentation in the digestive tract is considered as a potential risk provoking an intestinal disbiosis and the proliferation of pathogenic bacteria (Prohaszka and Baron, 1980; Ball and Aherne, 1987). Some of the products released by protein fermentation contributes to the SCFA production, but also release potential irritants of the intestinal mucosa, such as ammonia, skatol, or indol. In our study, we found the highest concentrations of isoacids and ammonia in the colonic digesta of the LF 20%CP diets and HF 16%CP. Our results confirm that a higher dietary CP content (20%CP) was associated with a higher protein fermentation in the colon; while a simultaneous increase in the amount of fiber into high CP diets increased the fermentation of carbohydrates (ie, the SCFA concentration), and allowed a reduction in the protein fermentation and the enterobacteria counts in feces.

In contrast, it is remarkable that the incorporation of fiber into the 16% CP diet increased the isoacid and ammonia concentrations, the viscosity of the colonic digesta, and the incidence of diarrhea. It could be speculated that a low protein diet coupled with a higher fiber diet might have altered the GIT function and increased the amount of substrates that escape digestion in the small intestine, predisposing pigs to diarrhea (Pluske *et al*., 2002). Unfortunately, we did not measure foregut digestibility or small intestine digesta parameters. However, as described above, we observed a lower relative weight of the small and large intestine, and a lower number of mucus-containing goblet cells in piglets fed the low CP diet than the high CP diet, with likely implications on the gut barrier function. These results were coupled with a higher count of intraepithelial lymphocytes in the colon epithelium and a higher serum concentration of the PigMAP in the low CP diets. Acute phase protein serum concentration is known to increase after inflammation caused by tissue damage or pathogen challenge (Piñeiro *et al*., 2009), the magnitude of the response being in general related to the degree of lesions or extent of the disease (Murata *et al*., 2004).

4.5. Conclusion

Present results suggest that a moderate inclusion of DF in the diet may allow increases in the productive performance and gut maturation of the piglets. However, its effect on the intestinal health may depend on the level of protein and essential AA in the diet, which appears to have a key role in the development and function of the intestinal tract. On the whole, protein levels of 20% CP supplemented with wheat bran (4%) and sugar beet pulp (2%) showed positive effects on the animal performance and less incidence of diarrhea and antibiotic interventions.

4.6. Literature cited

Adeola O and King DE (2006) Developmental changes in morphometry of the small intestine and jejunal sucrase activity during the first nine weeks of postnatal growth in pigs. *J Anim Sci* **84,** 112-118.

AOAC, Associ. Offic. Anal. Chem. (1995) Official methods of analysis. Associ. Offic. Anal. Chem.

Awati A, Williams BA, Bosch MW, *et al*. (2006) Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in feces of weanling piglets. *J Anim Sci* **84**, 2133-2140.

Ball RO and Aherne FK (1987) Influence of dietary nutrient density, level of feed intake and weaning age on young pigs. II. apparent nutrient digestibility and incidence and severity of diarrhea. *Can J Anim Sci* **67**, 1105-1115.

Bertschinger HU, Eggenberger E, Jucker H, *et al* (1979) Evaluation of low nutrient, high fiber diets for the prevention of porcine *Escherichia coli* enterotoxaemia. *Vet Microbiol* **3,** 281-290.

Bikker P, Dirkzwager A, Fledderus J, *et al*. (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J Anim Sci* **84**, 3337-3345.

BSAS, British Society of Animal Science (2003) Nutrient requirement standards for pigs. Penicuik, Midlothian, UK,

Buddington RK, Elnif J, Puchal-Gardiner AA, *et al*. (2001) Intestinal apical amino acid absorption during development of the pig. *Am J Physiol Regul Integr Comp Physiol* **280,** R241-247.

Carneiro M, Lordelo M, Cunha LF, *et al*. (2007) Microbial activity in the gut of piglets: II. effect of fiber source and enzyme supplementation. *Livest Sci* **108,** 262-265.

Castillo M, Martín-Orúe SM, Roca M, *et al*. (2005) The response of gastrointestinal microbiota to the use of avilamycin, butyrate and plant extracts in early-weaned pigs. *J Anim Sci* **84,** 2725-2734.

Diebold G, Mosenthin R, Piepho H, *et al*. (2004) Effect of supplementation of xylanase and phospholipase to wheat-based diet for weanling pigs on nutrient digestibility concentrations of microbial metabolites in ileal digesta and feces. *J Anim Sci* **82,** 2647-2656.

Figueroa JL, Lewis AJ, Miller PS, *et al*. (2002) Nitrogen metabolism and growth performance of gilts fed standard corn-soybean meal diets or low-crude protein, amino acid-supplemented diets. *J Anim Sci* **80,** 2911-2919.

Freire JPB, Peiniau J, Cunha LF, *et al*. (1998) Comparative effects of dietary fat and fiber in alentejano and large white piglets: Digestibility, digestive enzymes and metabolic data. *Livest Prod Sci* **53**, 37-47.

Gill BP, Mellange J, Rooke JA (2000) Growth performance and apparent nutrient digestibility in weaned piglets offered wheat-, barley- or sugar-beet pulp-based diets supplemented with food enzymes. *Anim Sci* **70**, 107-118.

Htoo JK, Araiza BA, Sauer WC, *et al*. (2007) Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs. *J Anim Sci* **85**, 3303-3312.

Jensen BB Jorgensen H (1994) Effect of dietary fiber on microbial activity and microbial gasproduction in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60**, 1897-1904.

Kim JC, Mullan BP, Hampson DJ, *et al*. (2007) Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhea and reduces indices of protein fermentation in the gastrointestinal tract. *Br J Nutr* **99**, 1217-1225.

Le Bellego L and Noblet J (2002) Performance and utilization of dietary energy and amino acids in piglets fed low protein diets. *Livest Prod Sci* **76,** 45-58.

Longland AC, Carruthers J, Low AG (1994) The ability of piglets 4 to 8 weeks old to digest and perform on diets containing two contrasting sources of non-starch polysaccharide. *Anim Pro* **58**, 465-480.

Mateos GG, Martin M, Latorre MA, *et al*. (2006) Inclusion of oat hull in diets for young pigs based on cooked maize or cooked rice. *Anim Sci* **82**, 57-63.

Murata H, Shimada N Yoshioka M (2004) Current research on acute phase proteins in veterinary diagnosis: An overview. *Vet Journal* **168**, 28-40.

Nofrarias M, Manzanilla EG, Pujols J, *et al*. (2006) Effects of spray-dried porcine plasma and plant extracts on intestinal morphology and on leukocyte cell subsets of weaned pigs. *J Anim Sci* **84**, 2735-2742.

Nyachoti CM, Omogbenigun FO, Rademacher M, *et al*. (2006) Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *J Anim Sci* **84**, 125-134.

Pierce KM, Callan JJ, McCarthy P, *et al*. (2007) The interaction between lactose level and crude protein concentration on piglet post-weaning performance, nitrogen metabolism, selected faecal microbial populations and faecal volatile fatty acid concentrations. *Anim Feed Sci Technol* **132,** 267-282.

Piñeiro C, Piñeiro M, Morales J, *et al*. (2009) Pig-MAP and haptoglobin concentration reference values in swine from commercial farms. *Vet J* **179,** 78-84,

Pluske JR, Pethick DW, Hopwood DE, *et al*. (2002) Nutritional influences on some major enteric bacterial diseases of pigs. *Nutr Res Rev* **15**, 333-371.

Prohaszka L and Baron F (1980) The predisposing role of high dietary protein supplies in enteropathogenic *E. coli* infections of weaned pigs. *Zentralbl Veterinarmed B* **27,** 222-232.

SAS Inc., 1999. SAS® User's Guide: Statistics, Version 8 Edition. SAS Inc., Cary, NC, USA.

Wellock IJ, Fortomaris PD, Houdijk JGM, *et al*. (2008) The consequences of non-starch polysaccharide solubility and inclusion level on the health and performance of weaned pigs challenged with enterotoxigenic *Escherichia coli*. *Br J Nutr* **99,** 520-530.

Wu, G. 1998. Intestinal mucosal amino acid catabolism. *J Nutr* **128,** 1249-1252.

Zhang H, Malo C, Boyle CR, *et al*. (1998) Diet influences development of the pig (*Sus scrofa*) intestine during the first 6 hours after birth. *J Nutr* **128,** 1302-1310.

Chapter 5

Effects of type of cereal and fibre level on growth and parameters of digestive maturation in young pigs. *Livest Sci*, 2010, **133**: 225-228.

5.1. Introduction

Formulation of diets for piglets is still a controversial area for the swine nutritionist. In particular, some decisions, such as which is the main cereal ingredient to use or which is the optimum level of fibre in the diet, are considered important issues affecting the palatability of the diet (Solà-Oriol *et al*., 2008), the feed intake (Mateos *et al*., 2006) and the gut maturation of the young piglet (Montagne *et al*., 2003). The election of the main cereal and fibrous ingredients have been also related with changes on the microbial fermentation pattern and with likely changes on the predisposition of piglets to diarrhea (Pluske *et al*., 2002). At this respect, protein fermentation in the digestive tract of piglets is known to release irritant products in the digesta content, such as ammonia, and it is considered one of the mechanisms that predispose piglets to disbiosis and diarrhoea (Aumaitre *et al*., 1995; Nollet *et al*., 1999). In order to reduce the protein fermentation some authors have suggested the inclusion of fermentable carbohydrates in the diet (Awati *et al*., 2006). Increasing the dietary FC content, through the inclusion of lactose (Pierce *et al*., 2007), wheat bran, sugar beet pulp or raw potato starch have been proved to stimulate lactic and butyric acid production in the small and large intestine (Bikker *et al*., 2006, Carneiro *et al*., 2007), and to reduce the counts of coliform bacteria in the small intestine (Bikker *et al*., 2006) and faeces (Pierce *et al*., 2007). Mateos *et al*. (2006) also described that the inclusion of moderate levels of fibre in the diet as oat hulls (2-4%) reduced the incidence of diarrhoea in diets based on cooked maize or cooked broken rice, but increased the feed intake with rice and reduced it with maize. The objective of the present study was to investigate the effects of incorporating moderate amount of fibrous ingredients (wheat bran and sugarbeet pulp) in two diets differing in the basal cereal, either rice, as a low fibre and palatable ingredient, or barley, with higher fibre content.

5.2. Materials and methods

A total of 144 piglets [BW, 7.6 ± 1.7 kg, (Large White x Landrace) x Pietrain] were weaned on day 26 and allocated in 36 pens (4 animals/pen) at the experimental facilities of IRTA Mas de Bover (Reus, Spain). During the first 14 days post weaning the animals received 2 different diets based on rice or barley (60%), vegetal protein (20%) and dairy products (15%). Individual BW and pen feed consumption was weekly registered.

On day 14 after weaning (40 days of age), the piglets were transported to the Universitat Autònoma de Barcelona facilities, weighted (9.07 ± 0.36 kg BW) and placed to 32 pens (3 animals/pen) considering the cereal fed by the animals. The pens were allotted on 4 treatments (Table 1) in a 2x2 factorial design, that included again the two types of cereals (Rice v. Barley) and two levels of Fibre (LF v. HF, Rice: from 2.83 to 3.61 of NDF, % of DM; and Barley: from 12.24 to 13.42 of NDF, % of DM). The HF content was obtained by including 4% of wheat bran (WB), the coarse outer covering of the wheat kernel and 2% of sugarbeet pulp (SBP). Diets were isoenergetic (10.32 MJ of ME/kg) and isonitrogenous (18.8% of CP) and formulated to satisfy the nutrient requirement standards for pigs (BSAS, 2003). Each pen had a feeder and a water nipple to ensure ad libitum feeding and free water access.

Animals received the experimental diets (Table 1) from day 14 to 35 post weaning. Individual BW and pen feed consumption was weekly registered. Animals were checked daily in order to evaluate the health status (by clinical evaluation and faecal scoring). On the last day of experiment, a sample of faeces was taken from the rectum of one piglet of each pen (total of 32 samples) for immediate traditional microbiology analyses. At the same day the animal with the intermediate weight of each pen was euthanized with an intravenous injection of sodium pentobarbital (200 mg/kg BW). Animals were bled, and the abdomen was immediately opened to tie and remove the whole gastrointestinal tract. The stomach, small intestine and the large intestine

were separated by double ties, weighed (with content), and sampled. Samples of proximal colon digesta were collected, and kept at -20ºC for further volatile fatty acids and ammonia quantification.

Chemical analyses of the diets (Table 5.1) were performed according to the Association of Official Analytical Chemists (AOAC, 1995) standard procedures. Ammonia was determined with the aid of a gas sensitive electrode (Crison ISE- 9665) combined with a digital voltmeter (Crison GLP 22). Three grams of digesta were diluted (1:2) with 0.16 M NaOH, and after homogenization, samples were centrifuged for 10 min (1500 x g). The ammonia released was measured in the supernatant as different voltage in mV (Adapted from Diebold *et al*., 2004). Short chain fatty acids were determined by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatization, as described by Jensen and Jorgensen (1994).

For bacterial counts, fresh digesta samples were suspended (10%, wt/vol) in a buffered peptone solution and subsequently homogenized. Thereafter, 10-fold dilutions were made with a buffered peptone physiological salt solution (CM509, Oxoid Ltd., Basingstoke, Hampshire, UK; containing peptone 10.0 g/L, sodium chloride 5.0 g/L, di-sodium phosphate 3.5 g/L, and potassium dihydrogen phosphate 1.5 g/L). For the enumeration of Enterococci, 100 µL of each dilution was pipetted as a drop on Chromocult® Enterococci-Agar (Merck KGaA, Darmstadt, Germany; dissolving 33g of agar/L of distilled water). The plates were incubated for 24 h at 37°C. The red colonies with a diameter of 0.5 to 2 mm were counted. For the enumeration of *E. coli* and total coliforms, 1 mL of solution was pipetted onto an *E. coli*/coliform count plate (3M Petrifilm, Europe Laboratoires 3M Santé, Cergy-Pontoise, France) with Violet Red Bile gel and an indicator of glucuronidase activity. The plates were incubated for 48 h at 37°C, and all blue *E. coli* colonies and total coliform colonies were counted following the manufacturer's instructions.

Table 5.1. Ingredients and analysed chemical composition (%) of the experimental diets.

	Type of Cereal			
Cereal	Rice		Barley	
Level of fibre	LF	HF	LF	HF
Ingredient , g/kg of feed				
Rice	593.3	539.3	-	-
Barley	-	-	583.0	509.4
Wheat bran	-	40.0	-	40.0
Sugar beet pulp	-	20.0	-	20.0
Whey D 68-15-8	147.2	147.2	142.2	142.2
Potato protein	83.6	83.6	50.0	50.0
Soybean meal, 44%CP	127.1	117.1	127.1	138.1
Animal fat	14.7	20.7	61.1	65.5
Calcium carbonate	5.4	5.6	6.8	6.8
Dicalcium phosphate	14.0	12.7	11.3	10.3
Sodium chloride	3.0	2.0	2.2	2.2
L-Lysine 99-78	3.0	3.1	5.3	4.9
DL-Metionine 99	1.8	1.8	2.6	2.6
L-Threonine 98	0.8	0.8	2.1	2.1
L-Tryptophan	0.9	0.9	1.0	1.0
Coline chloride, 50%	1.2	1.2	1.2	1.2
Vitamin and mineral*	4.0	4.0	4.0	4.0
Calculated content				
ME, MJ/kg	10.32	10.32	10.32	10.32
Lysine, %	1.4	1.4	1.4	1.4
Analysed chemical composition, % (DM basis)				
DM	88.78	88.86	91.01	90.98
Ash	5.39	5.11	5.97	5.78
GE (MJ/Kg DM)	16.56	16.80	18.29	18.37
Ether Extract	2.27	2.68	7.05	8.05
CP	18.69	18.03	19.15	19.31
NDF	2.83	3.61	12.24	13.42
ADF	1.36	1.47	4.01	4.32

*Supplied per kilogram of feed: 5000 IU of vitamin A, 2000 IU of vitamin D3, 15.0 mg of vitamin E, 1.3 mg of vitamin B1, 3.5 mg of vitamin B2, 1.5 mg of vitamin B6, 0.025 mg of vitamin B12, 10.0 mg of calcium pantothenate, 15.0 mg of niacin, 0.1 mg of biotin, 0.6 mg of folic acid, 2.0 mg of vitamin K3, 80.0 mg of Fe, 6.0 mg of Cu, 0,75 mg of Co, 150 mg of Zn, 60 mg of Mn, 0,75 mg of I, 0,10 mg of Se and 150 mg of Etoxiquín.

Data on productive performance, organ measurements, microbial counts, and fermentation parameters (VFA and ammonia) were subjected to factorial analysis, with initial body weight as the covariation factor (whenever significant), using the GLM procedure of SAS (1999). For all data analyzed, the pen was used as the experimental unit. The alpha level used for the determination of significance for all the analysis was 0.05. Data is presented as least square means (LS means) and standard error of the mean (SEM).

5.3. Results and Discussion

Average daily feed intake (ADFI), body weight (BW), average daily gain (ADG) and gain:feed efficiency (G:F) of piglets from d0 to 14 and from d14 to 35 post weaning are presented in Table 5.2. During the pre-starter period (d0-14), animals fed on the rice based diet showed a quantitative but not significant higher feed intake than animals fed on barley. Not significant differences were observed on the BW gain of the animals. During the following period (from d 14 to 35), the piglets fed on the rice based diet showed a higher ($P \leq 0.05$) ADFI than animals fed on the barley based diet. As a consequence, animals fed on the rice based diet tended to have a higher BW on day 35 post weaning than piglets fed on the barley based diet (19.6 vs. 18.5 kg, respectively). On the other hand, the supplementation of fibre did not cause significant changes on the animal performance of the piglets. Several reports have shown better performance indices in pigs fed rice based diets compared with other cereal, such as maize (Mateos *et al*., 2007) or barley (Pluske *et al*., 2002). Differences on the fibre content between cereals could explain the higher digestibility (Mateos *et al*., 2007) coefficients and higher feed intake (palatability) observed in piglets fed rice with respect to piglets fed sorghum, maize or rye (Solà-Oriol *et al*., 2008). In contrast, the moderate supplementation level of fibre used was not able to reduce the ADFI and consequently the productive performance. It was not observed significant differences on health status parameters (clinical signs, faecal score and mortality) between treatments.

Table 5.2. Effects of the type of cereal and the fibre inclusion level on the growth performance (BW, Kg.), the average daily feed intake (ADFI; g/animal and day), the average daily gain (ADG; g/animal/ and day) and the feed intake ratio (G:F) on piglets from d0 to 14 and from d14 to 35 post weaning.

		Diets							
	Cereal	Rice		Barley			*P values*		
	Fibre Level	Low	High	Low	High	SEM	*cereal*	*fibre*	*c x f*
BW (Kg.)	d0	7.64		7.60		0.04	0.369	-	-
	d14	9.12	8.87	9.23	9.07	0.36	0.246	0.117	0.723
	d35	19.34	19.78	18.37	18.71	1.51	0.067	0.474	0.911
ADFI (g)	d0-7	116		89		19.2	0.158	-	-
	d0-14	232		202		24.0	0.218	-	-
	d14-35	791	779	671	682	125	0.027	0.853	0.763
ADG (g)	d0-7	12		-23		27.9	0.225	-	-
	d0-14	114		93		25.0	0.400	-	-
	d14-35	487	510	443	461	97	0.351	0.484	0.835
G:F	d0-7	0.41		0.32		0.21	0.707	-	-
	d0-14	0.29		0.30		0.07	0.513	-	-
	d14-35	0.62	0.68	0.69	0.69	0.11	0.366	0.404	0.707

The results of the relative intestinal tract weight, the ammonia concentration, the volatile fatty acids (concentration and profile) in the proximal colon digesta, and the traditional microbiology for plate counts of Enterococci and coliforms at 35 days post weaning are summarized in Table 4. Changes on the cereal source and the fibre level in the diet promoted significant differences on the relative weight of the intestines. Piglets fed on barley based diet showed an increased ($P \leq 0.01$) on relative weight of the full total intestinal tract and large intestine. Diets supplemented with WB and SBP further increased the relative weight of the large intestine ($P \leq 0.05$). The results reflect that an increase on the fibre content promoted an increase on the gastrointestinal weight, likely associated with a higher weight of the gut contents (Anguita *et al*., 2007).

The fibre supplementation and the barley based diets determined a decrease ($P < 0.05$) on the ammonia concentration. Also, the barley based diets decreased the relative isoacids concentration ($P = 0.007$), which likely reflects a decrease on protein fermentation (Bikker *et al*., 2006). Previous reports have also confirmed that an increase in the amount of fermentable carbohydrates may reduce the protein fermentation (Awati *et al*., 2006, Bikker *et al*., 2006), with a likely benefit shift on the microbial populations. At this respect, pigs fed barley based diets tended ($P = 0.10$) to have a lower number of coliforms in faeces than piglets fed rice based diets; while the increase on the level of fibre in the diet with the WB and SBP supplementation increased ($P \leq 0.05$) the Enterococci counts, especially in the rice based diet. Considering that Enterococci are a lactic acid bacteria genus, the present results suggest that the WB and SBP promoted a beneficial shift on the gut microbiota.

Table 5.3. Effects of the type of cereal and the fibre inclusion level on the relative gastrointestinal tract (GIT, % of BW), on the ammonia concentration (mM/L), and on the volatile fatty acids (VFA, mM/L) of proximal colon digesta and on the number of Enterococci and coliforms (Log of CFU/ g faeces) on piglets of 35 days post weaning.

	Diets							
Cereal	Rice		Barley			*P values*		
Level of Fibre	Low	High	Low	High	SEM	*cereal*	*fibre*	*c x f*
TGI (% of BW)								
Total	9.9	10.5	12.6	13.1	1.38	<0.001	0.277	0.894
Large Intestine	3.8	4.2	5.7	6.6	0.86	<0.001	0.044	0.493
Ammonia (mM/L)	15.8	12.5	12.6	8.9	3.91	0.019	0.016	0.861
VFA (Total, mM/L)	113	129	129	121	31.9	0.744	0.716	0.308
VFA (Profile, %)								
Acetic	55.75	55.84	52.39	56.17	4.58	0.262	0.158	0.421
Propionic	24.52	25.39	30.30	28.32	2.98	0.001	0.621	0.206
Butyric	11.85	11.36	10.65	10.99	2.61	0.416	0.924	0.662
Isoacids[1]	2.33	1.70	1.03	0.97	0.98	0.007	0.328	0.424
Enterococci (log CFU/g)	4.508	5.891	4.109	4.885	1.114	0.110	0.015	0.397
Coliforms (log CFU/g)	6.708	6.419	6.156	5.859	0.941	0.100	0.386	0.991

[1]Sum of isobutiric and isovaleric.acids.

5.4. Conclusion

As a whole, the results confirm that, even formulating for a similar metabolizable energy content, piglets fed on the rice diet showed higher performance than piglets fed on barley, likely associated to the higher palatability of rice, or the higher intake of low fibre diets. However, the low fermentable content of rice also increased the metabolites of protein fermentation in the colon digesta and tended to increase the faecal excretion of coliforms. A moderate supplementation with wheat bran and sugarbeet pulp attenuates those effects by reducing the ammonia concentration and increasing the number of Enterococci.

5.5. Literature cited

Anguita M, Gasa J, Nofrarias M, *et al*. (2007) Effect of coarse ground corn, sugar beet pulp and wheat bran on the voluntary intake and physicochemical characteristics of digesta of growing pigs. *Livest Sci* **107**, 182-191.

AOAC, Associ. Offic. Anal. Chem. 1995. Official methods of analysis. Associ. Offic. Anal. Chem.

Aumaitre A, Peiniau J, Madec F (1995) Digestive adaptation after weaning and nutritional consequences in the piglet. *Pig News and Inform* **16**, 73N-79N.

Awati A, Williams BA, Bosch MW, *et al*. (2006) Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in feces of weanling piglets. *J Anim Sci* **84**, 2133-2140.

Bikker P, Dirkzwager A, Fledderus J, *et al*. (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J Anim Sci* **84**, 3337-3345.

BSAS, British Society of Animal Science. 2003. Nutrient requirement standards for pigs. Penicuik, Midlothian, UK,

Carneiro M, Lordelo M, Cunha LF, *et al*. (2007) Microbial activity in the gut of piglets: II. Effect of fiber source and enzyme supplementation. *Livest Sci* **108**, 262-265.

Diebold G, Mosenthin R, Piepho H, *et al*. (2004) Effect of supplementation of xylanase and phospholipase to a wheat-based diet for weanling pigs on nutrient digestibility and concentrations of microbial metabolites in ileal digesta and feces. *J Anim Sci* **82**, 2647-2656.

Jensen BB, Jorgensen H (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60**, 1897-1904.

Mateos GG, Martin M, Latorre MA, *et al*. (2006) Inclusion of oat hull in diets for young pigs based on cooked maize or cooked rice. *Anim Sci* **82**, 57-63.

Mateos GG, López E, Latorre MA, *et al*. (2007) The effect of inclusion of oat hulls in piglet diets based on raw or cooked rice and maize. *Anim Feed Sci Technol* **135**, 100-112.

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

Nollet H, Deprez P, Van Driessche E, *et al*. (1999) Protection of just weaned pigs against infection with F18+ *Escherichia coli* by non-immune plasma powder. *Vet Microbiol* **65**, 37-45.

Pierce KM, Callan JJ, McCarthy P, *et al*. (2007) The interaction between lactose level and crude protein concentration on piglet post-weaning performance, nitrogen metabolism, selected faecal microbial populations and faecal volatile fatty acid concentrations. *Anim Feed Sci Technol* **132**, 267-282.

Pluske JR, Pethick DW, Hopwood DE, *et al*. (2002) Nutritional influences on some major enteric bacterial diseases of pigs. *Nutr Res Rev* **15**, 333-371.

Solà-Oriol D, Roura E, Torrallardona D (2008) Use of double-choice feeding to quantify feed ingredient preferences in pigs. *Livest Sci* 1**23**, 129–137.

Chapter 6

Searching for anti-adhesive feedstuffs to block the attachment of enterotoxigenic *Escherichia coli* K88 to the porcine intestine.

6.1. Introduction

The weaning period is a very critical phase in the swine production due to the multifactorial stressors that predispose animals to opportunistic pathogens. One of these pathogens that take advantages on this scenario is the enterotoxigenic *E. coli* (ETEC) K88 that is an important cause of diarrhea in neonatal and recently weaned pigs (Fairbrother *et al*., 2005). This strain expresses F4 fimbriae, which are proteinaceous filamentous adhesins including repeating copies of a major fimbrial subunit FaeG and some additional minor subunits (Devriendt *et al*., 2010). This fimbria adheres to F4-specific receptors on the intestinal epithelium, subsequently colonizes small intestine and induces diarrhea (Nagy and Fekete, 2005).

Given the specificity of these mechanisms, it has been speculated the possibility of using molecules (mainly biologically active compounds) that could block the bacterial fimbriae and prevent the gastrointestinal colonization by ETEC. The family of these molecules is glycoproteins with different combinations of peptides and carbohydrates created by the glycosylation process and with an important role in the immune regulation (Marth and Grewal, 2008). Recently, this nutritional strategy was called as anti-adhesive therapy (Shoaf-Sweeney and Hutkins, 2009) and are a promising field of study aiming to reduce the antibiotic therapy in human (Ghazarian *et al*., 2011) and animals (Becker and Galletti, 2008).

The mechanisms proposed is that these substrate can act as “decoys” for the pathogenic bacteria and therefore occupy the fimbrial sites, blocking its adhesion to the intestinal epithelium favoring the bacteria to be excreted on feces (Shoaf-Sweeney and Hutkins, 2009).

For these reasons, the objective of the present study was the *in vitro* search for feedstuffs aiming to interact and block the adhesion of ETEC (K88) to the intestine, using a miniaturized model.

6.2. Material and Methods

6.2.1. Feedstuffs extract.

Table 6.1 presents the powder feedstuffs used in this study. Feedstuffs were ground and resuspended (w/v) using sterile PBS to reach 1% concentrations. Then, different suspensions were sonicated in water bath (3 times of 30 second each) and centrifuged (460 x g/5minutes) following Becker *et al*. (2007) described procedures.

Additionally, it was performed a physicochemical fractionation of some of the fibrous feedstuffs tested with the aim of identifying the fractions responsible of the blockage (wheat bran and locust bean). Different carbohydrate fractions were isolated following the procedures described by Maes and Delcour, (2002). This procedure makes possible to separate firstly the water soluble fraction of carbohydrates, through an amylase and protease digestion, and then from the unextractable cell wall material it is possible to alkaline extract two more different fractions. The fractions obtained and their main components are also presented in table 1.

6.2.2. E. coli growth

Two different *E. coli* strains were used in this experiment to elucidate the likely interaction between the feedstuffs and the bacterial fimbriae. The first one was an enterotoxigenic *E. coli* K88 (ETEC) isolated from a colibacillosis outbreak in Spain (Blanco *et al*., 1997), serotype (O149:K91:H10 [K-88]/LT-I/STb) that was gently provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The other one was a non-fimbriated *E. coli* (NFEC) (F4 -, F6 -, F18 -, LT1 -, ST1 -, ST2 +, Stx2e -) isolated from the faeces of post-weaning piglets and kindly donated by the Department of Animal Health and Anatomy from the Universitat Autònoma de Barcelona.

Table 6.1. Characterization of feedstuffs used in the adhesion trial.

#	*Product*	*Treat code*	*Dosis*	*Provider*	*Main compounds*	*References*
1	Neg control – Bovine Serum Albumin	NC	1%	Sigma	Protein – albumin	Becker and Galletti., 2008
2	D-Mannose	MAN	1%	Sigma	Mannose	Becker and Galletti., 2008
3	Natural porcine ileal mucus	MUC	100%	UAB	Mucopolisaccharides	Fang *et al*., 2000
4	Wheat bran extract	WB	1%	Local Mill	Arabinoxylans, PNA	Molist *et al*., 2011
5	Soybean hulls extract	SO	1%	Local Mill	Pectins	Molist *et al*., 2011
6	Sugar beet pulp extract	SBP	1%	Local Mill	Pectins	Molist *et al*., 2011
7	Locust bean extract	LB	1%	Armengol	Galactomannans	Becker and Galletti., 2008
8	Locust gum	LG	0.4%	Polygal	Galactomannans	Becker and Galletti., 2008
9	Guar gum	GG	0.4%	Polygal	Galactomannans	-
10	Casein glycomacropeptide	CGMP	1%	Arla Foods	Glycoproteins rich in sialic acids	Hermes *et al*., 2010
11	Lactobacilli EPS	EPS	1%	Sevilla	Exopolysaccharides	Wang *et al*., 2010
12	Mannanoligossacharides	MOS	1%	Alltech	Mannose, proteins	Becker and Galletti., 2008
13	*Aspergillus oryzae* fermentation extract	AO	1%	Molimen	Galactomannans	Hermes *et al*., 2010
14	Inulin	INU	1%	Orafti	Inulin	-
15	Fructoligossacharides	FOS	1%	Orafti	Oligofructose	-
16	Cranberry extract	CRA	1%	Cran Max	-	Liu *et al*., 2008
17	WB water extractable material	WB WEM	1%	Local Mill	Arabinoxylans (19.4%), β-glucans (3.1%)	Maes and Delcour, 2002
18	WB first alkaline extract after dialysis	WB AED1	1%	Local Mill	Arabinoxylans (64.8%)	Maes and Delcour, 2002
19	WB second alkaline extract after dialysis	WB AED2	1%	Local Mill	Arabinoxylans (65.6%)	Maes and Delcour, 2002
20	SO water extractable material	SO WEM	1%	Local Mill	Galactomannans	Ouhida *et al*., 2002
21	LB water extractable material	LB WEM	1%	Armengol	Galactomannans	-
22	LB first alkaline extract after dialysis	LB AED1	1%	Armengol	Galactomannans	-
23	LB second alkaline extract after dialysis	LB AED2	1%	Armengol	Galactomannans	-

Bacteria were cultured in unshaken Luria broth (Sigma, St Louis) at 37ºC and serially passage every 48h, at least five times. Bacterial cells from the culture were harvested and processed as earlier described (Becker *et al*., 2007).

6.2.3. Ileal porcine mucus isolation

This part of the experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval (permit number: CEAAH 746) from the Animal and Human Experimental Ethical Committee of this institution.

Twenty four piglets (20 days of age) were randomly selected from a commercial farm, with no historic of vaccination of the sows for *E. coli.* A sample of approximately 50 head bristles were carefully taken from each piglet and after washing in a sterile PBS buffer, the genomic DNA was extracted from the follicle using a 3% proteinase kappa solution diluted in a PCR buffer (1x). A genomic characterization test was performed to select pigs susceptible to ETEC K88 infection. This test relies on an XbaI polymorphism in intron 7 of the porcine *MUC4* gene. Primers and methods were those described by Jensen *et al*. (2006).

Subsequently, 5 piglets with a homozygous susceptible genotype were selected, weaned at 28 days of age and fed with a commercial feed. Additionally, animals were treated for 5 consecutive days with oral colistin sulfate (Coliplus® Solution, Divasa Farmavic SA, Barcelona, Spain) at 102,500 UI/kg of body weight with the aim of reducing the presence of coliforms in the mucus receptors. Then, the piglets were euthanized with an intravenous sodium pentobarbital overdose (200 mg/kg BW), bled and the abdomen was immediately opened. To extract the intestinal mucus, the procedures described by Fang *et al*. (2000) were followed. Briefly, the track of intermediate ileum was washed with sterile PBS until the buffer was clear. The gut was cut into about 2m-long sections and put into binding buffer (3.84 mM NaH_2PO_4, 6.16 mM Na_2HPO_4, 0.15 M NaCl, pH 7.2). After removal from the buffer, the sections were split along the mesenteric border. The mucus was collected by gently scraping with a glass slide and was transferred into the

binding buffer (20mL). All processes were performed on an ice-cold bath. The scraps from each section were pooled, mixed, and centrifuged at 10,000 × *g* at 4°C for 15 min to remove particulate material.

The supernatant containing the crude intestinal mucus was saved and preserved at -80ºC until its use. This natural porcine mucus, from ETEC K88-susceptible piglets, was used as a positive control in the *in vitro* adhesion experiment further described.

6.2.4. Anti-adhesive feedstuff screening with microplate systems.

The effect of feedstuffs to adhere to ETEC was determined using an adaptation of an *in vitro* adhesion test previously described (Becker *et al*., 2007). In this test, an overnight incubation with each feedstuffs extract was done in a 96-wells high-binding polystyrene microtitration plates (Microlon F plate 655 092; Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands).

Briefly, after the overnight incubation at 4ºC with feedstuffs, the plates were washed with sterile PBS to remove non-binding material. Afterwards, blocking of non-specific adhesion sites were done by incubating the plates with 1% of BSA + 0.5% of sodium azide in PBS (w/v) at 4ºC for 1h. Thereafter, plates were washed twice with sterile PBS. Bacteria (ETEC K88 and NFEC) were added to the plate wells after growth, washing and suspending in PBS to a final concentration of 3 to 5 x 10^8 CFU/mL in PBS. Finally, 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. The growing of the 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 optical density (OD) at a wavelength of 650 nm at intervals of 10 minutes for 12 hours. All readings were done in two independent assays and in triplicate per trial.

The technique is based on the fact that when bacteria grow, the medium becomes more opaque. Thus OD_{650nm} was measured every 10 minutes using a Spectrophotometer to monitor the

growth of bacteria. Each well described a sigmoid growth curve and growth detection time (h), defined as the time when bacteria start their exponential growth phase (OD = 0.05) and this timepoint was used to compare treatments (Becker *et al*., 2007). The more bacteria that are adhered to the wall of the well the shorter the growth detection time.

6.2.5 Calculations and statistical analysis

All statistical analysis were performed using SAS 9.2. The OD data from the adhesion assay were processed by nonlinear regression analysis, Gauss-Newton method, following the equations described by Becker *et al*. (2007). Calculated bacterial detection times ($t_{OD=0.05}$, h) were analyzed by ANOVA.

6.3. Results

6.3.1. Screening to select anti-adhesive feedstuffs against ETEC K88

Table 6.2 presents the growth detection time (h) for the two bacteria studied, when incubated with the different feedstuffs. In general, it was found differences between the ETEC and the NFEC, showing that fimbriae played an important role in this model. Considering the ETEC results, we could categorize the different feedstuffs into four different levels of adhesive capacity, where the WB, CGMP, and the EPS represented the highest adhesive capacity for this bacteria, with detection times values around 1h. The intermediate feedstuffs would be the MUC, SO, LG, GG and MOS, with detection times values around 2.5h. The LB, FOS and MAN presented similar results as observed for the NC, around 3.5; and the highest values were observed with the SBP, AO, INU and CRAN, representing the feedstuffs with less adhesive capacity in this model.

Regarding the NFEC results, it was found significant differences between treatments but of less magnitude than for the ETEC (ranges from 0.9 to 4.5 h in ETEC and from 3.0 to 5.5 in NFEC). None of the extracts gave lower detection values than the NC. The most important point

observed was the different behavior between the two bacteria for some particular feedstuffs, particularly WB, CGMP, EPS, GG and MOS that presented differences of more than 2 hours between both strains, representing certain specificity grade in favor to the ETEC.

Table 6.2. Detection times of bacterial growth t_{OD} = 0·05 (h) for *E. coli* K88, non-fimbriated *E. coli*, as a measure for adhesion in different feedstuffs extract solutions.

Treatments	***E. coli* K88** t_{OD} **(h)**	**NF *E. coli*** t_{OD} **(h)**
Negative control - BSA 1%/PBS	$3.8^{cd} \pm 0.09$	$3.3^{qr} \pm 0.37$
D-Mannose 1%	$4.0^{a-d} \pm 0.18$	$4.3^{n-p} \pm 0.15$
Natural porcine ileal mucus	$2.2^{f} \pm 0.02$	$4.3^{n-p} \pm 0.21$
Wheat bran 1%	$1.2^{g} \pm 0.07$	$4.0^{pq} \pm 0.60$
Soybean hulls 1%	$2.7^{ef} \pm 0.28$	$4.1^{op} \pm 0.13$
Sugar beep pulp 1%	$4.4^{ab} \pm 0.34$	$4.5^{m-p} \pm 0.09$
Locust bean 1%	$3.5^{d} \pm 0.10$	$4.6^{l-o} \pm 0.18$
Locust gum 1%	$2.3^{f} \pm 0.23$	$3.0^{r} \pm 0.13$
Guar gum 1%	$2.9^{e} \pm 0.36$	$5.5^{k} \pm 0.13$
Casein glycomacropeptide 1%	$1.0^{g} \pm 0.04$	$4.9^{k-n} \pm 0.19$
Lactobacillus exopolysac 1%	$0.9^{g} \pm 0.07$	$3.9^{pq} \pm 0.33$
Bio Mos 1%	$2.5^{ef} \pm 0.06$	$5.5^{k} \pm 0.25$
A. oryzae ferm extr 1%	$4.3^{a-c} \pm 0.16$	$5.1^{k-m} \pm 0.25$
Inulin 1%	$4.5^{a} \pm 0.15$	$5.0^{kl} \pm 0.14$
FOS 1%	$3.9^{b-d} \pm 0.15$	$5.1^{k-m} \pm 0.11$
Cranberry 1%	$4.5^{a} \pm 0.11$	$5.3^{kl} \pm 0.14$
SEM treat	**0.180**	**0.250**
P-value treat	**<0.0001**	**<0.0001**
SEM bacteria	**0.215**	
P-value bacteria	**<0.0001**	

6.3.2. The carbohydrate fractionation to enhance the adhesive capacity.

Table 3 presents the growth detection time (h) for the two bacteria, when incubated with the different feedstuffs fractions. We observed significant differences between the two bacteria. For NFEC none of the fraction reduced detection times compared to NC. However for ETEC, and similarly to previous table, WB and SO at 1 % reduce them significantly. Regarding different fractions, only the water extractable material (WEM) from WB (WB WEM) showed a reduction in

detection times compared to NC although of less magnitude than WB 1 %. Considering, the ETEC values, only the WB presented higher adhesive capacity compared to other treatments. The intermediate values were found with MUC, WB WEM, SO and LB. The MAN, WB AED1, WB AED2, SO, LB, LB AED1, and the LB AED2 treatments, presented similar results to the NC.

Table 6.3. Detection times of bacterial growth t_{OD} = 0·05 (h) for *E. coli* K88, non-fimbriated *E. coli*, as a measure for adhesion in different feedstuffs fractioning solutions.

Treatments	***E. coli* K88** t_{OD} **(h)**	**NF *E. coli*** t_{OD} **(h)**
Negative control - BSA 1%/PBS	$3.5^{ab} \pm 0.07$	$3.9^{kl} \pm 0.46$
D-Mannose 1%	$3.6^{a} \pm 0.13$	$3.8^{kl} \pm 0.24$
Natural porcine ileal mucus	$2.3^{de} \pm 0.09$	$3.0^{m} \pm 0.11$
Wheat bran 1%	$1.2^{f} \pm 0.04$	$3.6^{k-m} \pm 0.09$
WB WEM 1%	$2.3^{de} \pm 0.16$	$3.7^{k-m} \pm 0.48$
WB AED1 1%	$2.9^{a-d} \pm 0.20$	$4.0^{kl} \pm 0.36$
WB AED2 1%	$3.5^{ab} \pm 0.06$	$4.3^{k} \pm 0.04$
Soybean hulls 1%	$2.1^{e} \pm 0.19$	$3.7^{k-l} \pm 0.06$
Soybean hulls WEM 1%	$3.4^{ab} \pm 0.21$	$4.5^{k} \pm 0.10$
Locust bean 1%	$2.8^{a-e} \pm 0.10$	$4.0^{kl} \pm 0.30$
Locust bean WEM 1%	$3.4^{ab} \pm 0.41$	$3.8^{kl} \pm 0.10$
Locust bean AED1 1%	$3.0^{a-d} \pm 0.10$	$3.9^{kl} \pm 0.16$
Locust bean AED2 1%	$3.3^{a-c} \pm 0.30$	$3.8^{kl} \pm 0.51$
SEM treat	**0.251**	**0.370**
P-value treat	**<0.0001**	**<0.0001**
SEM bacteria	**0.231**	
P-value bacteria	**<0.0001**	

6.4. Discussion

The main objective of this study was to perform a preliminar search for natural ingredients that could bind the enteropathogen *E. coli*, as a previous step to positively influence the digestive process, reduce the intestinal pathogens load, and thus, improve animal's health.

The use of an *in vitro* model to evaluate the interactions between a potentially pathogenic bacteria and components of a diet, allowed the screening of more than 15 different ingredients with a wide variety of composition. The feedstuffs were selected according to its physicochemical characteristics and for their already reported capability to interact with intestinal pathogenic bacteria (see references in Table 1).

It is also well reported that the bacteria used in our model, uses the fimbriae expressed in its surface to attach specific intestinal receptors at the target cells (see reviews of Fairbrother *et al*., 2005; Nagy and Fekete, 2005). This is a prerequisite step to begin the intestinal colonization that ends with a hyper secretory diarrhea, mainly in young pigs.

From the adhesion results it can be observed that three feedstuffs (WB, CGMP and EPS) can potentially coat the plate and attract specifically the ETEC. This observation is in great accordance with our previously studies with WB (Molist *et al*., 2010, 2011) and CGMP (Hermes *et al*., 2010) using *in vivo* models; and with the *in vitro* study of Wang *et al*. (2010) for Lactobacilli EPS. This specific affinity of these feedstuffs to adhere the ETEC suggest the presence of particular compounds in their extracts, likely glycoproteins with affinity for F4 fimbriae that can attract the ETEC maintaining it attached to the well. However the specificity of some of these putative compounds to other ETEC specific lectins or adhesins different to F4 cannot be discarded.

However, a limitation of the *in vitro* model applied in this trial remains in the capability of the high-binding 96-well plate to be coated at the same extent by the different studied products. Thus, only when the result is positive we can confirm a clear binding of the substrate to the

bacteria. When the result is negative, or not attachment is observed to the bacteria, we can´t rule out there was not attachment to the bacteria, or the product simply was not attached to the wall's well itself. Thus, it is complicated to establish numerical comparisons among products. Finally it is important to differentiate the ability of feed ingredients to bind bacteria and the capability to block the adhesion to intestinal mucus.

Another objective of our study was to try the identification of fractions in these ingredients with a higher ability to bind ETEC. Thus, we planned the isolation of carbohydrate fractions from different ingredients, using enzymatic hydrolysis (mainly amylase and protease activity). The results showed that the isolated fraction showed lower binding activity than the raw extracts, likely suggesting that the enzymatic hydrolyses eliminated or modified the composition of molecules responsible for the coat of the well or for the adherence to ETEC. These results suggest that a complex glycoprotein could be evolved in the identification and characterization of lectin-like interactions in cell recognition as highlighted by recent reviews (Chow *et al*., 2010; Lane *et al*., 2010; Tiralongo and Moran, 2010; Ghazarian *et al*., 2011).

There is strong scientific evidence characterizing a wheat glycoprotein, supposedly inactivated by a protease treatment, called wheat germ agglutinin (WGA), which present a special ability to bind enterocytes of mice (Walter *et al*., 2004), pigs (Choi *et al*., 2003), and human enterocytes (Wirth *et al*., 1998). The high specificity of WGA for enterocytes seems to be due to its specific affinity to bind N-acetyl-D-glucosamine and sialic acid groups, as well as mucins from porcine stomach, being useful for drug delivery development (Güll *et al*., 2007). Moreover, it was recently considered as an adjuvant for oral vaccination against ETEC K88 (Vandamme *et al*., 2011). This knowledge could be useful indication for future applications as a nutritional strategy to reduce potentially pathogenic bacteria in young animals and it can at least in part explain the results found in our *in vitro* adhesion trial. Considering the finding results, more research is needed to isolate the moiety implicated on this attachment.

6.5. Conclusion

The techniques employed in this *in vitro* trial allowed in a simple way the screening of interactions between a fimbriated *E. coli* and different dietary ingredients. However, further studies should be conducted in order to identify the specific compunds in some ingredients, specially in the wheat bran, to better characterize candidates for a *in vivo* anti-adhesive therapy against intestinal pathogens.

6.6. Literature cited

Abbeele PV, Grootaert C, Possemiers S, *et al.*, (2009) *In vitro* model to study the modulation of the mucin-adhered bacterial community. *Appl Microbiol Biotechnol* **83**: 349–359

Becker PM, Galletti S, Hil PJR, *et al.* (2007) Validation of growth as measurand for bacterial adhesion to food and feed ingredients. *J Appl Microbiol* **103**, 2686-2696.

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

Blanco M, Blanco J, Gonzalez E, *et al.* (1997) Genes coding for enterotoxins and verotoxins in porcine *Escherichia coli* strains belonging to different O:K:H serotypes: Relationship with toxic phenotypes. *J Clin Microbiol* **35**: 2958-2963.

Choi BY, Sohn YS, Choi C, *et al.* (2003) Lectin histochemistry for glycoconjugates in the small intestine of piglets naturally infected with *Isospora suis*. *J Vet Med Sci* **65:** 389-382

Chow J, Lee SM, Shen Y, *et al.* (2010) Host–Bacterial symbiosis in health and disease. In: *Advances in Immunology*, pp.243-274 [Sidonia Fagarasan and Andrea Cerutti, editor]. Academic Press.

Devriendt B, Stuyven E, Verdonck BM, *et al*. (2010) Enterotoxigenic *Escherichia coli* (K88) induce proinflammatory responses in porcine intestinal epithelial cells. *Develop Comp Immunol* **34**: 1175-1182.

Fang L, Gan Z, Marquardt RR (2000) Isolation, affinity purification, and identification of piglet small intestine mucosa receptor for enterotoxigenic *Escherichia coli* K88ac fimbriae. *Infection and Immunity* **68**, 564-569.

Fairbrother JM, Nadeau É, Gyles CL (2005) *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* **6**, 17-39.

Ghazarian H, Idoni B, Oppenheimer SB (2011) A glycobiology review: Carbohydrates, lectins and implications in cancer therapeutics. *Acta Histochem* **113**, 236-247.

Güll I, Wirth M, Gabor F (2007) Development of a sensitive and reliable ELISA for quantification of wheat germ agglutinin. *J Immunol Met* **318**, 20–29

Hermes RG, Molist F, Pérez JF, *et al*. (2010) Casein glycomacropeptide (CGMP) in the diet of early weaned piglets reduces the *Escherichia coli* attachment to the intestinal mucosa and increases lactobacillar numbers in digesta. *J Animal Sci* **88**, E-Suppl. 582.

Jensen GM, Frydendahl K, Svendsen O, *et al.* (2006) Experimental infection with *Escherichia coli* O149:F4ac in weaned piglets. *Vet Microbiol* **115**, 243-249.

Lane JA, Mehra RK, Carrington SD, *et al.* (2010) The food glycome: A source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol* **142**, 1-13.

Liu Y, Gallardo-Moreno AM, Pinzon-Arango PA, *et al*. (2008) Cranberry changes the physicochemical surface properties of *E. coli* and adhesion with uroepithelial cells. *Colloids and Surfaces B: Biointerfaces* **65**, 35-42.

Maes C and Delcour JA (2002) Structural characterisation of water-extractable and water-unextractable arabinoxylans in wheat bran. *J Cereal Sci* **35**, 315–326.

Marth JD and Grewal PK (2008) Mammalian glycosylation in immunity. *Nat Rev Immunol* **8**, 874-887.

Molist F, Gómez de Segura A, Pérez JF, *et al.* (2010) Effect of wheat bran on the health and performance of weaned pigs challenged with *Escherichia coli* $K88^{+}$. *Livest Sci* **133**, 214-217

Molist F, Hermes RG, Gómez de Segura A, *et al*. (2011) Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *Br J Nut* **105**, 1592–1600

Nagy B and Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microb* **295**, 443-454.

Ouhida I, Pérez JF, Gasa J (2002) Soybean (*Glycine max*) cell wall composition and availability to feed enzymes. *J Agric Food Chem **50**,* 1933-1938.

Tiralongo J and Moran AP (2010) Bacterial lectin-like interactions in cell recognition and adhesion. In: *Microbial Glycobiology*, pp.549-565 [Otto Holst, Patrick J. Brennan, Mark von Itzstein, editors]. San Diego: Academic Press.

Vandamme K, Melkebeek V, Cox E, *et al.* (2011) Adjuvant effect of Gantrez®AN nanoparticles during oral vaccination of piglets against F4+enterotoxigenic *Escherichia coli*. *Vet Immunol Immunopathol* **139,** 148–155

Wang Y, Ganzle MG, Schwab C (2010) Exopolysaccharide synthesized by *Lactobacillus reuteri* decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes. *Appl Environ Microbiol* **76**, 4863-4866.

Walter F, Scholl I, Untersmayr E, *et al*. (2004) Functionalisation of allergen-loaded microspheres with wheat germ agglutinin for targeting enterocytes. *Biochem Biophys Res Commun* **315,** 281–287.

Wirth M, Hamilton G, Gabor F. (1998) Lectin-mediated drug targeting: quantification of binding and internalization of Wheat germ agglutinin and *Solanum tuberosum* lectin using Caco-2 and HT-29 cells. *J Drug Target* **6,** 95–104.

Chapter 7

Influence of dietary ingredients on *in vitro* inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). Submitted for publication on February, 2011. *Comp Immunol Microb*. Under review.

7.1. Introduction

Enteric diseases in piglets are the leading cause of mortality and a major cause of economic losses in the pig industry. Some factors involved are: a transient loss of the integrity and inflammation of the intestinal mucosa caused by anorexia after weaning, an immature digestive system unable to fully digest the new diet, and a sudden overgrowth of pathogenic microorganisms with the capacity to adhere to the intestinal mucosa (Fairbrother *et al*., 2005).

The main challenge in a young animal is to obtain a balanced microbial population to prevent the establishment of pathogenic microorganisms (Konstantinov *et al*., 2004). Animal producers have used a large array of antibiotics and drugs to obtain such stability and avoid intestinal diseases, but those antibiotics could leave residues in the meat of the animals, causing bacterial resistance to antibiotics and residual contamination of the food chain (Dibner and Richards, 2005). For this reason, the European Union banned the use of antibiotics as growth promoters and has regulated their clinical utilization since 2006. This regulation caused a huge interest to develop alternatives products and strategies capable to achieve the same efficacy as antibiotics (Lallès *et al*., 2009).

The first prerequisite step for the development of a bacterial gastrointestinal illness is the effective colonization of the intestinal tract by the bacteria. Thus, many pathogenic bacteria, including *E. coli*, possess mechanisms of adhesion to intestinal cells (Fairbrother *et al*., 2005). Enterotoxigenic *E. coli* (ETEC) K88 expresses F4 fimbriae, which are proteinaceous filamentous adhesins composed of repeating copies of the major fimbrial subunit FaeG and some additional minor subunits (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). F4 fimbriae are strongly immunogenic antigens, since an oral immunization of weaned pigs with purified F4 fimbriae induces a potent F4-specific intestinal immune response protecting the animals against a

subsequent F4+ ETEC challenge infection (Van den Broeck *et al*., 2000). However, this response is variable and depending on the presence of intestinal receptors for F4 fimbriae and the dose and form of the immunising antigen the small intestinal mucosal immune system may react with tolerance or hypersensitivity (Nagy and Fekete, 2005). Given the specificity of these mechanisms, it has been speculated that supplying exogenous molecules (e.g. biologically active nutrients) that could block fimbriae or its receptors can prevent gastrointestinal colonization by ETEC (Becker and Galleti, 2008).

The potential of certain compounds to inhibit the adherence of microorganisms, specifically *E. coli*, to the intestinal epithelium has been demonstrated previously. Molist *et al*. (2010) found that wheat bran (WB) could reduce adhesion of *E. coli* to the ileal mucosa after an ETEC K88 challenge in piglets. Brody (2000) reviewed the evidence that casein glycomacropeptide (CGMP), from bovine milk, has biological activity that binds *E. coli* toxins, inhibits bacterial and viral adhesion, promotes bifidobacteria growth and modulates immune system responses. Furthermore, in a previous study (Hermes *et al*., 2010) it was demonstrated that CGMP could inhibit the adhesion of ETEC K88 in the intestinal tract of piglets and reduce the diarrhea incidence. Castillo *et al*., 2008 found that the supplementation of 0.2% of yeast mannan-oligosaccharides (MOS) reduced the enterobacteria population in small intestine of piglets. Other feedstuffs that could potentially inhibit or diminish the adhesion of bacteria in the intestinal tract are the locust bean plant extract (LB), which is rich in galactomannans (Lazaridou *et al*., 2001) that could block bacterial adhesins, and the *Aspergillus oryzae* fermentation extract (AO) which possesses fucose-specific lectins (Matsumura *et al*., 2007) that could block carbohydrate structures on epithelium preventing ETEC to adhere to it.

The IPEC-J2 cell line, isolated from the jejunum of a neonatal pig, is a reliable cell line for the *in vitro* study of swine intestinal diseases (Schierack *et al*., 2006; Koh *et al*., 2008), and these cells specifically adhered to K88 ETEC (Koh *et al*., 2008). The objective of the present study was

to examine the ability of several feedstuffs to inhibit the adhesion of ETEC K88 to the IPEC-J2 cell line and to diminish their inflammatory response.

7.2. Material and Methods

7.2.1 Cell culture growth

IPEC-J2 (epithelial cell derived from cells isolated from the jejunum of a neonatal pig and maintained as a continuous culture), were kindly donated by the Professor Anthony Blikslager (North Carolina State University, Raleigh, NC, USA). Cells were grown in Dulbecco's modified eagle medium (DMEM)/Ham's F-12 (1:1) medium (HyClone Lab., Inc, South Logan, UT), supplemented with fetal bovine serum (5%, JR Scientific, Inc. Woodland, CA); Penicilin-Streptomicin (1%, Invitrogen Life Technologies, Carlsbad, CA); insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL) added as ITS supplement (1%, Invitrogen Life Technologies, Carlsbad, CA) and maintained in an atmosphere of 5% CO2 at 37ºC until confluence (about 3 days). Cells were used between passages 45 and 55 and were routinely tested to be free of mycoplasma contamination.

The IPEC-J2 cells were seeded in 75-ml flasks to amplify the cells number and then 2-4 x 10^5 cells were seeded into 6-well plates in a 2-ml volume (for the gene expression assay), or into 96-well flat bottom plates in a 200 µL volume (for the adhesion assay). Cells were allowed to adhere for 24 hours and were washed and re-fed every other day until confluence (3 to 4 days). One day prior to the experiment, standard culture media containing antibiotics was replaced by an antibiotic-free media.

7.2.2. E. coli strains

Two strains of *E. coli* were used in this study, an enterotoxigenic F4 fimbria type *E. coli* K88ac (MUN 287, Berberov *et al*., 2004) and a non-fimbriated *E. coli* strain (G58-1, Berberov *et*

al., 2004). Both bacteria were kindly donated by the Professor Rodney A. Moxley (University of Nebraska, Lincoln; NE, USA) and were seeded in Luria Bertani Broth: 37ºC/18 hours under low agitation followed by two passages on a CO_2-independent media (GIBCO 18045-088, Invitrogen Life Technologies, Carlsbad, CA) to reach a concentration of 1 to 2 x 10^8 CFU/ml before added to the cell plates. Bacterial solution was adjusted by OD 1 to 2 x 10^8 CFU/ml before being used in each experiment and agar plate counts (on Luria agar), were used to confirm the number of bacteria in the adjusted solution.

7.2.3. Feedstuffs solutions

Feedstuffs described in Table 7.1 were used in this study. All products were powdered except for WB that was finely ground in an analytical grinder A10S (IKA, Staufen, Germany). Feedstuffs were diluted (w/v) using sterile PBS to reach 0.1, 0.2, 0.4 and 0.8% concentrations. Then, the different dilutions were sonicated in a water bath (3 times/30 second each) and centrifuged (460 g/5 minutes) as described by Becker *et al*. (2007). Extracts were frozen at -20ºC until their utilization in the following assays.

7.2.4. Adhesion trial

The effect of feedstuffs on the numbers of adhered ETEC per well was determined as follows. The IPEC-J2 was seeded in 96-well flat bottom plates as described above. After confluence, media was removed and replaced with a CO_2-independent medium (GIBCO 18045-088, Invitrogen Life Technologies, Carlsbad, CA). The *E. coli* (K88 or non-fimbriated) used in this experiment were prepared as described above. Briefly, the adhesion test consisted of 30 minutes incubation of the different feedstuffs (1mL) with the bacteria (1mL) at room temperature mimicking the delay before chyme reaches the small intestine. Then, 350 µL of this mixture were added to each well, followed by another 30 min incubation (at 37ºC and 5% of CO_2) with the cell monolayer. Then the wells were thoroughly washed three times with sterile PBS, in order to

remove the non-adhered bacteria and the residue of the different feedstuffs. Finally, CO_2-independent medium was added to allow the growth of the adhered bacteria and to keep the cells alive. Experiments were performed in triplicate. The technique is based on the fact that when bacteria grow, the medium becomes more opaque. Thus OD_{650nm} was measured every 10 minutes using a Spectrophotometer (Versa Max microplate reader, Molecular Devices, Sunnyvale, CA) to monitor the growth of bacteria. Each well described a sigmoid growth curve and growth detection time (h), defined as the time when bacteria start their exponential growth phase (OD = 0.05) and this time point was used to compare treatments (Becker *et al*., 2007). The more bacteria that are adhered to the cells the shorter the growth detection time. Because the adherence measure was based on bacterial growth, effects of feedstuffs on bacterial growth per se were tested in order to dismiss any possible anti-bacterial, bacteriostatic or growth-promoting effects on the ETEC strain. Thus, all solutions containing feedstuffs with the bacteria were plated in Luria agar: 37ºC for 48 hours to check for any differences in bacterial growth.

Table 7.1. Characterization of feedstuffs used in this study.

#	*Product*	*Treat code*	*MW*	*Provider*	*Main compounds*	*References*
1	Wheat bran extract	WB	?	Local Mill	Arabinoxylans, PNA	Molist *et al*., 2011
2	Locust bean extract	LB	?	Armengol	Galactomannans	Becker and Galletti., 2008
3	Casein glycomacropeptide	CGMP	?	Arla Foods	Glycoproteins rich in sialic acids	Hermes *et al*., 2010
4	*Aspergillus oryzae* fermentation extract	AO	?	Molimen	Galactomannans	Hermes *et al*., 2010
5	Mannanoligossacharides	MOS	?	Alltech	Mannose, proteins	Becker and Galletti., 2008

7.2.5. Response of epithelial cells to exposure to E. coli and different feedstuffs

1mL of the feedstuffs solutions (described above) and 1mL of the *E. coli* strains were incubated for 30 min at room temperature. Next, the 2 mL of feedstuff-bacteria solutions were added to confluent monolayers of IPEC-J2, as described above, and incubated for 2 h at 37ºC with 5% of CO_2. A positive control (PC) containing of 1mL of sterile PBS with 1mL of the *E. coli* strains was included as a reference treatment of the innate inflammatory response to the challenge. Experiments were performed in triplicate. Cells from all treatments were washed twice with sterile PBS and subjected to RNA extraction by adding 1mL of TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) to each well and followed by the centrifuge-based extraction as described by the manufacturer. Contaminating DNAs were removed by digestion using the Turbo DNA-free kit (Applied Biosystems, Carlsbad, CA, USA). The RNA samples were pooled (triplicate), according to the treatments and stored at -20ºC until use. RNA quality was assessed by microcapillary electrophoresis using an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). RNA was quantified by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE, USA).

7.2.6. Reverse transcription and quantitative real-time PCR.

The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to obtain cDNA for SYBR-Green real time RT-PCR, following the manufacturer`s instructions. Briefly, reverse transcription was carried out in a 20 µl final volume that included 4 µl of 5× iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, 1 µg of RNA template, and nuclease-free water to complete the final volume.

Quantitative real time RT-PCR was used to quantify the gene products of interest (TLR-4, TLR-5, IL-1β, IL-8, IL-10, and TNF-α) relative to the quantity of Cyclophilin A mRNA in total RNA isolated from cultured IPEC-J2 (see Table 7.2 for primers description). The PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (200 nM),

12.5µL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 5µL of the cDNA sample. Assays using non-template controls and samples were performed using the iQ5 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling parameters included 30 cycles of 30 s at 94ºC, 30 s at 57ºC and 45 s at 72ºC. Standard curves for each TLR, cytokines and Cyclophilin A were obtained using tenfold serially dilutions of a reference cDNA.

The relative abundance of TLR-4, TLR.5, IL-1β, IL-8, IL-10, and TNF-α was measured according to the previously described protocols of Arce *et al*. (2010), Collado-Romero *et al*. (2010) and Mariani *et al*. (2009) and using the Cyclophilin A as a reference gene, to calculate the relative abundance of the target genes.

7.2.7. Calculations and statistical analysis

All statistical analysis were performed using SAS 9.2 (SAS Inc, Cary, NC, USA). The OD data from the adhesion assay were processed by nonlinear regression analysis, Gauss-Newton method, following the equations described by Becker *et al.* (2007). Calculated bacterial detection times ($t_{OD=0.05}$, h) were analyzed by ANOVA and linear trends were studied for dose response effect.

The relative expression of inflammatory-related genes was determined following Arce *et al*. (2010), using the relative expression ratio (*R*) of each target gene (TLRs and cytokines) calculated based on *E* and Ct deviation of a sample versus a control and expressed in comparison to the reference gene (Cyclophilin-A).

$$R = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control-sample)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control-sample)}}}$$

Where, R = ratio; E = efficiency; Ct = threshold cycle. Cycle at which PCR amplification reaches a significant value; ΔCt = Ct(control) – Ct(sample).

Table 7.2. Primers for real-time polymerase chain reaction.

Oligo name	***Sequence (5' - 3')***	***Efficiency***	***R^2***	***Reference***
Cyclophilin-A-for	CCTGAACATACGGGTCCTG	2.00	0.98	[1]
Cyclophilin-A-rev	AACTGGGAACCGTTTGTGTTG			
TLR-4-for	GCCATCGCTGCTAACATCATC	1.98	0.99	[1]
TLR-4-rev	CTCATACTCAAAGATACACCATCGG			
TLR-5-for	CAGCGACCAAAACAGATTGA	2.00	0.99	[2]
TLR-5-rev	TGCTCACCAGACAGACAACC			
IL-1β-for	GGCCGCCAAGATATAACTGA	1.99	0.98	[1]
IL-1β-rev	GGACCTCTGGGTATGGCTTTC			
IL-8-for	TTCGATGCCAGTGCATAAATA	2.02	0.97	[1]
IL-8-rev	CTGTACAACCTTCTGCACCCA			
IL-10-for	CAGATGGGCGACTTGTTG	2.00	0.99	[2]
IL-10-rev	ACAGGGCAGAAATTGATGAC			
TNF-α-for	CGCCCACGTTGTAGCCAATGT	2.00	0.99	[1]
TNF-α-rev	CAGATAGTCGGGCAGGTTGATCTC			

[1] Arce *et al*. (2010)
[2] Collado-Romero *et al*. (2010)

Gene expression data was analyzed by ANOVA comparing the different feedstuffs and doses using the Tukey correction for multiple comparisons with an alpha level of significance of 0.05. Linear trends in gene expression depending on the dose of feedstuffs were analyzed for those feedstuffs that differed from the positive control and as well as between doses of the feedstuff.

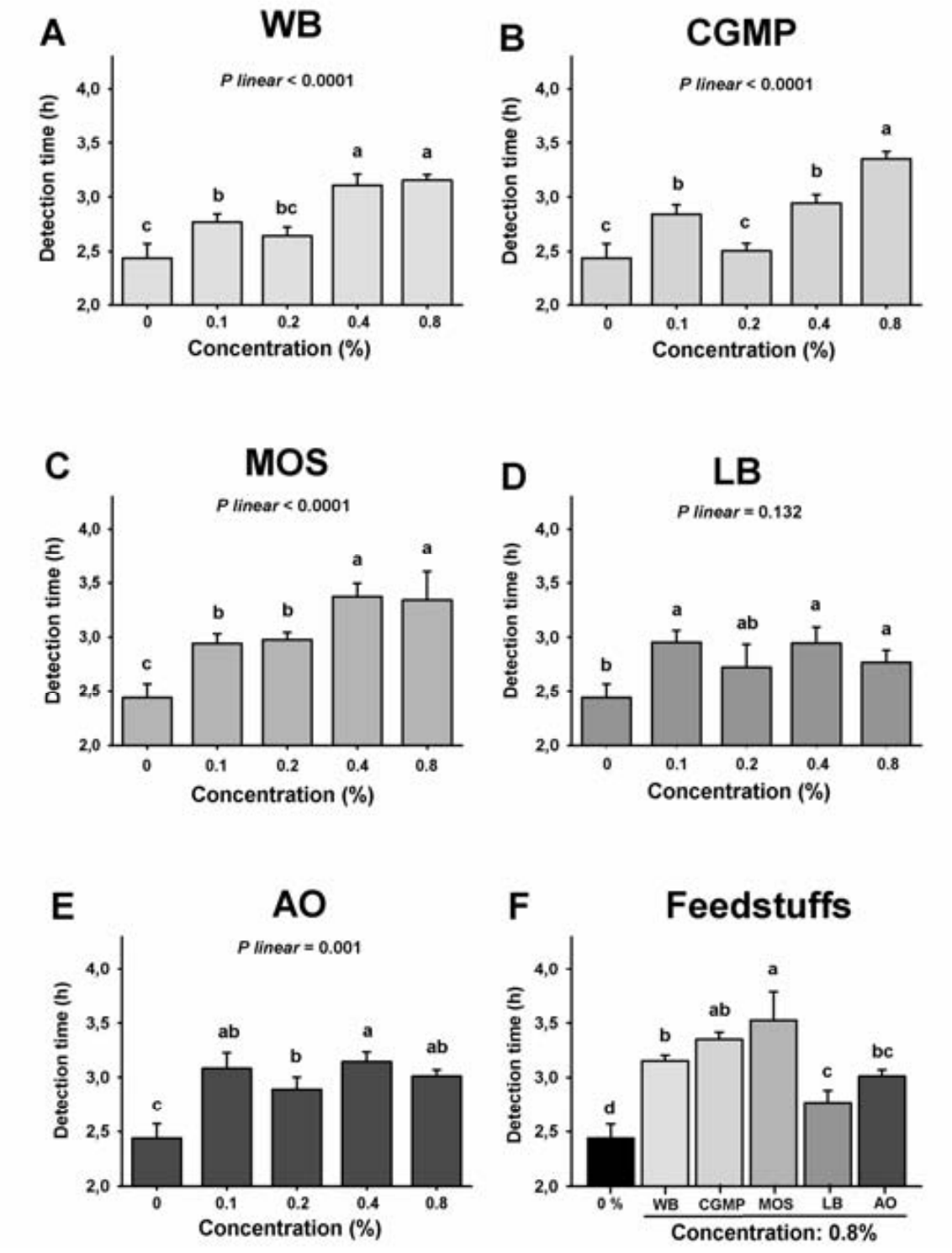
7.3. Results

7.3.1. Adhesion trial – Blocking of E. coli binding receptors

In order to test the differential adhesion between both types of *E. coli* (the fimbriated *E. coli* strain MUN 287 and the non-fimbriated strain G58-1), adhesion tests with the two different strains were performed. It was observed that the fimbriae played an important role in bacterial adhesion, because the fimbriated *E. coli* resulted in shorter detection times ($t_{OD=0.05}$, h) and therefore had higher adhesion rates than the non-fimbriated strain (2.44 vs. 2.97 h, $P = 0.04$, SEM = 0.423, data not shown). No effects of feedstuffs on bacterial growth per se were observed (data not shown).

Figure 7.1 presents the adhesion assay results of ETEC K88 co-incubated with increasing doses of each tested feedstuff (Fig 1.A-E). Furthermore, figure 1.F presents a comparison between the highest doses of the studied feedstuffs. Most of the treatments and doses studied reduced the adhesion of ETEC K88 to IPEC-J2 cells. For WB, CGMP and MOS, adhesion of ETEC K88 decreased linearly with increasing feedstuff concentrations (r = 0.84, 0.88, 0,85, respectively; $P <$ 0.001). On the other hand, for LB and AO the highest inhibition effect was seen already at the lowest concentration, 0.1%. Comparing the highest tested dose with the negative control, the lowest adhesion level was reached with MOS followed by CGMP, although all feedstuffs were effective when compared to the negative control.

Figure 7.1. Dose response relationships of the ability of various feedstuffs to block the attachment of *E. coli* K88 to a monolayer of IPEC-J2 cells.



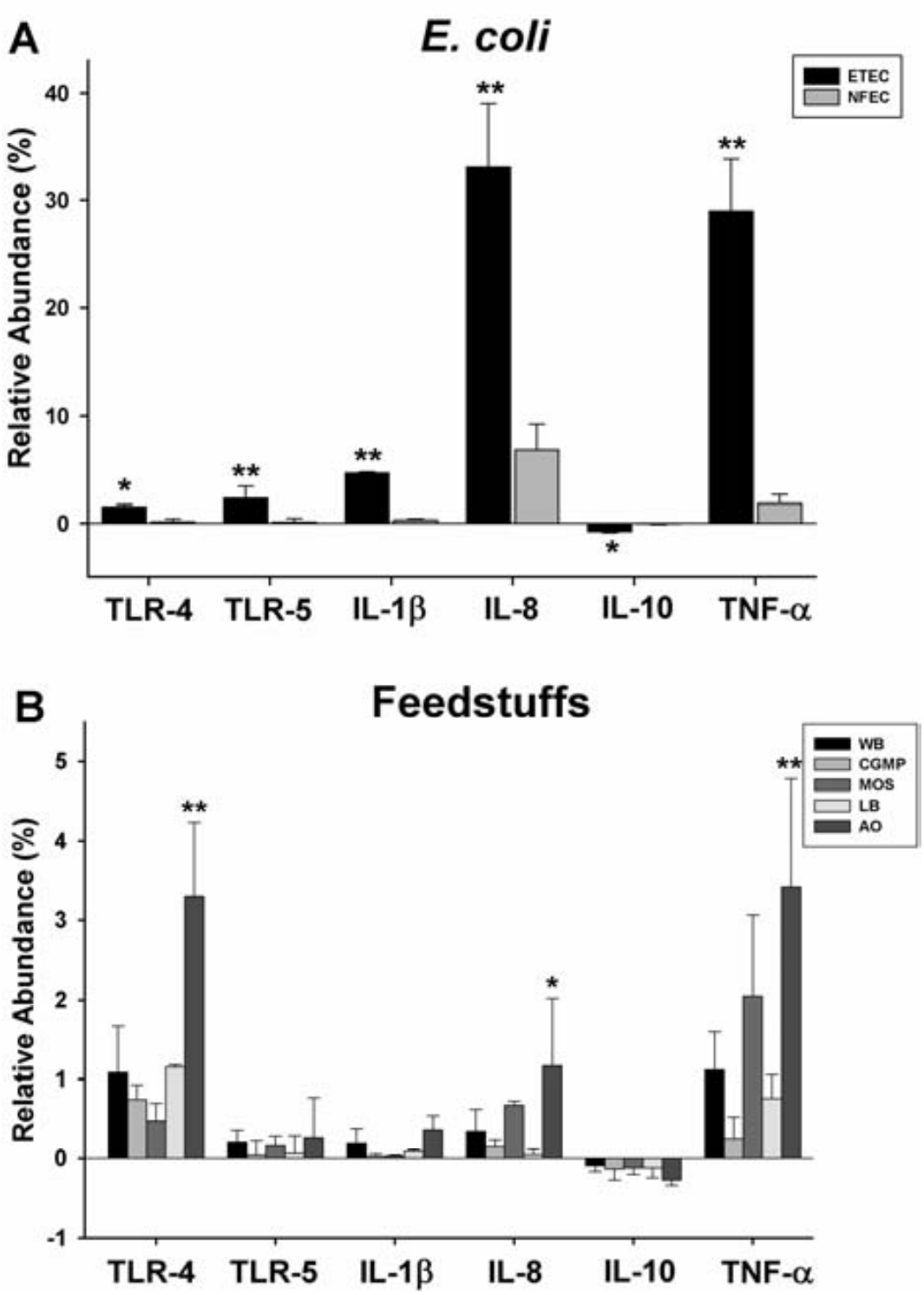
(A) WB, wheat bran; (B) CGMP, casein glycomacropeptide; (C) MOS, mannan-oligosaccharides; (D) LB, locust bean; (E) AO, *Aspergillus oryzae* fermentation extract and (F) Feedstuffs comparison at highest dose tested (0.8%). Detection time: The length of time (h) required to detect the growth of attached *E. coli*. The longer the detection time, the fewer attached *E. coli*. ^{abcd} different letters mean significant differences ($P < 0.05$) between doses (A-E) or feedstuffs (F). Experiments were performed in triplicate. Error bars represent the standard deviation of the mean.

7.3.2. Effect of ETEC or NFEC challenge, and/or different feedstuffs on the inflammatory response of IPEC-J2 cells

Figure 7.2 shows the relative mRNA expression by IPEC-J2 in response to a challenge with the two different *E. coli* strains (Fig. 2.A) or to the different feedstuffs studied in the absence of *E. coli.* (Fig. 2.B). Non-fimbriated *E. coli* did not induce any change in mRNA expression of IPEC-J2 cells for the analyzed TLRs or cytokines compared to non challenged cells. Fimbriated ETEC stimulated higher mRNA expression of TLR-4 ($P < 0.01$), TLR-5 ($P < 0.001$), IL-1β ($P < 0.001$), IL-8 ($P < 0.001$), IL-10 ($P < 0.001$), and TNF-α ($P < 0.001$) compared to non-fimbriated strain. Among ingredients, AO generated higher mRNA expression of TLR-4 ($P < 0.001$), IL-8 ($P = 0.023$), and TNF-α ($P = 0.006$) compared to other treatments.

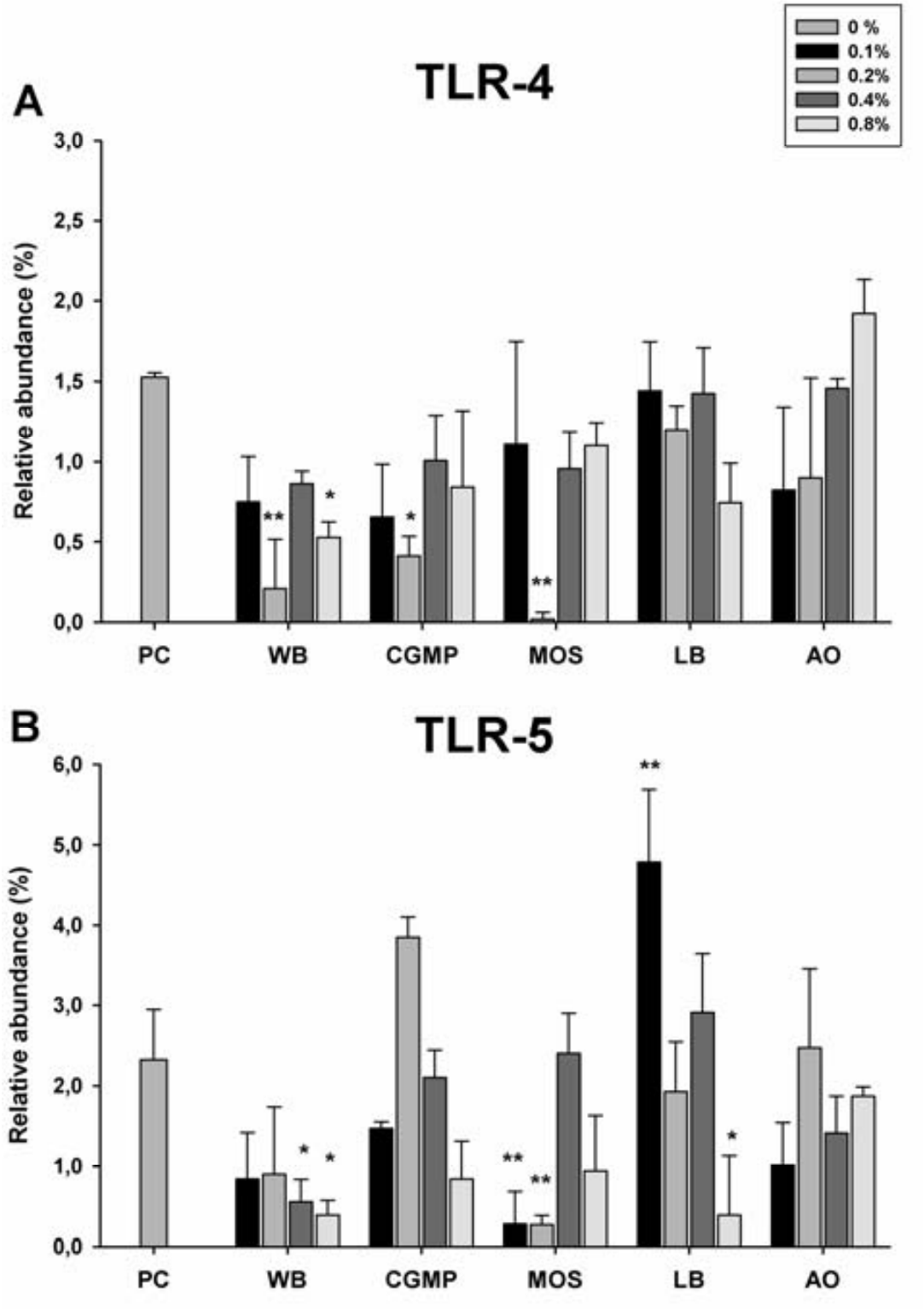
Figure 7.3 shows the relative expression of TLR-4 (A) and TLR5 (B) genes in response to *E. coli* K88 challenge that was previously co-incubated with sterile PBS as positive control or with different feedstuffs at different doses. When compared to PC, expression of TLR-4 was decreased by WB at doses of 0.2% ($P = 0.001$), and 0.8% ($P = 0.039$), CGMP at 0.2% ($P = 0.011$), and MOS at 0.2% ($P < 0.001$). Regarding TLR-5 expression, it was decreased compared to PC by WB at 0.4 and 0.8% ($P = 0.044$ and 0.017, respectively), MOS at 0.1 and 0.2 % ($P = 0.008$ in both cases), and LB at 0.8% ($P = 0.016$). Treatment LB at 0.1% increased expression ($P < 0.001$) compared to PC. There were no differences in TLR-4 and 5 expressions between the other doses of feedstuffs studied compared to positive control.

Figure 7.2. Innate immune response of IPEC-J2 cells.



Toll-like receptor 4 (TLR-4) and 5 (TLR-5); Interleukin-1β (IL-1β), 8 (IL-8), and 10 (IL-10); and tumor necrosis factor- α (TNF-α,). (A) Response of IPEC-J2 to a challenge with enterotoxigenic *E. coli* (ETEC) K88 and to non-fimbriated *E. coli* (NFEC). (B) Response of non-challenged cells to different feedstuff at 0.8% (WB, wheat bran; CGMP, casein glycomacropeptide; MOS, mannan-oligosaccharides; LB, locust bean; AO, *Aspergillus oryzae* fermentation extract). Experiments were performed in triplicate. Error bars represent the standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$.

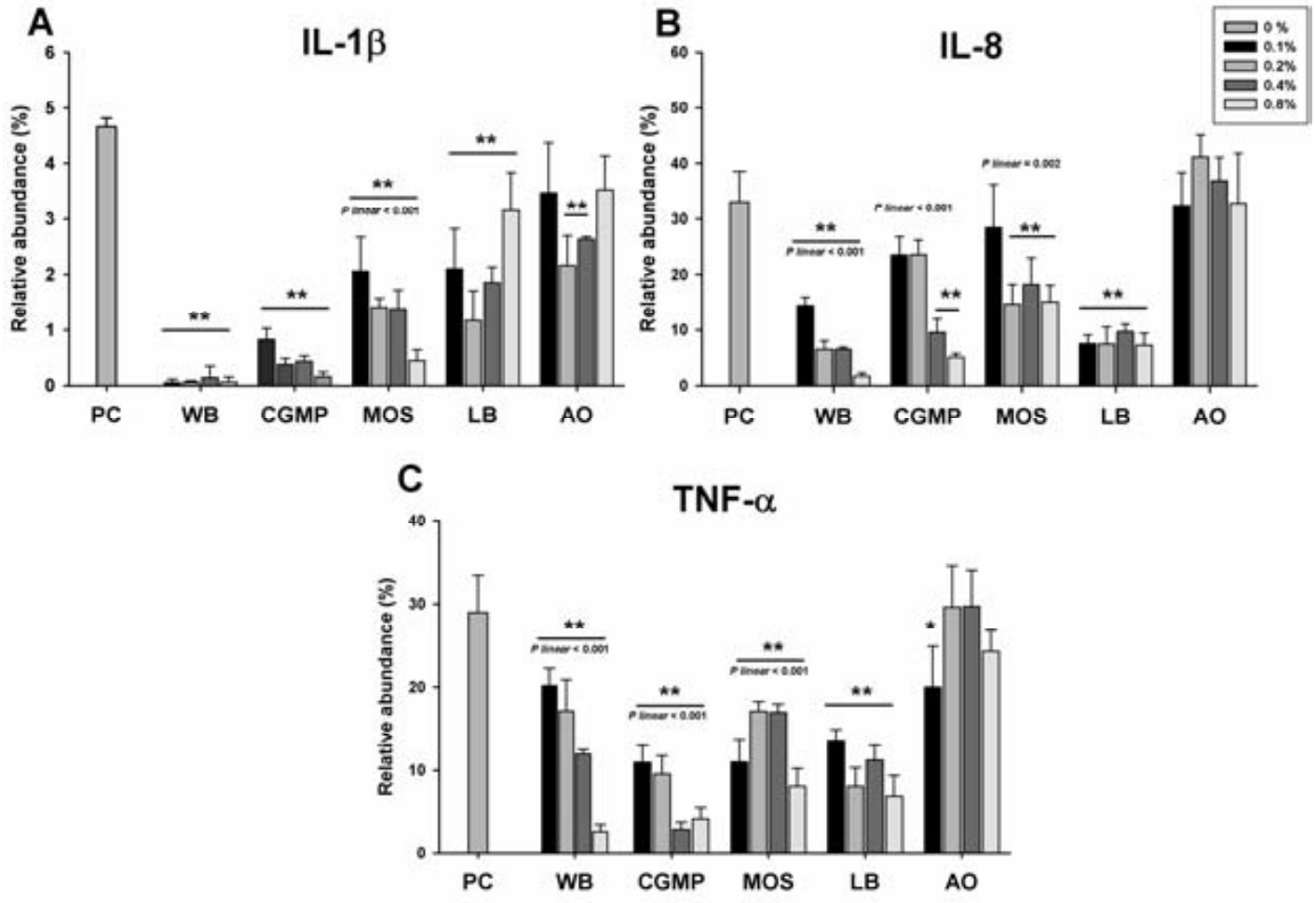
Figure 7.3. Toll-like immune response of IPEC-J2 cells to an ETEC K88 challenge in the presence of different feedstuffs.



(PC, positive control, where no feedstuff was added but sterile PBS, and cells were ETEC challenged; WB, wheat bran; CGMP, casein glycomacropeptide; MOS, mannan-oligosaccharides; LB, locust bean; AO, *Aspergillus oryzae* fermentation extract)**.** (A) Toll-like receptor 4 (TLR-4) relative abundance (%). (B) Toll-like receptor 5 (TLR-5) relative abundance (%). Experiments were performed in triplicate. Error bars means the standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$.

Cytokine IL-1β, IL-8, and TNF-α gene expressions are presented in the Fig 7.4A, 7.4B, and 7.4C, respectively. All doses of WB, CGMP, MOS, and LB, and doses 0.2 and 0.4 of AO decreased IL-1β expression ($P < 0.001$ except for LB at 0.8% that had $P = 0.008$) compared to PC. Decrease in IL-1β expression was linear as MOS dose increased ($P < 0.001$, r = 0.80). Chemokine IL-8 expression was decreased compared to PC by all doses of WB and LB ($P < 0.001$ in all cases), CGMP at 0.4 and 0.8% ($P < 0.001$ in both cases), and MOS at 0.2, 0.4, and 0.8% ($P < 0.01$ in all cases). Decrease in IL-8 expression was linear as WB, CGMP, and MOS dose increased (r = 0.75, $P < 0.001$; r = 0.90, $P < 0.001$; and r = 0.63, $P = 0.002$, respectively). Concerning TNF-α, its expression was decreased compared to positive control at all doses of WB ($P = 0.034$ for 0.1% and $P < 0.001$ for other doses), CGMP ($P < 0.001$ for all doses), MOS ($P < 0.001$ for all doses), and LB ($P < 0.001$ for all doses), and 0.1% of AO ($P = 0.028$). Decrease in TNF-α expression was linear as WB, and MOS dose increased (r = 0.91, $P < 0.001$; r = 0.65, $P < 0.001$, respectively).. No differences were observed between feedstuffs for IL-10 (data not shown).

Figure 7.4. Innate immune response of IPEC-J2 cells to an ETEC K88 challenge in the presence of different feedstuffs.



(PC, positive control, where no feedstuff was added but sterile PBS, and cells were ETEC challenged; WB, wheat bran; CGMP, casein glycomacropeptide; MOS, mannan-oligosaccharides; LB, locust bean; AO, *Aspergillus oryzae* fermentation extract)**.** (A) Interleukin-1 beta (IL-1β) relative abundance (%). (B) Interleukin-8 (IL-8) relative abundance (%). (C) Tumor necrosis factor-alpha (TNF-α) relative abundance (%). Experiments were performed in triplicate. Error bars means the standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$.

7.4. Discussion

Feed ingredients can modify intestinal health (Wismar *et al*., 2010) by a variety of mechanisms, including direct effects on enterocytes or by preventing the attachment of pathogenic bacteria to enterocytes. The first aim of this study was to investigate the capability of certain nutritional ingredients to interfere with the adhesion of *E. coli* K88 to the intestinal epithelium. Our results confirmed that *E. coli* K88 attached to the IPEC-J2 cells in a higher extent than the non-fimbriated *E. coli*. Previous studies using IPEC-J2 also observed specific adherence of ETEC K88 (Koh *et al*., 2008; Geens and Niewold, 2010), suggesting the presence of F4-receptors for this bacterial strain in this cell line, and the ability to develop an inflammatory response to ETEC K88 challenge (Pavlova *et al*., 2008). Devriendt *et al*., (2010) showed that ETEC K88 caused an over expression of IL-6 and IL-8, probably due to a stimulation of TLR-5 mediated signalling cascade upon flagellin detection. In our study, the ETEC challenge increased the expression of TLR-4 and TLR-5 in IPEC-J2 compared to the non-challenged control group, which may explain the over expressions of IL-1β, IL-8 and TNF-α.

The F4 intestinal receptor is composed of *N*- and *O*-glycans containing galactose, glucose, sialic acid, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and fucose (Grange *et al*., 1998). ETEC may recognize these ligands likely through β-linked galactose (Grange *et al*., 2002). Interestingly, these receptors are found on intestinal cells of newborn piglets, but they disappear as the animals develop and grow (Conway *et al*., 1990). Our working hypothesis was that mimicking the specific binding sites in the epithelium by means of certain molecules in dietary ingredients (glycoproteins and/or oligosaccharides) would selectively prevent the adhesion of ETEC. Several investigators (Marquardt *et al*., 1999; Sarabia-Sainz *et al*., 2009) have attempted to identify immunoglobulins with the ability to interact with the ETEC K88 receptors, but only a few have focused on the possible effect of feedstuffs (Becker and Galletti, 2008). In our study, all feedstuff treatments reduced adhesion compared to a negative control. In the case of WB, CGMP and MOS

we observed a dose-response reduction in adhesion. The water-soluble fraction of the WB is mainly composed by arabinoxylans (Kamal-Eldin *et al*., 2009), intensively studied for their capability to promote lactobacilli and bifidobacteria growth, probably due to feruloyl oligosaccharides present in the arabinoxylans (Yuan *et al*., 2005). In recent studies, we observed that WB in the diet reduced the *E. coli* number in the intestinal tract *in vivo* (Molist *et al*., 2009 and 2010). This reduction could be due to the competitive exclusion exerted by promoting lactic bacteria and also the capability of certain soluble compounds of wheat bran to bind specifically to ETEC K88, as shown *in vitro* by Molist *et al*. (2011).

Milk contains a variety of active glycoproteins with different functional properties as lactadherin, identified in porcine milk as an inhibitor of F4 ETEC attachment to intestinal villi (Shahriar *et al*., 2006). CGMP, a glycoprotein extracted from bovine milk whey has a potential anti-adhesive activity on *E. coli* too (Malkoski *et al*., 2001 and Nakajima *et al*., 2005). In a previous study (Hermes *et al*., 2010), the inclusion of CGMP in the diet reduced the adhesion of ETEC K88 to the intestinal mucosa of young pigs challenged with ETEC K88. One of the mechanisms that could be involved in CGMP-ETEC interaction is the highly complex *O*-linked glycosylation of CGMP (Boutrou *et al*., 2008) which is key for recognition by carbohydrate-binding molecules (Kooyk and Rabinovich, 2008) and is similar to F4 intestinal receptor (Grange *et al*., 1998 and 2002).

Results obtained with MOS, LB, and AO fermentation extracts, all of them rich in galactomannans, could be explained by a mannose-adhesin interaction. Inhibition of the adhesion of ETEC by mannose residues has been repeatedly reported (Leusch *et al*., 1991; Naughton *et al*., 2001). The presence of mannans may act as “glycodecoys” for this type of pathogenic bacteria (Zinger-Yosovich and Gilboa-Garber, 2009). MOS is an extract from yeast cell walls that has already shown beneficial effects in piglets. Castillo *et al*., (2008) reported that dietary MOS inclusion reduced the jejunal numbers of enterobacteria of early-weaned pigs and White *et al*. (2002) observed a reduction on the fecal coliform counts after an ETEC K88 challenge in piglets.

Becker and Galletti (2008) measured *in vitro* adhesion of several enteropathogens to different feed ingredients rich in mannans and concluded that yeast MOS show a high inhibitory effect on ETEC K88 adhesion, compared to other sources. Locust bean extract has been reported to reduce intestinal disorders in human (Aksit *et al*., 1998), and AO presents a very unique composition (Mennink-Kersten *et al*., 2004) with a cell wall containing high amounts of mannoproteins (Levitz, 2004). Both are promising molecules due to the inhibition of adhesion but further investigations must be done to ascertain beneficial effect in young pigs. It must be remarked the fact that these compounds showed not linear response to dose increases which could mean that lower doses than the ones used in the present experiment could give a similar result.

Because we found that these feed ingredients blocked ETEC attachment, we further evaluated the inflammatory response of the IPEC-J2 cell line when challenged with a pathogenic *E. coli*, either alone or in the presence of the different feedstuffs. Schierack *et al*. (2006) and Arce *et al*. (2010) extensively characterized this cell line and validated its use as a model for *in vitro* intestinal mucosa studies, especially since this cell line express the F4 receptor (Geens and Niewold, 2010). Stimulation of TLR-4 and TLR5 by ETEC (Devriendt *et al* 2010; Moue *et al*., 2008) leads to secretion of IL-1β, IL-8 and TNF-α (Alexander and Rietschel, 2001) by intestinal epithelial cells. IL-10 is probably later expressed in order to control inflammation. The TLR-4 is implicated in the mediation of inflammatory response to gram-negative bacteria (Takeuchi *et al*., 1999), and the TLR-5 is involved in the recognition of flagellin, a monomeric constituent of bacterial flagella that is present in some strains of ETEC K88 (Devriendt *et al*., 2010). In this study, the ETEC challenge promoted an increased expression of both TLRs and of IL-1β, IL-8, and TNF-α. Pre-treatment of ETEC with the different extracts studied resulted in inconsistent changes in the expression of TLRs but their effects on IL-1β, IL-8 and TNF-α were quite consistent. The lack of effects on IL-10 may be due to a short interval between the challenge and the measurement of cytokine expression since IL-10 is normally expressed later in inflammation.

Pre-treatment of *E. coli* with WB reduced IL-1β, IL-8 and TNF-α expression. This reduction could be due to a lower expression of TLR-4 inducing a lower stimulation from bacteria. This could be mediated by the previously shown reduction in the attachment of bacteria. However, these changes could also be due to an indirect immunomodulatory role of WB on the enterocytes. Phytic acid (Weglarz *et al*., 2007) or wheat arabinoxylans (Hughes *et al*., 2007) have been suggested to have immunomodulatory effects *in vivo* (Cao *et al*., 2011; Zhou *et al*., 2010), likely due to prebiotic effects. Treatments CGMP and MOS also reduced IL-1β, IL-8, and TNF-α expression and showed some effects in TLR-4 and TLR-5 expression. Some studies have reported the ability of CGMP to diminish inflammatory response in colitis-induced animals through the reduction of pro-inflammatory cytokines, such as IL-1β and TNF-α (Requena *et al*., 2008). Concerning MOS a direct effect on epithelial cells would be expected to induce expression of inflammatory cytokines (Brown 2006; Gantner *et al*., 2003) due to β-glucans receptors or mannose binding lectins expressed by epithelial cells (Carmona *et al*., 2010; Uemura *et al*., 2002). However, the ETEC K88 anti-adhesive effect of MOS (Becker and Galletti, 2008) is probably responsible for the lower inflammatory cytokine expression in our study. Treatment LB reduced the expression of IL-1β, IL-8, TNF-α, and TLR-5 but not TLR-4. To our knowledge, the LB galactomannans immunomodulatory effect has not been previously reported and it is not clear if the decreased pro-inflammatory cytokines expression were solely due to the inhibitory effect of ETEC K88 adhesion to the epithelial cells.

Contrary to the other treatments, AO increased TLR-4, IL-8, and TNF-α expression when we incubated IPEC-J2 with AO in the absence of *E. coli*. These results suggest that the AO likely presents some immunogenic compound that interact with enterocytes and trigger inflammatory response. It is possible that this effect is due to galactomannans present in the membrane of the *Aspergillus* (Verweij *et al*., 1998; Levitz, 2004). Thus, AO did not affect expression of the studied genes after the challenge except for couple of cases that could be the result of a combination of a

reduction in *E. coli* adhesion and a direct immune stimulatory effect. Perhaps lower doses of AO could still prevent adhesion of ETEC without pro-inflammatory effects.

7.5. Conclusion

We were able to demonstrate the ability of WB, CGMP, MOS, LB and AO to interfere in the pathogenic process of the ETEC K88, particularly reducing the adhesion of the pathogen to the epithelium and in some cases modifying the innate immune response. In sight of the results, WB seems the best candidate ingredient, out of those studied, to be further tested *in vivo* due to its consistent effect on all the parameters. However, CGMP, MOS and LB should also be explored further; and AO should be further studied to elucidate the origin of its pro-inflammatory effect on porcine intestinal epithelial cells.

7.6. Literature cited

Aksit S, Çaglayan S, Cukan R *et al*. (1998) Carob bean juice: A powerful adjunct to oral rehydration solution treatment in diarrhoea. *Paediatr Perinat Epidemiol* **12**, 176-181.

Alexander C and Rietschel ET (2001) Invited review: Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* **7**, 167-202.

Arce C, Ramírez-Boo M, Lucena C, *et al*. (2010) Innate immune activation of swine intestinal epithelial cell lines (IPEC-J2 and IPI-2I) in response to LPS from *Salmonella* typhimurium. *Comp Immunol Microbiol Infect Dis* **33**, 161-174.

Becker PM, Galletti S, Roubos-van den Hil PJ, *et al*. (2007). Validation of growth as measurand for bacterial adhesion to food and feed ingredients. *J Appl Microbiol* **103**, 2686-2696.

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

Berberov EM, Zhou Y, Francis DH, *et al*. (2004) Relative importance of heat-labile enterotoxin in the causation of severe diarrheal disease in the gnotobiotic piglet model by a strain of enterotoxigenic *Escherichia coli* that produces multiple enterotoxins. *Infect Immun* **72**, 3914-3924.

Boutrou R, Jardin J, Blais A, *et al*. (2008) Glycosylations of κ-casein-derived caseino-macropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J Agric Food Chem* **56**, 8166-8173.

Brody EP. (2000) Biological activities of bovine glycomacropeptide. *Br J Nutr* **84**, 39-46

Brown GD (2006) Dectin-1: A signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* **6**, 33-43.

Cao L, Liu X, Qian T, *et al*. (2011) Antitumor and immunomodulatory activity of arabinoxylans: A major constituent of wheat bran. *Int J Biol Macromol* **48**, 160-164.

Carmona EM**,** Lamont JD**,** Xue A, *et al*. (2010) Pneumocystis cell wall β-glucan stimulates calcium-dependent signaling of IL-8 secretion by human airway epithelial cells. *Respir Res* **11**, 95-99.

Castillo M, Martín-Orúe SM, Taylor-Pickard JA, *et al*. (2008) Use of mannanoligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: Effects on microbiota and gut function. *J Anim Sci* **86**, 94-101.

Collado-Romero M, Arce C, Ramirez-Boo M, *et al*. (2010) Quantitative analysis of the immune response upon *Salmonella* typhimurium infection along the porcine intestinal gut. *Vet Res* **41**, 23-35.

Conway PL, Welin A, Cohen PS (1990) Presence of K88-specific receptors in porcine ileal mucus is age dependent. *Infect Immun* **58,** 3178-3182.

Devriendt B, Stuyven E; Verdonck F, *et al*. (2010) Enterotoxigenic *Escherichia coli* (K88) induce proinflammatory responses in porcine intestinal epithelial cells. *Dev Comp Immunol* **34**, 1175-1182.

Dibner J and Richards J (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poult Sci* **84**, 634-643.

Fairbrother JM, Nadeau E, Gyles CL (2005) *Escherichia coli* in postweaning diarrhea in pigs: An update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* **6**, 17-39.

Gantner B N, Simmons RM, Canavera SJ, *et al*. (2003) Collaborative induction of inflammatory responses by dectin-1 and toll-like receptor 2. *J Exp Med* **197,** 1107-1117.

Geens MM and Niewold TA (2010) Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli*, *Escherichia coli*, and *E. coli* lipopolysaccharide. *Comp Funct Genomics* **2010**, 1-11.

Grange PA, Erickson AK, Anderson TJ, *et al*. (1998) Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of *Escherichia coli*. *Infect Immun* **66**, 1613-1621.

Grange PA, Mouricout MA, Levery SB, *et al*. (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**, 2336-2343.

Hermes RG, Molist F, Pérez JF, *et al*. (2010) Casein glycomacropeptide (CGMP) in the diet of early weaned piglets reduces *Escherichia coli* attachment to the intestinal mucosa and increases lactobacillus numbers in digesta. *J Anim Sci* **88**(E-Suppl2), 582.

Hughes SA Shewry PR, Li L, *et al*. (2007) *In vitro* fermentation by human fecal microflora of wheat arabinoxylans. *J Agric Food Chem* **55,** 4589-4595.

Kamal-Eldin A, Lærke HN, Knudsen KEB, *et al*. (2009) Physical, microscopic and chemical characterisation of industrial rye and wheat brans from the nordic countries. *Food Nutr Res* 2009, **53**. 1-11.

Koh SY, George S, Brözel V (2008) Porcine intestinal epithelial cell lines as a new in vitro model for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli*. *Vet Microbiol* **130**, 191-197.

Kooyk Y, Rabinovich GA (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol* **9**, 593-601.

Konstantinov SR, Favier CF, Zhu WY (2004) Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. *Anim Res* **53**, 317-324.

Lallès JP, Bosi P, Janczyk P, *et al*. (2009) Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: A review. *Animal* **3**, 1625-1643.

Lazaridou A, Biliaderis CG, Izydorczyk MS (2001) Structural characteristics and rheological properties of locust bean galactomannans: A comparison of samples from different carob tree populations. *J Sci Food Agric* **81,** 68-75.

Leusch H, Drzeniek Z, Markos-Pusztai Z, *et al*. (1991) Binding of *Escherichia coli* and *Salmonella* strains to members of the carcinoembryonic antigen family: Differential binding inhibition by aromatic α-glycosides of mannose. *Infect Immun* **59**, 2051-2057.

Levitz SM (2004) Interactions of toll-like receptors with fungi. *Microb Infect* **6**, 1351-1355.

Malkoski M, Dashper SG, O'Brien-Simpson NM, *et al*. (2001) Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrob Agents Chemother* **45**, 2309-2315.

Mariani V, Palermo S, Fiorentini S, *et al*. (2009) Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. *Vet Immunol Immunopathol* **131**, 278-284.

Marquart RR, Jin LZ, Kim JW, *et al*. (1999) Passive protective effect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. *FEMS Immunol Med Microbiol* **23**,283-288

Matsumura K; Higashida K, Ishida H, *et al*. (2008) Carbohydrate Binding Specificity of a Fucose-specific Lectin from *Aspergillus oryzae. J Biol Chem* **282**, 15700-15708.

Mennink-Kersten MA, Donnelly JP, Verweij PE (2004) Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *Lancet Infect Dis* **4**, 349-357.

Molist F, Hermes RG, Gómez de Segura A, *et al*. (2011) The interaction between wheat bran and therapeutic doses of zinc oxide may reduce their effects on the intestinal microbiota of early weaning piglet. *Br J Nutr* **105,** 1592–1600.

Molist F, Gómez de Segura A, Pérez JF (2010) Effect of wheat bran on the health and performance of weaned pigs challenged with *Escherichia coli* K88. *Livest Sci* **133**, 214-217.

Molist F, Gómez de Segura A, Gasa J, *et al*. (2009) Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. *Anim Feed Sci Technol* **149**, 346-353.

Moue M, Tohno M, Shimazu T, *et al*. (2008) Toll-like receptor 4 and cytokine expression involved in functional immune response in an originally established porcine intestinal epitheliocyte cell line. *Biochim Biophys Act* **1780**, 134-144.

Nagy B and Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microbiol* **295**, 443-454.

Nakajima K, Tamura N, Kobayashi-Hattori K, *et al*. (2005) Prevention of intestinal infection by glycomacropeptide. *Biosci Biotechnol Biochem* **69**, 2294-2301.

Naughton PJ, Mikkelsen LL, Jensen BB (2001) Effects of nondigestible oligosaccharides on *Salmonella enterica* serovar typhimurium and nonpathogenic *Escherichia coli* in the pig small intestine *in vitro*. *Appl Environ Microbiol* **67,** 3391-3395.

Pavlova B, Volf J, Alexa P (2008) Cytokine mRNA expression in porcine cell lines stimulated by enterotoxigenic *Escherichia coli*. *Vet Microbiol* **132**, 105-110.

Requena P, Daddaoua A, Martínez-Plata E (2008) Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17. *Br J Pharmacol* **154**, 825-832.

Sarabia-Sainz AI, Ramos-Clamont G, Candia-Plata MM, *et al*. (2009) Biorecognition of *Escherichia coli* K88 adhesin for glycated porcine albumin. *Int J Biol Macromol* **44**, 175-181.

Schierack P, Nordhoff M, Pollmann M, *et al*. (2006) Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. *Histochem Cell Biol* **125**, 293-305.

Shahriar F, Ngeleka M, Gordon JR, *et al*. (2006) Identification by mass spectroscopy of F4ac-fimbrial-binding proteins in porcine milk and characterization of lactadherin as an inhibitor of F4ac-positive *Escherichia coli* attachment to intestinal villi *in vitro*. *Dev Comp Immunol* **230**, 723-734.

Takeuchi O, Hoshino K, Kawai T, *et al*. (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443-451.

Uemura K, Saka M, Nakagawa T (2002) L-MBP is expressed in epithelial cells of mouse. Small intestine. *J Immunol* **169**, 6945-6950

Van den Broeck W, Cox E, Oudega E, *et al*. (2000) The F4 fimbrial antigen of *Escherichia coli* and its receptors. *Vet Microbiol* **71**, 223-44.

Verweij PE, Poulain D, Obayashi T, *et al*. (1998) Current trends in the detection of antigenaemia, metabolites, and cell wall markers for the diagnosis and therapeutic monitoring of fungal infections. *Med Mycol* **36**, 146–155.

Weglarz L, Wawszczyk J, Orchel A, *et al*. (2007) Phytic acid modulates in vitro IL-8 and IL-6 release from colonic epithelial cells stimulated with LPS and IL-1β. *Dig Dis Sci* **52**, 93-102.

Wismar R, Brix S, Frøkiær H, *et al*. (2010) Dietary fibers as immunoregulatory compounds in health and disease. *Ann NY Acad Sci* **1190**, 70-85.

White LA, NewmanMC, Cromwell GL, *et al*. (2002). Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. *J Anim Sci* **80**, 2619-2628.

Yuan X, Wang J, Yao H (2005) Feruloyl oligosaccharides stimulate the growth of *Bifidobacterium bifidum*. *Anaerobe* **11**, 225-229.

Zhou S, Liu X, Guo Y (2010) Comparison of the immunological activities of arabinoxylans from wheat bran with alkali and xylanase-aided extraction. *Carbohydr Polym* **81**, 784-789.

Zinger-Yosovich, K, Gilboa-Garber N (2009) Blocking of *Pseudomonas aeruginosa* and ralstonia solanacearum lectins by plant and microbial branched polysaccharides used as food additives. *J Agric Food Chem* **57**, 6908-6913.

Chapter 8

Casein glycomacropeptide in the diet may reduce the *E. coli* attachment to the intestinal mucosa of early weaned piglets after an ETEC K88 challenge. Submitted for publication on February, 2011. *Plos One*. Under review.

8.1. Introduction

Adherence of bacteria to intestinal epithelium is known to be a prerequisite step for the colonization and infection of the gastrointestinal tract by many pathogens. Some strains of toxigenic *E. coli* have developed mechanisms of adhesion to intestinal (Fairbrother *et al*., 2005) or renal cells (Korhonen *et al*., 1986). In particular, enterotoxigenic *E. coli* (ETEC) strains adhere to receptors on the intestinal epithelium by proteinaceous surface appendages, called fimbriae (Grange *et al*., 2002). The ETEC expressing K88 fimbrial antigen is the most common pathogroup in young pigs (Grange *et al*., 2002), and the glycoproteins, sialoglycoproteins or glycosphingolipids are considered the main receptors for different K88 (ab, ac or ad) fimbrial variants (Jin and Zhao, 2000; Nagy and Fekete, 2005; Van den Broeck *et al*., 2000).

The potential of certain compounds to inhibit the adherence of microorganisms, specifically *E. coli*, to the intestinal epithelium has been studied *in vitro* by various authors. Schwertmann *et al*. (1999) described the potential of different milk glycoproteins to block the fimbriae of *E. coli*; Grange *et al*. (2002) described different proteins and glycoesfingolipids from pig serum; and Naughton *et al*. (2001) described different types of prebiotics (nondigestible oligosaccharides) capable of reducing the numbers of *E. coli* in jejunal organ cultures of pigs. Some reports suggest that milk contains glycoconjugates that have structural homology to the glycan moieties of the intestinal mucosal cell surface and may act as competitive inhibitors of pathogen binding to their glycoconjugate receptors. Examples include oligosaccharides containing α1,2 linked fucosylated oligosaccharides (Newburg *et al*., 2004) or glycoproteins containing sialic compounds (Schwertmann *et al*., 1999).

Caseins are the most abundant bovine milk proteins. α_{s1}-, α_{s2}-, β-, and κ-casein are the four major types (Swaisgod, 1992). The casein glycomacropeptide (CGMP) is a glycoprotein originating from the C-terminal portion of κ-casein during cheese manufacture. Chymosin, an enzyme added to milk, hydrolyses κ-casein into para-casein (residues 1-105), which remains with

the curd, and CGMP (residues 106-169), which is removed, becoming the most abundant protein/peptide in whey proteins (20 to 25%). The CGMP is entirely free of the aromatic amino acids (Thomä-Worringer *et al*., 2006), which has permitted its use on human phenylketonuria diets (La Clair *et al*, 2009). Furthermore, threonine constitutes 18% of the total amino acid content and a large portion is glycosylated resulting in a sialic acid content of around 4.2% (Nakano *et al*., 2007).

Several reviews describe the biological activities of CGMP (Brody, 2000; Lonnerdal, 2003; Krissansen, 2007). Probably one of the most studied effects has been its interaction with the microbiota through the activity of carbohydrate moieties present in the molecule. Some authors have reported that CGMP binds the cholera toxin of *Vibrio cholera* (Kawasaki *et al*., 1992) and promotes the growth of *Bifidobacteria* (Idota *et al*., 1994) and *Lactococcus* species (Bouhallab *et al*., 1993) *in vitro* but inhibiting the growth of *Bacillus subtilis*, *Salmonella enterica* serovars Typhimurium and Enteriditis in Luria-Bertani medium (Wong *et al*., 2006). Moreover, other studies have shown that CGMP inhibits the adhesion of pathogenic *E. coli* (Newburg, 1997; Rhoades *et al*., 2005) to the mucosal surface or its growth *in vitro* (Malkoski *et al*., 2001). In addition, CGMP has been described as showing immunosuppressive and immunostimulatory properties (Li and Mine 2004), and anti-inflammatory activity in rats with induced colitis (Daddaoua *et al*., 2005).

However, the activity of CGMP *in vivo*, especially in the distal segments of the gastrointestinal tract, has not been well explored. Peptides derived from CGMP were detected in the intestinal lumen and blood after ingestion of milk products in humans (Chabance *et al*., 1998; Ledoux *et al*., 1999) and animals (Fosset, *et al*., 2002), which suggests that some CGMP fragments may resist protein digestion, probably due to its *O*-glycosylation (Boutrou *et al*., 2008), and reach the distal segment of the gastrointestinal tract.

The objective of this study was to confirm the ability of CGMP to block the attachment of ETEC to the ileum mucosa *in vitro* (Trial 1), and to assess whether dietary CGMP can prevent the

digestive and productive disturbances provoked by an enterotoxigenic *Escherichia coli* challenge in weanling piglets (Trial 2).

8.2. Material and Methods

8.2.1. Trial 1. In vitro inhibition trial

8.2.1.1. Chemicals. The Lacprodan CGMP-10® was kindly donated by Arla Foods (Viby J, Denmark). Reagents used in this study were: OCT (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), fluorescein isothiocyanate (FITC; Sigma, St. Louis, USA), laminin (Vector Laboratories Inc., CA, USA; 20 µg/ml in PBS) and Luria agar (Sigma, St. Louis). All other reagents and solvents were of the highest available purity and used as purchased.

8.2.1.2. Bacterial strain and culture conditions. An ETEC *E. coli* K88 (F4+, LT1+, ST1+, ST2+) strain associated with post-weaning diarrhea in pigs was kindly donated by Dr. Ignasi Badiola (CReSA, Barcelona, Spain). Bacteria were cultured three times overnight at 37ºC on Luria agar in order that the bacteria could express their virulent factors.

8.2.1.3. Tissue samples. Two 25 day old piglets were fed a commercial diet and treated with colistin (gram negative antibiotic) over 3 days to reduce the microbial load in the gastrointestinal tract of the animals. After the antibiotic treatment, piglets were euthanized, and 2 cm long sections of ileum were collected. The intestinal tissue samples were aseptically removed, washed in phosphate buffered saline (PBS, pH 7.1), covered with OCT Tissuetek, and immediately snap frozen in liquid nitrogen as described previously (Edelman *et al*., 2003). Frozen sections of 5 µm in thickness were cut in a Leica cryostat (Leica Instruments GmbH, Nussloch, Germany), mounted on SuperFrost Plus glass slides (KeboLab, Finland) and stored at -20ºC until use. Before adhesion inhibition testing, sections were fixed for 10 min at room temperature with cold 3.5 % paraformaldehyde in PBS and then washed three times with 50 ml of PBS.

8.2.1.4. Adhesion inhibition trial. For the adhesion inhibition studies bacteria were previously conjugated with FITC (Sigma, St. Louis, USA) as described earlier (Nowicki *et al*., 1986; Edelman *et al*., 2003). Before adding the bacteria to tissue, the FITC-labeled bacteria were incubated with CGMP (LACPRODAN CGMP-10®; Arla Foods, Viby J, Denmark) on crushed ice for 30 min. at different concentrations: 0 (control), 0.5, 1.5 or 2.5 mg/ml in PBS. After that, bacteria were added to tissue sections and incubated for 1 h at room temperature. To visualize tissue compartments and to localize adhesion sites, we double stained tissue sections using an indirect immunofluorescence method to detect the extracellular matrix regions of the tissue. After washing, the sections were incubated first with a polyclonal anti-laminin antiserum (Virkola *et al*., 1993) and then with tetramethyl rhodamine (TRITC)-conjugated swine anti-rabbit immunoglobulin G (diluted 1:100 in PBS). The adherent bacteria on tissue sections were examined with an Olympus BX50 (Hamburg, Germany) fluorescence microscope equipped with filters for FITC and TRITC and the images were digitally recorded using the Image-Pro Plus program, version 4.0 (Media Cybernetics).

8.2.2. Trial 2. In vivo trial Inclusion of CGMP in the diet of ETEC challenged piglets.

8.2.2.1. Chemicals. The Lacprodan CGMP-10® as a dietary source of CGMP was kindly donated by Arla Foods (Viby J, Denmark). This product has a crude protein content of 83-87% from which 75-85% is CGMP, a maximum content of lactose and fat of 2 and 0.5% respectively and 6.5% for ash. Its sialic acid content is estimated in 4.2%. All other chemicals used in this study were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), unless otherwise specified. All reagents and solvents were of the highest available purity and used as purchased.

8.2.2.2. Animals and housing. This experiment was performed at the Experimental Unit of the Universitat Autònoma de Barcelona and received prior approval (permit number: CEAAH 746) from the Animal and Human Experimental Ethical Committee of this institution. The treatment, management, housing, husbandry and slaughtering conditions conformed to the

European Union Guidelines. Furthermore, the trial was conducted as a Level 2 – High Risk Biosecurity Procedures, with appropriate training of the involved personnel.

A total of 72 piglets [(Large White x Landrace) x Pietrain] from a commercial farm (Coll Suri, Manlleu, Spain) were weaned at 24 ± 3 days of age with an average body weight of 6.9 ± 0.46 kg. Piglets were transported to the Universitat Autònoma de Barcelona facilities and placed in 3 rooms of 8 pens each (24 pens, 3 animals/pen). Each pen (3 m^2) had a feeder and a water nipple to provide food and water for *ad libitum* consumption. The weaning rooms were equipped with automatic heating and forced ventilation. The experiment was conducted during the winter season (February) with an average inside room temperature of 30 ± 2 ºC.

The experiment was conceived as a 2×2 factorial design that included two diets (control vs. CGMP), and an ETEC challenge or not. Two rooms were used for the microbial challenge and one room for the non-challenged animals. The two experimental diets were randomly distributed between the pens of each room.

Diets (Table 1) were isoenergetic and isonitrogenous and formulated to satisfy the nutrient requirement standards for pigs (BSAS, 2003). In the CGMP-diets, Lacprodan CGMP-10® (74% of purity) was added at 2% (w/w), representing about 1.5% of CGMP. This dose was based on the previous *in vitro* assay results where 0.25% presented the best inhibition of ETEC adhesion to the ileum epithelium samples. For the translation of this dose to the diet we assumed that the CGMP would be partially digested (around 40 %) and diluted in the ileal digesta (approximately 20 % DM).

8.2.2.3. Bacterial strain. The bacteria strain used in this study was isolated from a colibacilosis outbreak in Spain (Blanco *et al*., 1997), serotype (O149:K91:H10 [K-88]/LT-I/STb), and was provided by the *E. coli* Reference Laboratory, Veterinary Faculty of Santiago de Compostela (Lugo). The infection inoculum was prepared by 16h-incubation at 37ºC in Luria broth with slow agitation (1*g*) in an orbital incubator (WY-100, Comecta S.A., Barcelona, Spain).

8.2.2.4. Experimental Procedures. Animals received the experimental diets (Table 1) over 15 days: a control diet (0% CGMP) and a CGMP diet (1.5% CGMP), replacing a similar amount of soy protein and wheat gluten from the control diet. After one week of adaptation, a single 2-ml oral dose ($1 \times 10^9 - 10^{10}$ CFU/ml) of the ETEC (K88) strain was administered to the challenged animals or a single 2-ml oral dose of sterile Luria broth to the non-challenged animals. Individual body weight and pen feed consumption was registered weekly. Animals were checked daily to evaluate their status after the *E. coli* challenge. Briefly, the rectal temperature was measured every 2 days and clinical signs (i.e. dehydration, apathy and diarrhea) were monitored daily. The diarrhea incidence was measured as the percentage of animals in each pen that presented inconsistent to liquid feces. The mortality rate was also recorded.

Four days after the ETEC challenge (day 11) and on the final day of the experiment (day 15 after weaning), one animal from each pen was euthanized. On day 11 from each pen the pig closest to the medium weight was selected; on day 15 the heavier of the two remaining was taken. Animals were bled and euthanized with an intravenous sodium pentobarbital overdose (200 mg/kg BW), and the abdomen was immediately opened. Samples of ileum and proximal colon digesta were collected for bacterial counts and four other subsamples were maintained at -20ºC for further analyses including: quantification of microbial groups by quantitative PCR (qPCR), and determination of volatile fatty acids (VFA), ammonia, and protein concentrations in digesta. Moreover, for analyses of the enterobacteria attached to the ileum mucosa, 5-cm long sections of ileum were collected from each animal, washed thoroughly three times with sterile PBS, opened longitudinally and scraped with a microscopy glass slide to obtain the mucosa scrapes content. For histological study, sections of 3 cm from the ileum were removed, opened longitudinally and fixed by immersion in Carnoy solution as described by Swidsinski *et al*. (2005). Another ileum section of 3 cm was removed, opened longitudinally, placed in cassettes recovered with OCT Tissuetek® cryoprotective solution (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands), frozen in

liquid nitrogen-cooled isopentane and maintained at -80ºC for further analyses using Fluorescence *In Situ* Hybridization (FISH).

8.2.2.5. Analytical Procedures. Chemical analyses of the diets including, dry matter (DM), ash, gross energy (GE), crude protein (CP), ether extract, neutral detergent fiber (NDF) and acid detergent fiber (ADF) were performed according to the AOAC standard procedures (1995).

For bacterial counts, the content of ileal mucosa scrapes were seeded in eosin methylene blue (EMB) agar (Scharlab, S.L., Barcelona, Spain). The plates were incubated for 24 h at 37°C and the manufacturer's instructions for the quantification of the colonies were followed.

The DNA from ileal and colon digesta was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). Lactobacilli and enterobacteria were quantified by real time PCR using SyBR Green dye following the procedure described by Castillo *et al*. [51]. For *E. coli* (K88) real time PCR quantification a new procedure was implemented. For this, the selected target gen was that codifying the F4 fimbria of *E. coli* K-88. PCR products (439 bp) obtained using the primers 5`- GCACATGCCTGGATGA-CTGGTG-3` and 5`-CGTCCGCAGAAGTAACCCCACCT-3` (Castillo *et al*., 2006) and DNA obtained from pure cultures of the challenge strain (QIAamp DNA Mini Kit, Qiagen, West Sussex, UK) were used for the construction of the standard curves. The PCR product was purified with the commercial kit DNA purification system (Promega Biotech Ibérica, Spain) and the concentration measured using a Qubit™ Fluorometer (Invitrogen, Carlsbad, CA, USA). The products obtained were also sequenced (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) to confirm it, and number of copies calculated. Serial dilutions were performed and 10^4, 10^5, 10^6, 10^7 and 10^8 copies of the gene per reaction were used for calibration.

To quantify *E. coli* K-88 the following pair of primers were designed using the Primer Express Software (Applied Biosystems, CA, USA): 5`- CAGAAATGGGAATGGAA-AGTTG-3` and 5`-CCATTGGTCAGGTCATTCAATACA-3` (Setia *et al*., 2009). Real-time PCR was performed with the ABI 7900 HT Sequence Detection System (PE Biosystems, Warrington, UK)

using optical grade 96-well plates. The PCR reaction was performed on a total volume of 25 µl using the SYBR-Green PCR Core Reagents kit (PE Biosystems). Each reaction included 2.5 µl 10x SYBR Green buffer, 3 µl MgCl2 (25 mM), 2 µl dNTPs (2.5 mM), 0.25 µl AmpErase UNG (1 U/µl), 0.125 µl AmpliTaq Gold (5 U/µl), 1 µl of each primer (12.5 µM) and 2 µl of DNA samples. The reaction conditions for amplification of DNA were 95 ºC for 10 min, 40 cycles of 95ºC for 15 s, and 60 ºC for 1 min. To determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification.

The minimum level of detection of the method, considering the amount of DNA included in each reaction, was established in 3.249 (± 0.419) log of 16S rRNA gene copies/g of FM sample, compared to a non-template control dissociation curve.

VFA were determined by gas chromatography, after submitting the samples to an acid-base treatment followed by an ether extraction and derivatization, as described by Jensen and Jorgensen (1994).

Ammonia was determined with the aid of a gas sensitive electrode (Crison ISE- 9665, Crison Instruments, S.A., Barcelona, Spain). Three grams of digesta were diluted (1:2) with 0.16 M NaOH, and after homogenization, samples were centrifuged (1500 x *g*) for 10 min. Subsequently, the ammonia released from the samples was measured in the supernatants using a digital voltmeter (Crison GLP 22) (Diebold *et al*., 2004).

The crude protein measurement on colon digesta was performed in a combustion analyzer (TruSpec CN, LECO Corporation, Madrid, Spain).

Tissue samples for morphological measures were dehydrated and embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin. Morphological measurements were performed with a light microscope (BHS, Olympus, Spain), according to published parameters by Nofrarías *et al*. (2006).

Serum was obtained from 10 ml blood into the tubes (without anticoagulant) and by centrifugation of blood at 3,000 x *g*, 15 min at 4ºC. Serum urea concentration was measured by

Glutamate Dehydrogenase (GLDH) reaction, using the Olympus System Reagent® (Olympus, Ireland) and using a Olympus AU400 (series 3112676, Germany) device. The Tumor Necrosis Factor-α (TNF-α) and the Interferon-γ (IFN-γ) concentrations were determined by Quantikine® Porcine TNF-α and IFN-γ kits, respectively (R&D Systems, Abingdon, United Kingdom). Pig Major Acute-phase Protein (Pig-MAP) concentration was determined by a sandwich type ELISA (Pig MAP kit ELISA, Pig CHAMP Pro Europe S.A., Segovia, Spain), according to the manufacturer's instructions.

The FISH technique was performed by modifying the protocol described by Swidsinski *et al*. [49]. Briefly, triplicate frozen ileum samples were sliced (5 µm thick) on a Leica CM 1900® (Leica Microsystems GmbH, Wetzlar, Germany) cryostat. The tissue samples were placed in Superfrost Gold Plus® (Thermo Fisher Scientific, Waltham, MA, USA) and fixed with 4% (w/v) paraformaldehyde solution for 30 minutes. Oligonucleotide probes were synthesized by TIB Molbiol (Berlin, Germany), using carbocyanite-3 (Cy3) and FITC dyes, added at the 5` end to the EC1531 probe, and the EUB338, NON338 probes, respectively. A hybridization buffer (0.9M NaCl, 20mM Tris-HCl pH 7.4, 2% formamide and 0.1% SDS) was used at 50ºC for 45 minutes. Furthermore, a 4,6-diamidino-2-phenylindole (DAPI) staining was used to mark all the eukaryotic cells nuclei. The *in situ* quantification of mucosal bacteria was visualized with a Confocal Laser Microscope (Fluoview FV1000, Olympus GmbH, Hamburg, Germany) and photo-documented with an Olympus camera and software (FV-ASW, version 1.7c, Olympus GmbH, Hamburg, Germany). Quantification was performed when the hybridization signals were clear and morphologically distinguishable as bacterial cells by at least triple-color identification with universal and group-specific FISH probes and DAPI staining and by the absence of cross-hybridization or hybridization using the NON338 nonsense probe. From each triplicate sample, the percentage of villi with adhered bacteria was determined by the same person who was blind to the treatments.

8.2.2.6. Statistical Analyses. The effect of experimental treatments on performance, the incidence of diarrhea, and analytical data were tested with a factorial ANOVA analysis using the GLM procedure of SAS (1999).

For fermentation products, diarrhea incidence, percentage of infected ileal villi and enterobacteria in mucosa scrapes, the following model was used:

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$,

where Y_{ijk} is the dependent variable, μ is the overall mean, α_i is the effect of diet, β_j is the effect of day, $(\alpha\beta)_{ij}$ is the interaction between diet and day and $\varepsilon \sim N(0,\sigma^2_\varepsilon)$ represents the unexplained random error.

Whenever a non-significant effect was found for day or interaction, they were secondary excluded from the model and results shown as global means per experimental diet. For other parameters, data were analyzed and shown by sampling day ($Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$)

For all analyzed data, the pen was used as the experimental unit. The alpha level used for the determination of significance for all the analysis was 0.05. The statistical trend was also considered for *P* values > 0.05 and < 0.10. Data are presented as least squares means and SEM. When treatment effects were established ($P < 0.05$), treatment least squares means were separated using the probability of differences (PDIFF) function adjusted by Tukey-Kramer.

8.3. Results

8.3.1. In vitro inhibition trial (Trial 1):

Results (Figure 8.1. AI) confirmed that *E. coli* can adhere strongly to the apical surface of villus ileum enterocytes. A weak adhesion was also seen in the lamina propria under the villus epithelium as identified by staining with anti-laminin antibodies (Fig.8.1. AII). The adhesion of *E. coli* K88 was reduced by CGMP (Fig. 8.1. B-D). The increased concentration of the inhibitor

resulted in a gradual decrease in the number of *E. coli* attached to the epithelial surface (Fig. 8.1. B-D). The highest concentration of CGMP (2.5 mg/ml; Fig. 8.1. D) was needed for *E. coli* K88 inhibition on the apical epithelium of the ileum. However, it could not inhibit adherence to lamina propria regions of the ileum as seen in Fig. 8.1. B-D.

8.3.2. Animal Performance and the health status (Trial 2).

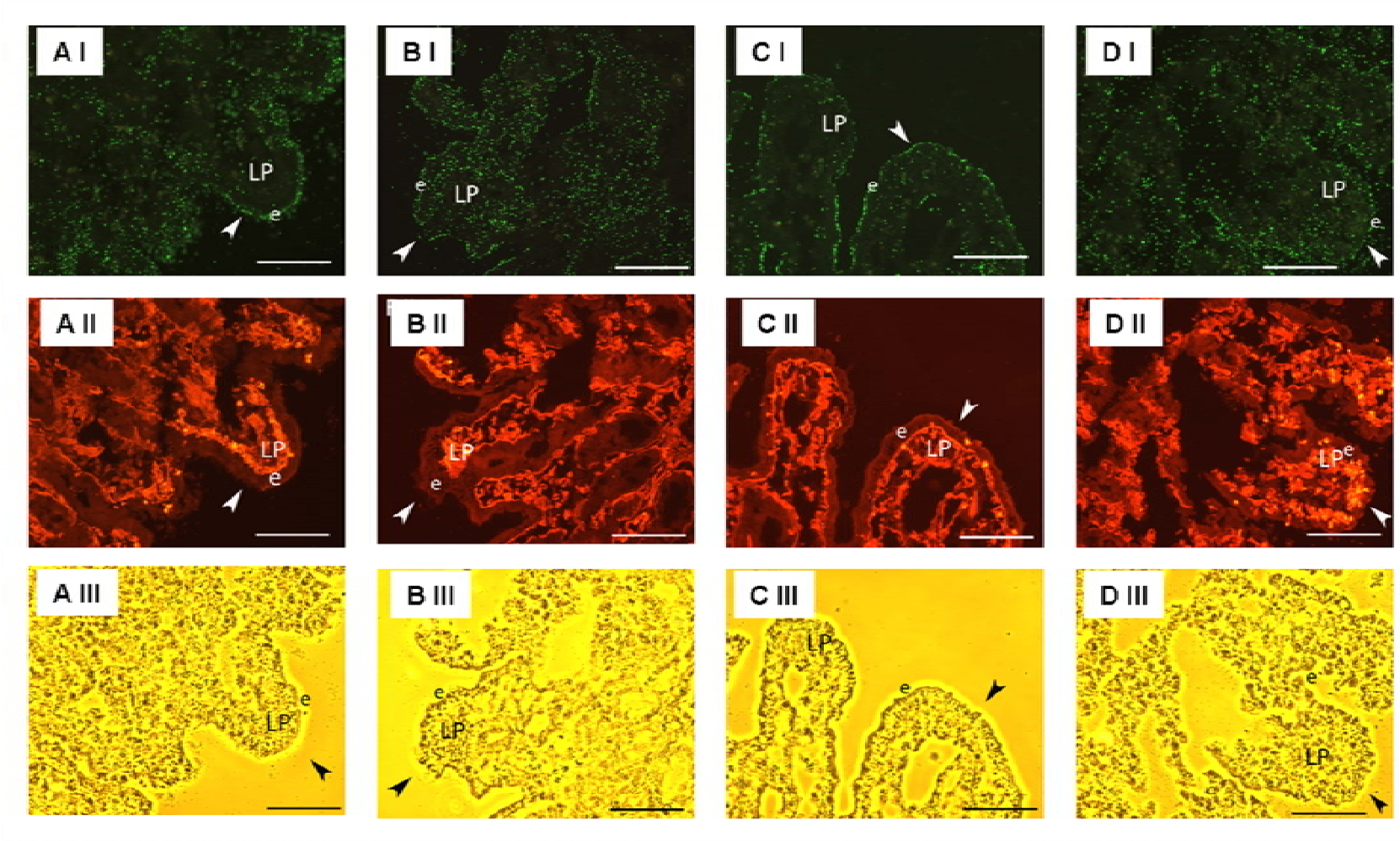
Neither experimental diet nor ETEC challenge affected feed intake and growth (data not shown). The average final body weight on day 15 after weaning was 9.6 ± 1.44 kg.

No significant differences were observed in mortality rate (experimental average = 8%) between diets (Table 8.1). Furthermore, no significant increase was observed in the body temperature recorded (data not shown).

8.3.3. The acute immune response (Trial 2).

Table 8.2 shows the Pig MAP and TNF-α serum concentrations. The administration of CGMP did not affect the Pig Map and TNF-α concentrations. On the other hand, the ETEC challenge tended (P = 0.092) to increase the Pig MAP concentration (P = 0.092), and increased TNF-α concentration (P = 0.038) 4 days after infection. The IFN-γ results were below (<39 pg/ml) the kit assay sensitivity used (data not shown).

Figure 8.1. Inhibition of *E. coli* K88 adherence to the piglet ileum epithelium with CGMP.



The tissue sections were double stained with FITC-labelled bacteria (panels I) and laminin (panels II). Panels III show the tissue by phase contrast microscopy. The CGMP was test in the following concentration: 0 (A); 0.5 (B); 1.5 (C); 2.5 mg/ml (D). Arrows indicate the epithelial surface. Symbol “e” denotes epithelium and symbol “LP” lamina propria. Size bars 100 μm.

Table 8.1. Ingredients and analyzed chemical composition of the experimental diets.

	Diets	
	Control	**CGMP**
Ingredients, g/kg of feed (as fed basis)		
Corn	332.2	330.0
Barley	211.6	219.9
Soy Protein	90.0	80.6
Lacprodan CGMP-10[1]	-	20.0
Fishmeal	40.0	40.0
Wheat Gluten	58.1	44.5
Full-fat Whey	100.0	100.0
Sweet Whey	130.0	130.0
L-Lysine	6.8	6.8
DL-Metionine	1.5	1.4
L-Threonine	2.1	0.0
L-Tryptophan	0.7	0.8
Calcium carbonate	9.3	8.7
Dicalcium phosphate	10.3	10.0
Sodium chloride	3.4	3.4
Vitamin and mineral*	4.0	4.0
Analysed chemical composition, g/kg of feed (as fed basis)		
Dry matter	91.2	90.9
Ash	5.0	4.9
Gross energy (Kcal/Kg of DM)	4243	4201
Crude protein	20.7	20.7
Ether extract	6.8	7.0
Neutral detergent fiber	6.7	7.2
Acid detergent fiber	2.1	2.4

[1] 74% of purity, Arla Foods, Viby J, Denmark.

***** Supplied per kilogram of feed: 13000 IU of vitamin A, 1800 IU of vitamin D3, 60.0 mg of vitamin E, 3.0 mg of vitamin K3, 2.0 mg of vitamin B1, 6.0 mg of vitamin B2, 3.0 mg of vitamin B6, 0.02 mg of vitamin B12, 35.0 mg of niacin, 15.0 mg of calcium pantothenate, 0.12 mg of biotin, 1 mg of folic acid, 20.0 mg of Fe (as iron chelate), 120.0 mg of Cu (20.0 mg as copper chelate and 100.0 mg as copper sulfate), 110 mg of Zn (20.0mg as zinc chelate and 90.0 mg as zinc oxide), 45.0 mg of Mn (5.0mg as manganese chelate and 40.0 mg as manganese oxide), 0.30 mg of Se (as sodium selenite) 0,10 mg of Co (as cobalt carbonate), 1 mg of I (as calcium iodate) and 2.5 mg of ethoxyquin.

Table 8.2. Effects of 1.5% dietary inclusion of CGMP on pig-major acute phase protein (Pig-MAP) and tumor necrosis factor-α (TNF-α) of weaning piglets challenged or not with ETEC (K88) at 4 and 8 days post challenge (PC).

		Diets							
		Control		**CGMP**			***P-values***		
	Challenge	**No**	**Yes**	**No**	**Yes**	**SEM**	***diet***	***chall***	***d x c***
Pig MAP (µg/mL)	4 d PC	667	692	580	861	206.3	0.353	0.092	0.192
	8 d PC	525	644	573	514	218.4	0.467	0.771	0.363
TNF-α (pg/mL)	4 d PC	54.1	79.3	51.1	108.7	40.13	0.382	0.038	0.394
	8 d PC	83.0	71.8	74.0	66.3	37.19	0.663	0.571	0.971

8.3.4. Changes in the intestinal morphometry (Trial 2).

Table 8.3 presents the results of the ileum morphology (4 days post challenge). The ETEC challenge tended (P = 0.054) to increase crypt depth, reduced the villus:crypt ratio and also significantly increased the intraepithelial lymphocyte numbers ($P < 0.05$). On the other hand, the CGMP inclusion increased ($P < 0.05$) the crypt depth but did not modify the villus:crypt ratio. No significant differences were observed in the ileum histology of piglets 8 days post challenge (data not shown).

Table 8.3. Effects of 1.5% dietary inclusion of CGMP on ileum histology (4 days post challenge); and enterobacteria number (Log of CFU/g of content) on ileum mucosa scrapes of weaning piglets (whole experimental period) challenged or not with ETEC (K88).

		Diets							
		Control		**CGMP**			***P-values***		
	Challenge	**No**	**Yes**	**No**	**Yes**	**SEM**	***diet***	***chall***	***d x c***
Ileum histology	VH[1]	348.8	318.4	359.5	318.1	64.75	0.898	0.215	0.847
	CD[2]	207.3	231.7	237.5	254.9	23.55	0.015	0.054	0.735
	Ratio[3]	1.7	1.4	1.5	1.3	0.295	0.364	0.043	0779
	IEL[4]	5.4	5.7	4.1	6.2	1.286	0.484	0.046	0.110
Ileum mucosa scrapes	Enterob	11.87	9.79	5.12	4.91	3.343	0.020	0.625	0.689

[1]VH: Villus Height (μm), [2]CD: Crypt Depth (μm), [3] Villi:Crypt ratio, [4] IEL: Intraepithelial lymphocytes (cells/100 μm).

8.3.5. Changes in the lumen microbial activity (Trial 2).

Microbial counts of lactobacilli, enterobacteria and *E. coli* K88 in ileum and proximal colon digesta, measured by real time PCR are presented in Table 8.4. The administration of CGMP increased the lactobacilli numbers eight days post challenge in ileum and proximal colon digesta of both challenged and non-challenged pigs, decreased the enterobacteria in ileum digesta of challenged pigs on day 4 (*P* interaction = 0.006) and tended to reduce this population in both groups on day 8 after challenge (*P* = 0.089). On the other hand, the *E. coli* challenge decreased the lactobacilli numbers in the ileum digesta at four days post challenge in all the animals and increased the enterobacteria on day 4 post challenge in the ileum and proximal colon digesta, only in the animals receiving the control diet.

Regarding the *E. coli* K88 quantification by Real Time PCR, it was not able to detect these microorganisms in any of the non-challenged animals; however, it was measurable in the challenged group. Ileal mean values decreased significantly from day 4 to 8 (7.93 vs. 6.17 log of 16S rRNA gene copies/g of FM sample, P = 0.004) but not colonic samples. No differences were observed between experimental diets in any section.

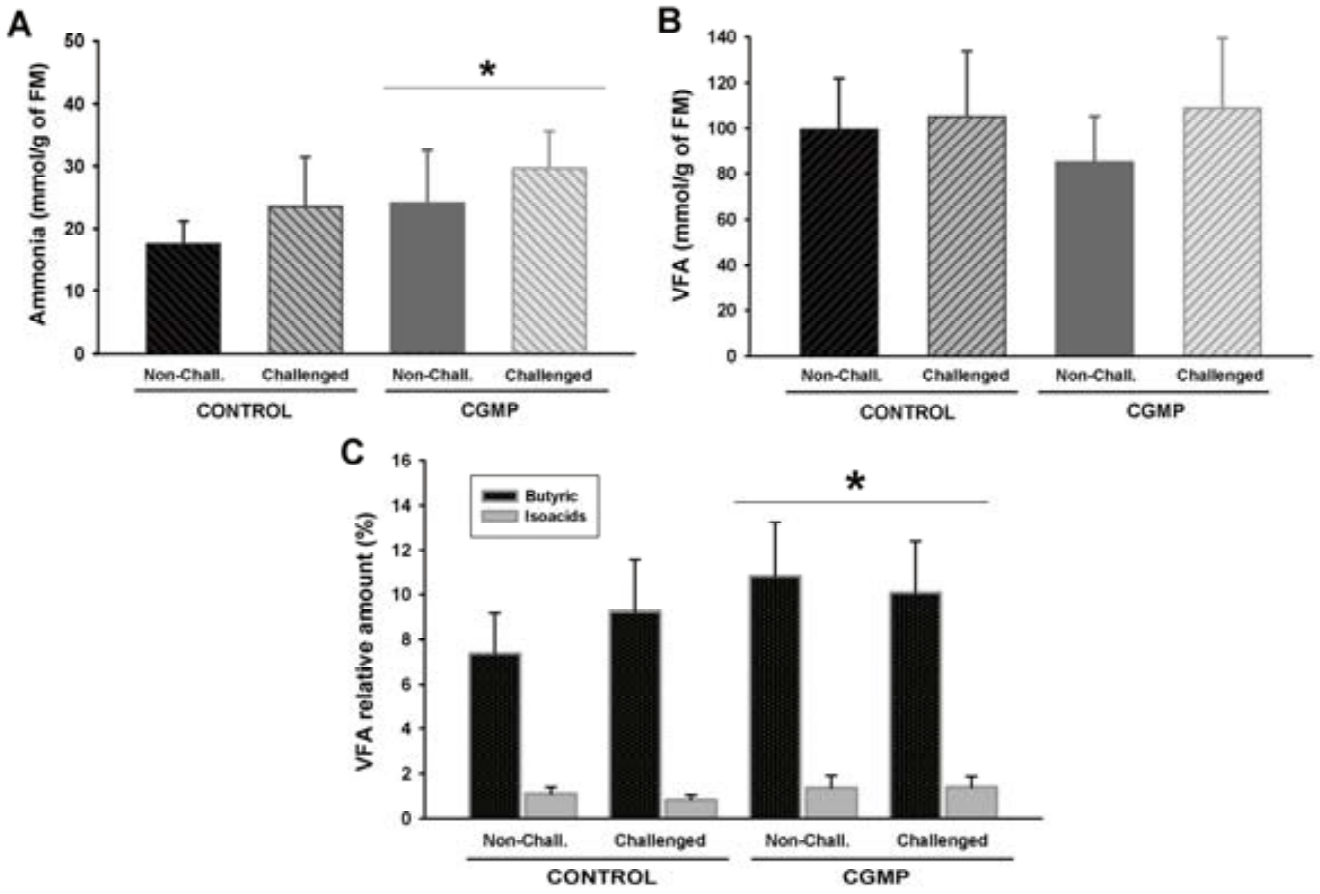
Table 8.4. Effects of 1.5% dietary inclusion of CGMP on the counts of lactobacilli, enterobacteria and *E. coli* K88 (log of 16S rRNA gene copies/g of FM sample) of weaning piglets challenged or not with ETEC (K88).

		Diets							
		Control		**CGMP**			***P-values***		
	Challenge	**No**	**Yes**	**No**	**Yes**	**SEM**	***diet***	***chall***	***d x c***
Lactobacilli									
Ileum digesta	4d PC	10.92	9.82	10.83	10.16	0.777	0.595	0.019	0.553
	8d PC	10.16	10.04	10.87	10.37	0.536	0.037	0.202	0.424
Proximal colon digesta	4d PC	11.12	11.45	11.62	11.48	0.579	0.297	0.707	0.365
	8d PC	10.86	11.35	11.78	11.62	0.462	0.007	0.428	0.122
Enterobacteria									
Ileum digesta	4d PC	8.01	10.06	8.69	8.34	0.874	0.022	0.033	0.006
	8d PC	10.16	9.64	9.48	8.49	1.285	0.089	0.204	0.681
Proximal colon digesta	4d PC	9.48	11.40	10.88	11.05	0.733	0.441	0.004	0.012
	8d PC	10.23	10.36	9.61	10.06	1.218	0.396	0.588	0.771
***E. coli* K88**									
Ileum digesta	4d PC	ND[1]	8.11	ND	7.73	1.680	0.674	-	-
	8d PC	ND	6.33	ND	6.01	0.592	0.302	-	-
Proximal colon digesta	4d PC	ND	10.06	ND	8.90	2.071	0.283	-	-
	8d PC	ND	8.88	ND	9.15	0.701	0.450	-	-

[1]ND: not detected. Minimum detection level of the method: 3.249 (± 0.419) log of 16S rRNA gene copies/g of FM sample.

Fig. 8.2 presents the ammonia, VFA concentration and the relative concentrations of butyric and isoacids in the proximal colon digesta. Experimental factors did not cause significant differences in the total VFA concentration, but changed the VFA profile and the ammonia concentrations. The CGMP diet increased ($P < 0.05$) the relative butyric and isoacids profiles, as well as the ammonia concentration in the proximal colon digesta.

Figure 8.2. Fermentation products on proximal colon.

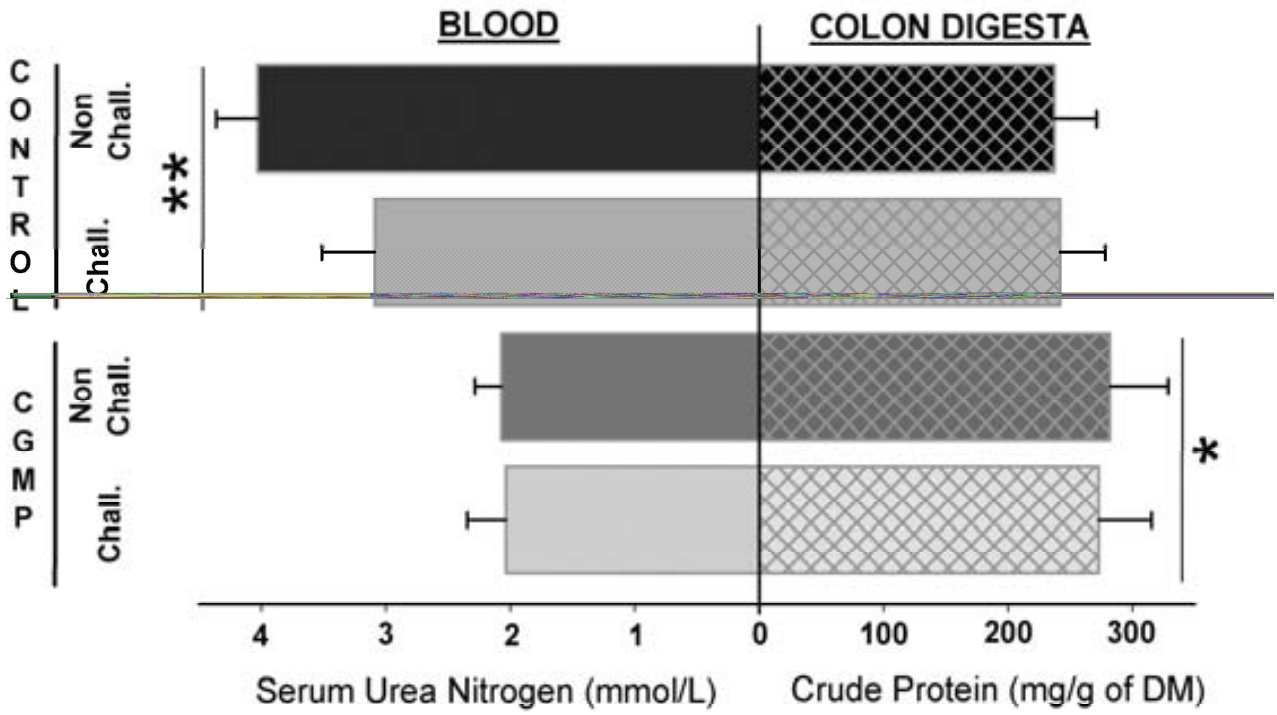


(A) Ammonia concentration (mmol/g of FM) of proximal colon digesta 8 days post challenge (B) Volatile fatty acid concentration (mmol/g of FM) and (C) Butyric and branched chain fatty acids relative amounts (%) of proximal colon digesta in the whole experimental period. * *Pdiet* < 0.05.

8.3.6. Monitoring the protein digestion (Trial 2)

Fig. 8.3 presents the serum urea nitrogen and the crude protein concentrations at day 4 post-challenge. The administration of CGMP diet decreased ($P < 0.01$) the serum urea concentration, simultaneously with an increase ($P < 0.05$) in the crude protein concentration of the proximal colon digesta. No differences were found 8 days post-challenge (data not shown).

Figure 8.3. Monitoring the protein digestion. Serum urea nitrogen (mmol/L) and crude protein concentration (mg/g of DM) of weaning piglets challenged or not with ETEC (K88).



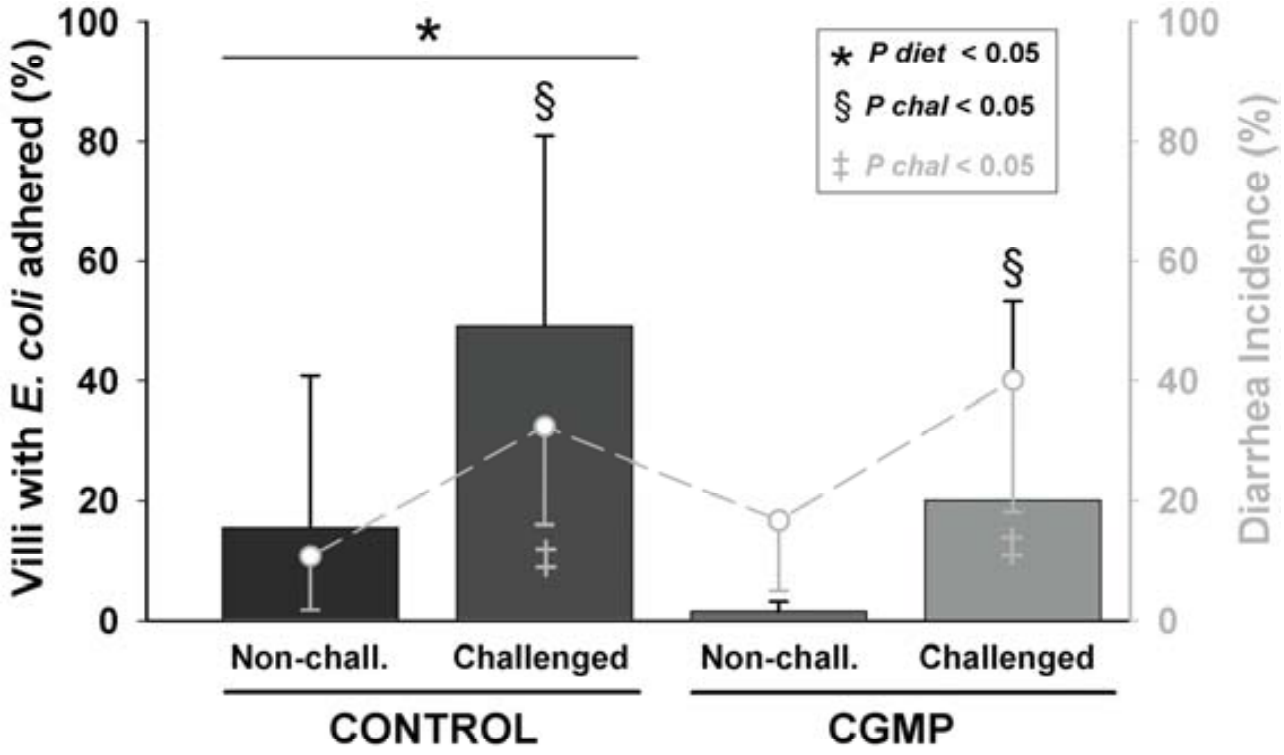
(Left Bars) Serum urea nitrogen concentration (mmol/L) and (Right Bars) Crude protein concentration (mg/g of DM) in the proximal colon digesta of animals 4 days post challenge. * *Pdiet* < 0.05 and ** *Pdiet* < 0.01.

8.3.7. Ileal adhesion (Trial 2).

In the Fig. 4 are presented the percentage of ileal villi with *E. coli* adhered (Bars) and the diarrhea incidence of animals (Lines) in both sampling days. Table 3 also shows the number of enterobacteria adhered in the ileum mucosa in the experimental period.

The CGMP diet reduced up to 6 log units the enterobacteria number of mucosa scrape contents ($P = 0.05$) and also the percentage of villi with *E. coli* adhered ($P < 0.05$), but did not reduce the diarrhoea incidence. In any case, the diarrhoea promoted by ETEC challenge ($P < 0.05$) was not severe enough for antibiotic intervention during the experimental period.

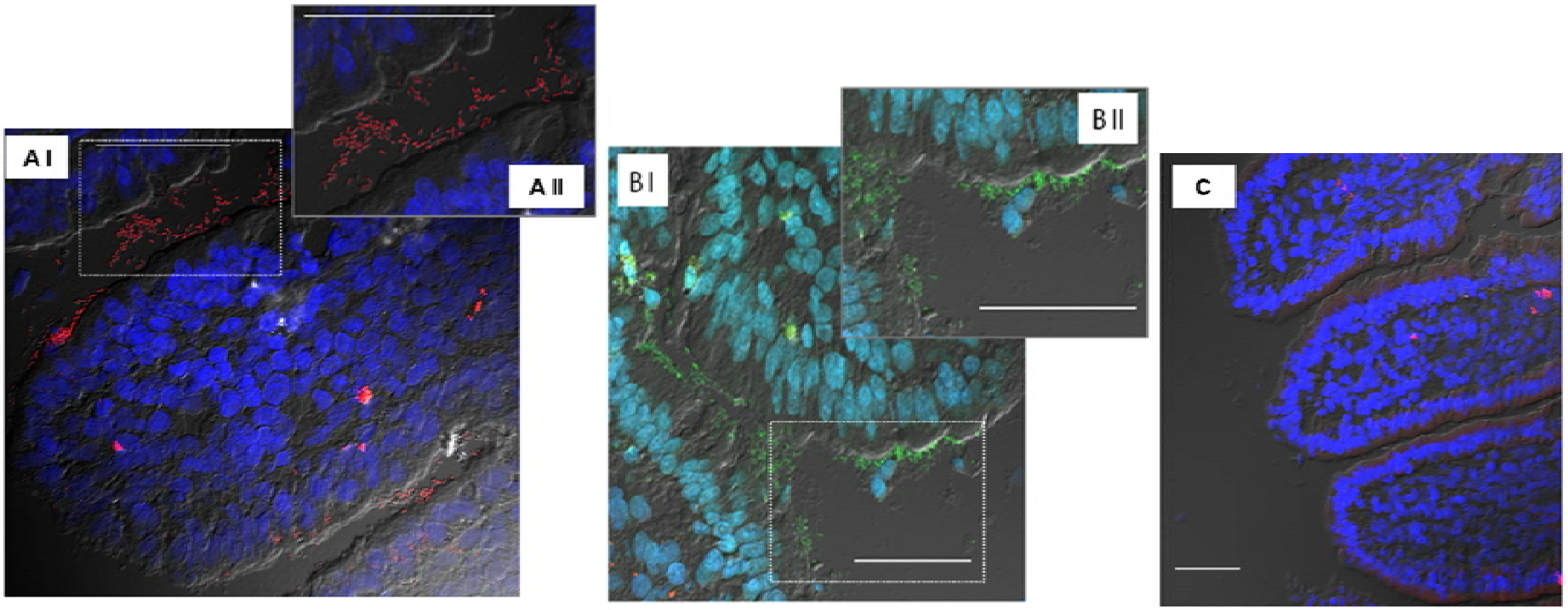
Figure 8.4. *In situ* villi adherence of *E. coli* and the diarrhea incidence.



(Bars) Percentage of ileal villi with *E. coli* adhered and (Lines) Percentage of animals that presented diarrhea in the experimental period.

To better illustrate the ileal *in situ* monitoring of *E. coli* adhesion, Fig. 8.5 shows a positive sample stained with CY3 *E. coli* specific probe (A panel), where *E. coli* bacteria adhered to the ileal mucosa, and also another positive sample stained with FITC universal bacteria specific probe (B panel) and a negative sample (C panel) without bacteria adhered.

Figure 8.5. *E. coli* adhesion to the ileum mucosa measured by FISH technique.



(A panel) positive, merge picture with DAPI staining (fluorescent blue, intestinal cells nucleus), CY3 staining (EC 1531 probe, fluorescent red, *E. coli* cells) and transmitted light (intestinal villi). (B panel): positive, merge picture with DAPI staining (fluorescent blue, intestinal cells nucleus), FITC staining (EUB 338 universal bacteria probe, fluorescent green, *E. coli* cells) and transmitted light (intestinal villi). (C panel): negative, merge picture with DAPI staining (fluorescent blue, intestinal cells nucleus) and transmitted light (intestinal villi). Olympus Laser Confocal Microscope (Fluoview FV1000, Olympus GmbH, Hamburg, Germany). Size bars 50 μm.

8.4. Discussion

8.4.1. CGMP as inhibitor of the E. coli attachment to the intestinal mucosa.

In the first *in vitro* assay we clearly observed the adhesion of ETEC *E. coli* K88 on the apical pole of ileum villus enterocytes of the post-weaning piglet. Anderson *et al*. (1980), described K88 fimbrial binding to purified brush border membranes of porcine small intestine and later it was shown that glycoproteins, sialoglycoproteins or glycosphingolipids were recognized by different K88 fimbrial variants. The fimbrial K88ac variant is the most prevalent and clinically important variant of ETEC and tends to colonize the jejunum and ileum in neonatal and weaned piglets (Fairbrother *et al*., 2005; Nagy and Fekete, 2005).

Because adhesion to host cells is the key first step in causing microbial infections, it should be possible to prevent them by blocking the adhesion sites. In humans, milk oligosaccharides have been reported to act as soluble receptors for bacterial adhesions (Fairbrother *et al*., 2005), by blocking their binding sites and preventing them from becoming attached to the mucosa cells. These oligosaccharides generally have a lactose moiety at the reducing end of the molecule and often contain a fucose and/or sialic acid moiety at the non-reducing end, which confers the biological activities of human milk in breastfeeding infants (Newburg *et al*., 2004; Brody, 2000). In the present study, the CGMP (4.2% of sialic acid) showed an effective blocking activity of the *E. coli* K88 attachment to the ileum mucosa. Other authors have also described the ability of CGMP (Strömqvist *et al*., 1995; Brück *et al*., 2003) and also several sialic acids ingredients (Korhonen *et al*., 1986; Schroten *et al*., 1993) to inhibit the adhesion of different pathogenic bacteria to the epithelial tissue *in vitro*. A striking example of the successful application of receptor analogues was obtained by Mouricout *et al*. (1990) who protected colostrum-deprived newborn calves from a lethal dose of enterotoxigenic *E. coli* K99 by oral administration of sialylated glycoproteins. In our work, the CGMP reduced the counts of enterobacteria in the ileum

digesta and mucosa scrapes, as well as the percentage of intestinal mucosa samples with *E. coli* attached to the villi.

8.4.2. Digestive effects of the CGMP in early weaned piglets.

The CGMP diets increased the crude protein, ammonia and isoacids concentrations in colonic digesta, suggesting a higher amount of undigested protein reaching the hindgut and being fermented by the bacteria, leading to production of isoacids and ammonia (Blachier *et al*., 2007).These results could reflect a lower digestion of the total dietary protein and/or specifically glycosylated proteins from the CGMP in the small intestine (Boutrou *et al*., 2008) or an increased endogenous protein secretion (Ledoux *et al*., 1999) into the intestinal lumen. The higher ammonia concentration in the hindgut is coherent with the lower serum urea concentration found in the piglets fed the CGMP.

To explain this lower plasmatic urea concentration, a higher transfer from plasma to colon as a source of nitrogen for bacteria growth could also be considered. However this hypothesis does not seem plausible bearing in mind the increase observed in isoacids and total protein concentration in the hindgut. It is difficult to be certain whether the higher concentration of protein originated from the undigested dietary protein, the CGMP or endogenous protein. Regarding the digestion of CGMP, it has been previously observed that glycoproteins are less digested along the gastrointestinal tract than their unglycosylated homologs, likely due to their large number of *O*-glycosidic linkages which confer a unique conformation that reduces the digestion by the endopeptidases (Boutrou *et al*., 2008). Moreover, its amino acid sequence, which is free from aromatic amino acids and other essential amino acids (arginine, cysteine, histidine, tryptophan and tyrosine) (Brody, 2000), could make difficult the activity of endogenous proteases that are preferably cleaved by these amino acids, such as trypsin, chymotrypsin and pepsin (Erickson and Kim, 1990). However, particularly regarding the proteolysis of bovine CGMP which, it has been established, can be almost completed by trypsin *in vitro* (Shammet etl al., 1992); in our laboratory

we confirmed it as nearly complete (98.6%) compared to 99.2% for sweet whey sources, following Boisen and Fernández (1997) *in vitro* protein digestion protocol. This data would also indicate a higher flux of dietary or endogenous protein to the hindgut; however, it is also possible that *in vitro* models may not account for the specific characteristics of CGMP digestion in very young animals.

In humans, it has been reported that bovine and human CGMP fractions occur in the plasma of 5 day-old newly-born infant and adult humans after ingestion of milk and yogurt (Chabance *et al*., 1998). The CGMP plasma concentrations were higher in 5-days-old infants than in adults, which suggests that the newborn gut is likely to be more permeable to peptides or that gastric CGMP digestion is limited in infants by low proteolytic enzyme concentrations and relatively high pH levels. A lower digestion of milk glycosylated proteins could also be an evolutionary adaptation for preservation of active functional molecules in newly born mammals. However, CGMP could also have had an effect on digestion physiology and indirectly on dietary protein digestion or in the secretion of endogenous protein. Casein-glycomacropeptide is known to inhibit gastric acid secretion, gastrin secretion and gastrointestinal motility in dogs, suggesting that proteolysis could be depressed by the presence of CGMP in the stomach (Stan *et al*., 1983). In summary, from our results we cannot confirm which exactly were the effects of CGMP in increasing the concentration and fermentation of protein in the hindgut, however it seems plausible that CGMP could have an effect on the physiology of the digestion of proteins along the gastrointestinal tract.

8.4.3. The effect of CGMP in the intestinal health and microbiota.

The challenge with ETEC *E. coli* promoted a mild diarrhea during the first day after infection. However, the animals recovered quickly without antibiotic treatment. Most of the parameters were significantly affected on day 4 but not day 8 days after ETEC challenge. We observed an increase on the TNF-α and the Pig-MAP acute protein, which indicates an acute

immune response to the pathogen (Piñeiro *et al*., 2009), but this response was not modified by the experimental diet. Furthermore, the challenge caused an increase of crypt depth, a reduction of the ratio villus height:crypt depth, and an increase in the number of intraepithelial lymphocytes, which were not modified by the administered diet.

Further, the experimental ETEC challenge reduced the lactobacilli and increased enterobacteria in the ileum and colon digesta of the animals. The response to enterobacteria was, however, dependent on the diet, as *E. coli* challenge increased enterobacteria in animals fed the Control diet, but not in piglets fed the CGMP treatment. On the other hand, CGMP stimulated the lactobacilli population and decreased the number of enterobacteria in the ileum and colon digesta. Some authors have also reported a growth-promoting effect of CGMP on both *Bifidobacteria* (Naughton *et al*., 2001; Janer *et al*., 2004) and *Lactococcus* species (Bouhallab *et al*., 1993) *in vitro.* The decrease observed in enterobacteria could be explained by a competitive effect of the lactobacilli population on enterobacteria but also by a direct effect of CGMP on their growth. This could be due to a reduction on gut colonization by blocking the receptor analog to the intestinal mucosa, as it has been demonstrated *in vitro* in this and other studies (Newburg *et al*., 1997), or by direct inhibition of the growth of the bacteria as described *in vitro* for *E. coli* by Malkoski *et al*. (2001).

Several investigators studied the potential ability of CGMP to exert a prebiotic effect on the intestinal microbiota. Bruck *et al*. (2006) suggested its inclusion in infant formulae to simulate the beneficial bacteriological effect of breast milk and Nakajima *et al*. (2005) its potential to prevent intestinal infection caused by *Salmonella enteritidis* and enterohemorrhagic *E. coli*.

8.5. Conclusion

In conclusion, our results suggest that the inclusion of 1.5% of CGMP in piglet diets result in a higher amount of protein reaching and fermenting in the large intestine of piglets, likely as a consequence of a lower digestion of the total and/or glycosilated proteins of the diet in the small intestine or by a higher endogenous nitrogenous excretion. Present results also confirm the inhibitory effect of CGMP on *E. coli* attachment to the intestinal mucosa *in vitro* and *in vivo*, and the ability of CGMP to reduce the overgrowth of enterobacteria in the digestive tract of piglets after an ETEC oral challenge. The ability of CGMP to nourish a healthy gut microbiota make it a potential prebiotic ingredient for use in infant formulas, or functional foods that could prevent intestinal infection caused by *Escherichia coli* or potentially other enteropathogens.

8.6. Literature Cited

Anderson MJ, Whitehead JS, Kim YS (1980) Interaction of *Escherichia coli* K88 antigen with porcine intestinal brush border membranes. *Infect Immun* **29**, 897-901.

Blachier F, Mariotti F, Huneau JF, *et al*. (2007) Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amino Acids* **33**, 547-562.

Blanco M, Blanco J, Gonzalez E, *et al*. (1997) Genes coding for enterotoxins and verotoxins in porcine *Escherichia coli* strains belonging to different O:K:H serotypes: Relationship with toxic phenotypes. *J Clin Microbiol* **35**, 2958-2963.

Boisen S, Fernández JA (1997) Prediction of the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. *Anim Feed Sci Technol* **68**, 277-286.

Bouhallab S, Favrot C, Maubois JL (1993) Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactococcus lactis* subsp lactis. *Lait* **73**, 73-77.

Boutrou R, Jardin J, Blais A, *et al*. (2008) Glycosylations of κ-casein-derived caseino-macropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J Agric Food Chem*. **56**, 8166-8173.

Brody EP (2000) Biological activities of bovine glycomacropeptide. *Br J Nutr* **84**, 39-46.

Brück WM, Graverholt G, Gibson GR (2003) A two-stage continuous culture system to study the effect of supplemental α-lactalbumin and glycomacropeptide on mixed cultures of human gut bacteria challenged with enteropathogenic *Escherichia coli* and *Salmonella* serotype typhimurium. *J Appl Microbiol* **95**, 44-53.

Brück WM, Redgrave M, Tuohy KM, *et al*. (2006) Effects of bovine alfa-lactalbumin and casein glycomacropeptide-eniched infant formulae on faecal microbiota in healthy term infants. *J Pediatr Gastr Nutr* **43**, 673-679.

BSAS, British Society of Animal Science (2003) Nutrient requirement standards for pigs. Penicuik, Midlothian, UK: British Society of Animal Science.

Castillo M, Martín-Orúe SM, Manzanilla EG, *et al*. (2006) Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet Microbiol* **114**, 165-170.

Chabance B, Marteau P, Rambaud JC, *et al*. (1998) Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **80**, 155-165.

Daddaoua A, Puerta V, Zarzuelo A, *et al*. (2005) Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J Nutr* **135**, 1164-1170.

Diebold G, Mosenthin R, Piepho H, *et al*. (2004) Effect of supplementation of xylanase and phospholipase to a wheat-based diet for weanling pigs on nutrient digestibility and concentrations of microbial metabolites in ileal digesta and feces. *J Anim Sci* **82**, 2647-2656.

Edelman S, Leskelä S, Ron E, *et al*. (2003) *In vitro* adhesion of an avian pathogenic *Escherichia coli* O78 strain to surfaces of the chicken intestinal tract and to ileal mucus. *Vet Microbiol* **91**, 41-56.

Erickson RH and Kim YS (1990) Digestion and absorption of dietary protein. *Annu Rev Med* **41**, 133-139.

Fairbrother JM, Nadeau É, Gyles CL (2005) *Escherichia coli* in postweaning diarrhea in pigs: An update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* **6**, 17-39.

Fosset S, Fromentin G, Gietzen DW, *et al*. (2002) Peptide fragments released from phe-caseinomacropeptide *in vivo* in the rat. *Peptides* **23**, 1773-1781.

Grange PA, Mouricout MA, Levery SB, *et al*. (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**, 2336-2343.

Idota T, Kawakami H, Nakajima I (1994) Growth-promoting effects of N-acetylneuraminic acid-containing substances on bifidobacteria. *Biosci Biotechnol Biochem* **58**, 1720-1722.

Janer C, Peláez C, Requena T (2004) Caseinomacropeptide and whey protein concentrate enhance B*ifidobacterium lactis* growth in milk. *Food Chem* **86**, 263-267.

Jensen BB, Jorgensen H (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl Environ Microbiol* **60**, 1897-1904.

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

Kawasaki Y, Isoda H, Tanimoto M, *et al*. (1992) Inhibition by lactoferrin and k-casein glycomacropeptide of binding of cholera toxin to its receptor. *Biosci Biotechnol Biochem* **56**, 195-198.

Korhonen TK, Parkkinen J, Hacker J, *et al*. (1986) Binding of *Escherichia coli* S fimbriae to human kidney epithelium. *Infect Immun* **54**, 322-327.

Krissansen GW (2007) Emerging health properties of whey proteins and their clinical implications. *J Am Coll Nutr* **26**, 713-723.

LaClair CE, Ney DM, MacLeod EL, *et al*. (2009) Purification and use of glycomacropeptide for nutritional management of phenylketonuria. *J Food Sci* **74**, 199-206.

Ledoux N, Mahé S, Dubarry M, *et al*. (1999) Intraluminal immunoreactive caseinomacropeptide after milk protein ingestion in humans. *Nahrung* **43**, 196-200

Li EWY, Mine Y (2004) Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. *J Agric Food Chem* **52**, 2704-2708.

Lonnerdal B (2003) Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr* **77**, 1537-1543.

Malkoski M, Dashper SG, O'Brien-Simpson NM, *et al*. (2001) Kappacin, a novel antibacterial peptide from bovine milk. *Antimicrob Agents Chemother* **45**, 2309-2315.

Mouricout M, Petit JM, Carias JR, *et al*. (1990) Glycoprotein glycans that inhibit adhesion of *Escherichia coli* mediated by K99 fimbriae: Treatment of experimental colibacillosis. *Infect Immun* **58**, 98-106.

Nagy B and Fekete PZ (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microbiol* **295**, 443-454.

Nakajima K, Tamura N, Kobayashi-Hattori K, *et al*. (2005) Prevention of intestinal infection by glycomacropeptide. *Biosci Biotechnol Biochem* **69**, 2294-2301.

Nakano T, Ikawa N, Ozimek L (2007) Detection of sialylated phosphorylated kappa-casein glycomacropeptide electrophoresed on polyacrylamide gels and cellulose acetate strips by the thiobarbituric acid and malachite green dye reactions. *J Agric Food Chem* **55**, 2714-2726.

Naughton PJ, Mikkelsen LL, Jensen BB (2001) Effects of nondigestible oligosaccharides on salmonella enterica serovar typhimurium and nonpathogenic *Escherichia coli* in the pig small intestine *in vitro*. *Appl Environ Microbiol* **67**, 3391-3395.

Newburg DS (1997) Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J Nutr* **127**, 980-984.

Newburg DS, Ruiz-Palacios GM, Altaye M, *et al*. (2004) Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology* **14**, 253-263.

Nofrarias M, Manzanilla EG, Pujols J, *et al*. (2006) Effects of spray-dried porcine plasma and plant extracts on intestinal morphology and on leukocyte cell subsets of weaned pigs. *J Anim Sci* **84**, 2735-2742.

Nowicki B, Holthöfer H, Saraneva T, Rhen M, *et al*. (1986) Location of adhesion sites for P-fimbriated and for 075X-positive *Escherichia coli* in the human kidney. *Microb Pathog*. **1**, 169-180.

Official Methods of Analysis (1995) Association of Analytical Communities.

Piñeiro C, Piñeiro M, Morales J, *et al*. (2009) Pig-MAP and haptoglobin concentration reference values in swine from commercial farms. *Vet J* **179**, 78-84.

Rhoades JR, Gibson GR, Formentin K, *et al*. (2005) Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. *J Dairy Sci* **88**, 3455-3459.

SAS (1999) User`s guide. 8th ed. Cary, NC, USA. SAS Inc.

Shammet KM, Brown RJ, McMahon DJ (1992) Proteolytic activity of proteinases on macropeptide isolated from kappa-casein. *J Dairy Sci* **75**, 1380-1388.

Schroten H, Plogmann R, Hanisch F, *et al*. (1993) Inhibition of adhesion of S-fimbriated *Escherichia coli* to buccal epithelial cells by human skim milk is predominantly mediated by mucins and depends on the period of lactation. *Acta Paediatrica* **82**, 6-11.

Schwertmann A, Shroten H, Hacker H, Kunz C (1999) S-fimbriae from *Escherichia coli* bind to soluble glycoproteins from human milk. *J Pediat Gastroenterol Nutr* **28**, 257-63.

Setia A, Bhandari SK, House JD, *et al*. (2009) Development and *in vitro* evaluation of an *Escherichia coli* probiotic able to inhibit the growth of pathogenic *Escherichia coli* K88. *J Anim Sci* **87**, 2005-2012.

Stan EY, Groisman SD, Krasilshchikov KB, *et al*. (1983) Effect of κ-casein on glycomacropeptide on gastrointestinal motility in dogs. *Bull Exp Biol Med* **96**, 10-2.

Strömqvist M, Falk P, Lennart Hansson SB, *et al*. (1995) Human milk κ-casein and inhibition of *Helicobacter pylori* adhesion to human gastric mucosa. *J Pediatr Gastroenterol Nutr* **21**, 288-296.

Swaisgood HE (1992) Chemistry of the caseins. In: Fox PF, editor. London, England: Elsevier Science Publishers Ltd.

Swidsinski A, Weber J, Loening-Baucke V, *et al*. (2005) Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease. *J Clin Microbiol* **43**, 3380-3389.

Thomä-Worringer C, Sørensen J, López-Fandiño R (2006) Health effects and technological features of caseinomacropeptide. *Int Dairy J* **16**, 1324-1333.

Van den Broeck W, Cox E, Oudega B, *et al*. (2000) The F4 fimbrial antigen of *Escherichia coli* and its receptors. *Vet Microbiol* **71**, 223-44.

Virkola R, Parkkinen J, Hacker J, *et al*. (1993) Sialyloligosaccharide chains of laminin as an extracellular matrix target for S fimbriae of *Escherichia coli*. *Infect Immun* **61**, 4480-4484.

Wong PYY, Nakamura S, Kitts DD (2006) Functional and biological activities of casein glycomacropeptide as influenced by lipophilization with medium and long chain fatty acid. *Food Chem* **97**, 310-317.

Chapter 9

General discussion.

Nowadays, the animal production industry is facing the challenge of getting optimal animal performance, with less health complications and minimal environment impact. Nutritionists are in charge of selecting feed ingredients, which it will constitute the main input for the meat chain industry.

However, their tasks are not easy. If in one hand, nutritionists tend to include in the diet chemical ingredients and additives to maximize the animal production; on the other hand, consumers are concerned about the use of chemical substances that may leave residues in the meat, milk and egg. The pressure caused by the consumers, and new enforced laws had prompted the search for feeding strategies that present no risk of residue transfer to the final products and decrease the microbial resistance to therapeutic antibiotics. Thus, a huge amount of scientific information has been generated in the last decade to address for more “natural” alternatives to the chemical compounds. Probably, the main topic working area has been the weaning period of piglets because it is the most critical phase in swine production due to enteric diseases.

Regarding the nutritional approaches, different authors have worked with the requirements of the animals at each development phase and with the characteristics of major ingredients, as well as with the likely role of feed additives for improving performance and health of the animals. Recently, Lange *et al*. (2010); proposed a division of this large number of feed additives according to its mechanisms of action: (1) enhancing the pig's immune response (e.g. immunoglobulin; ω-3 fatty acids, yeast derived ß-glucans), (2) reducing pathogen load in the pig's gut (e.g. organic and inorganic acids, high levels of zinc oxide, essential oils, herbs and spices, some types of prebiotics, bacteriophages, and anti-microbial peptides), (3) stimulate establishment of beneficial gut microbes (e.g. probiotics and some types of prebiotics), and (4) stimulate digestive function (e.g. butyric acid, gluconic acid, lactic acid, glutamine, threonine, cysteine, and nucleotides). The list is very large. Then, a big challenge remains to search for the best combination of feed ingredients and nutritional approaches to obtain the most effective alternative for in-feed antibiotics.

In general, the main focus of these alternative feed additives is to improve the intestinal health. Therefore, they usually present a direct or indirect related impact on the intestinal microbiota. If this is taken into account, we could state that there is basically two ways of action, which likely interact each other. They are:

1) The growth inhibition of potentially pathogenic microbiota, through natural antimicrobial feed additives (organic acids and essential oils) or by the promising research of anti-adhesive feed substrates that may specifically interact with pathogens and reduce its colonization in the gastrointestinal tract; and/or:

2) The growth stimulation of potentially beneficial microbiota, either added in the diet (probiotics) or by the stimulation of its growth by prebiotics (special non digestible oligosaccharides or dietary fiber).

This thesis has centered the attention in the first chapters on fibrous ingredients, and in the following chapters on special feed additives that can have an impact on both cited mechanisms of action. The elucidation of its effects is further discussed.

9.1. The role of the fibrous ingredients in the promotion of gut's health.

There is strong scientific information that supports the beneficial impact of the dietary fiber on the gastrointestinal function and health. However, many of these studies also reported the negative impact of DF supplementation on food intake. This effect could be particularly detrimental in the diet for young pigs when the feed intake must be stimulated. That is the reason why DF supplemented diets is usually offered for pregnant sows to control hunger, stereotype behavior and weight status (Danielsen and Vestergaard 2001) and rarely for young animals, where high digestible ingredients are preferred (Tokach *et al*., 2003). However, due to the beneficial effects on the gut's physiology, some research groups have focused their investigations in the last decade on the use of moderate amounts of DF in the early weaned piglets (Pluske, 2006), evaluating its effects on the digestive parameters, growth and on animal`s health.

The first two trials of this thesis aimed to evaluate the inclusion of DF in the diet of piglets as a way to produce beneficial changes on the intestinal physiology of the young animals. The hypothesis was that fiber could generate a positive impact on the gut microbiota and fermentation, and reduce the detrimental protein fermentation. It was also important to check the lack of interference in the feed intake. From the results of a series of experiments presented at the doctoral thesis of Molist (2010), we decided to work on two sources of fibrous ingredients and its optimal supplementation level (a more soluble and fermentable fiber source, sugar beet pulp at 2%; and an insoluble fiber source, wheat bran at 4%). These two sources were used in combination in trials 1 and 2 in order to better elucidate their effect on animal performance and health.

Trial 1 was designed with the objective of evaluating the interaction between dietary protein and fiber (two levels of DF supplementation and two levels of protein). In this study, we used a large number of techniques to fully characterize the effects of these factors on gut function, health and animal performance. We hypothesized that the fiber supplemented diet could mitigate the protein fermentation in high CP diets. Others researchers (Awati *et al*., 2006; Bikker *et al*., 2006; Piva *et al*., 2006; Kim *et al*., 2008) have studied the inclusion of fibrous ingredients in the diet of young pigs and reported the reduction in the production of toxic metabolites caused by the protein fermentation. Protein fermentation can generate detrimental substances for the host animal, such as amines and ammonia (Nollet *et al*., 1999; Kim *et al*., 2008), predisposing animals for the occurrence of digestive disorders (Biker el al., 2006). Results of Trial 1 are in great accordance with those presented by these researchers and reinforce the role of DF on the reduction of protein fermentation with the inclusion of dietary fiber. The proposed mechanisms by which DF present this effect might be the change on the microbial fermentation along the GIT from protein to carbohydrate (Awati *et al*., 2006).

Regarding the negative impact of DF on animal performance (Freire *et al*., 1998; Wellock *et al*., 2008), it seems to be strongly dependent on the level of fiber included in the diet. In Trial 1, the moderate DF supplementation did not reduce feed intake whereas increased the growth of the

animals. These results, like other studies, could confirm the role of fiber on the maintenance of gut function, as a previous step to an optimal performance (Mateos *et al*., 2006). We also observed a reduction in the potentially pathogenic population of coliforms and enterobacteria that leaded to an increase on the lactobacilli:enterobacteria ratio in the intestinal microbiota, as it was also reported before by others (Konstantinov *et al*., 2004; Bikker *et al*., 2006). An increase in lactobacilli, with a subsequent decrease in the coliforms in the GIT, could be described as a process of colonization resistance.

Trial II incorporated in the diets the same combination of DF in two different cereal based diets. Rice or barley were chosen as main cereals, considering their differences on palatability and fiber content (Solà-Oriol *et al*., 2009). We observed that a barley-based diet, combined with a higher content of fiber, reduced the feed intake and consequently the growth of the animals. However, the inclusion of a moderate amount of fiber in the rice-based diet reduced the production of potentially toxic metabolites (e.g. amines, isoacids and ammonia) from protein fermentation. These results and others (Mateos *et al*., 2006) could suggest that an optimal range of fiber may be necessary in the piglet feeding to improve the intestinal adaptation, reduce intestinal disorders and environmental impact. Several reports have shown better performance indices in pigs fed rice based diets compared to other cereals, such as maize (Mateos *et al*., 2006) or barley (Pluske *et al*., 2002). This has been attributed to a higher digestibility (Mateos *et al*., 2006) and palatability (Solà-Oriol *et al*., 2009) of the rice-based diets. However, the supplementation of insoluble dietary fiber may decrease postweaning diarrhea in dietary situations where there may be a misbalance of carbohydrate to protein entering the hindgut (Kim *et al*., 2008).

In order to elucidate the whole impact of DF on the intestinal microbiota and function we decided to focus our research on the likely role of fibrous ingredients, and others feedstuffs from natural sources, on the GIT parameters and its influence on intestinal infections, using colibacilosis challenge models.

An intriguing observation was the consistent results obtained with the wheat bran supplementation throughout the different experiments, as summarized in Table 9.1. From these results it was observed that WB reduced coliforms and enterobacteria in the gastrointestinal tract and faeces of early weaned piglets. These results were also associated in some experiments with a significant change on the water retention capacity of digesta in the colon and with an increase on fermentation, both likely having and effect on the coliforms growth (Williams *et al*., 2001). These observations intrigued our curiosity to assess the affinity that this DF can possibly have for the *E coli* strains of bacteria and how likely binding mechanisms could explain the mode of action of this fibrous ingredient.

Table 9.1. Summarized results of the *in vivo* effects on intestinal microbiota by the use of dietary fiber (DF) in the diet of young pigs

Type of DF	Inclusion level	Results	Reference
Wheat bran (WB) + Sugar beet pulp (SBP)	WB at 4% + SBP at 3%	It reduced the enterobacteria number in caecum digesta.	Molist *et al*., 2009
Wheat bran (WB) + Sugar beet pulp (SBP)	WB at 4% + SBP at 2%	It reduced coliforms and tended to reduce the enterobacteria population in feces.	Hermes *et al*., 2009
Wheat bran	4%	It reduced the *E. coli* population in ileum digesta.	Molist *et al*., 2010a
Wheat bran	4%	It tended to reduce enterobacteria population in feces.	Molist *et al*., 2010b
Wheat bran (WB) + Sugar beet pulp (SBP)	WB at 4% + SBP at 2%	It mostly reduced the coliform population in feces.	Hermes *et al*., 2010a
Wheat bran	4%	It mostly reduced the coliform and *E. coli* populations in feces.	Molist *et al*., 2011

In the following experiments we used an ETEC strain (highly pathogenic for young pigs), to assess *in vitro* and *in vivo* the role of different feed ingredients on the infective process. The strain used was an F4 fimbriated *E. coli* that attacks young animals by the recognition of specific intestinal receptors in the enterocytes and by a firmly attachment on the membrane of the host cells (Fairbrother *et al*., 2005). The F4 intestinal receptor is composed by *N*- and *O*-glycans containing galactose, glucose, sialic acid, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine and fucose (Grange *et al*., 1998). ETEC may recognize this adhesion-ligand interaction, likely through β-linked galactose (Grange *et al*., 2002).

In the light of this knowledge we speculated that if it would be possible to find feedstuffs mimicking the specific binding sites of the epithelium by means of certain molecules, mostly glycoproteins and/or oligosaccharides. In this way it would be possible to selectively prevent the adhesion of ETEC to the intestinal mucosa. Several investigators (Grange *et al*., 2002; Marquardt *et al*., 1999; Sarabia-Sainz *et al*., 2009) have attempted to isolate and identify immunoglobulins with the ability to interact with the ETEC K88 receptors, but only a few have focused on the possible effect of feedstuffs (Becker and Galletti, 2008). Moreover, recent studies suggest that one of the modes of action of prebiotics could include the ability to act as a "decoy" for pathogen binding cellular receptors in the gut (Shoaf-Sweeney and Hutkins, 2009) as candidates to be used in therapies to prevent intestinal pathologies (Lane *et al.*, 2010).

9.2. Anti-adhesive feedstuffs as a feeding strategy to prevent pathogenic bacteria.

Most pathogenic bacteria begin the infective process by the identification of receptors in the membrane of the target host cell, the attachment and its proliferation (Shoaf-Sweeney and Hutkins, 2009). However these bacteria must overcome the immune defenses, compete with other microorganisms of the intestinal microbiota and find nutritional substrates for its growth. For that reason, some groups of bacteria have developed a high complex identification system of molecules which allow its proliferation and survival in the host organism (Shoaf-Sweeney and

Hutkins, 2009). It is composed by membrane receptors called adhesins and can make possible the identification of specific affinity points, composed by specific combinations of high complex sugars and proteins by the glycosylation process (Marth and Grewal, 2008). On the other hand, the glycosylation process explain the difference among the so-called lectins, a class of sugar-specific and cell-agglutinating proteins that can be found in all living beings and are of great importance of the cells to communicate with the environment, ensuring its survival (Sharon, 2009)

Some researchers have focused their work on the identification of these adhesins and lectins aiming for isolation of molecules able to interfere and avoid the attachment of pathogens to the host and preventing the infective process. It was recently called as the anti-adhesive therapy (Lane *et al*., 2010) and seems to be a promise field of study, mainly because these lectins are presumably found in natural products such as animals, microbial or plant tissues (Sharon, 2009) and may be considered in the development of alternative feed additives to antibiotic growth promoters.

But bacterial adhesion is highly complex and seems to be a multifactorial event which it is probable that a cocktail of oligosaccharides and glycoproteins with anti-adhesive properties may be the best solution (Ghazarian *et al.*, 2011). However, cost-effective technologies capable of isolating and enriching dietary anti-adhesive agents are still under investigation, as well as improved methods for the delivery of these products to their site of action (Lane *et al*., 2010).

In order to investigate in this intriguing field of study, we performed in trial III a screening of different products, carefully selected by its likely anti-adhesive properties for the GIT pathogenic bacteria. In this experiment we used a miniaturized model to evaluate the adhesion of fimbriated bacteria to the feedstuffs substrates tested. The obtained results allowed us to identify the affinity of the ETEC bacteria to certain substrates such as the wheat bran (WB), the exopolysaccharides from lactobacilli (EPS) and the bovine casein glycomacropeptide (CGMP).

The results found with the WB were coherent and could explain, at least partially, the previously results obtained by Molist (2010) with challenged and non challenged piglets. The

results obtained evidenced the existence of some soluble compounds in the wheat bran to adhere specifically to ETEC K88. Besides we did not identify exactly which compound is directly implicated, the protease treatment employed in this trial seems to inactivate its moiety responsible for that specificity. Contrarily, other investigators reported the anti-adhesive capacity of arabinoxylans (highly presented in the WB fractioning extracts), also working with an *in vitro* adhesion model to study the mucin-adhered bacterial community (Abbeele *et al*., 2009), but on the other hand, a wheat glycoprotein, supposedly inactivated by a protease treatment, called wheat germ agglutinin (WGA), present a special ability to bind enterocytes of mice (Walter *et al*., 2004), pigs (Choi *et al*., 2003), and human enterocytes (Wirth *et al*., 1998). The high specificity of WGA for enterocytes it has being useful for lectin-mediated drug delivery development (Güll *et al*., 2007), where interestingly, it was considered as an adjuvant for oral vaccination against ETEC K88 (Vandamme *et al*., 2011). This knowledge could be useful indication for future applications as a nutritional strategy to reduce potentially pathogenic bacteria in young animals and it can at least in part explain the results found in our *in vitro* adhesion trial. Considering the finding results, more research is needed to isolate the moiety implicated on this attachment.

Regarding the potential of EPS to interfere *E. coli* adhesion, Wang *et al*. (2010), recently reported the *in vitro* capability of the EPS produced by strains of *Lactobacillus reuteri* to inhibit the ETEC-induced hemagglutination of porcine erythrocytes. The EPS is currently being evaluated for commercially production, since its availability is limited and highly complex (Champagne *et al*., 2007). The EPS used in this trial was obtained from the biofilm formed on the olive manufacturing, which represents a promising field of study for the Spanish agricultural industry.

Contrary to the EPS, the CGMP is a milk derivate product commercially available. Its use as an anti-adhesive agent was tested also in trial V, in which an *in vivo* experiment was performed challenging the animals with a pathogenic *E. coli* strain. Other studies have shown that CGMP inhibits the adhesion of pathogenic *E. coli* (Newburg, 1997; Nakajima *et al*., 2005; Rhoades *et al*.,

2005) to the mucosal surface or its growth *in vitro* (Malkoski *et al*., 2001), likely due to its high syalylated content (Martín-Sosa *et al*., 2002).

However, a limitation of the *in vitro* model applied in trial III remains in the capability of the high-binding 96-well plate to be coated at the same extent by the different studied products. Thus, only when the result is positive we can confirm a clear binding of the substrate to the bacteria. When the result is negative, or not attachment is observed to the bacteria, we can not rule out that the product could not be attached to the wall's well itself. Thus, it is complicated to establish numerical comparisons among products. Finally it is important to differentiate the ability of feed ingredients to bind bacteria and the capability to block the adhesion to intestinal mucus.

For that reason we decided to choose 5 feedstuffs to study their blocking ability using a more complex *in vitro* model composed by a monolayer culture of intestinal epithelial cells (IPEC-J2), isolated from jejunum of neonatal pigs challenged with a F4-fimbriated ETEC. This cell line has been extensively characterized and used as a reliable model for the *in vitro* study of swine intestinal diseases (Schierack *et al*., 2006; Koh *et al*., 2008), it likely express the F4 intestinal receptors (Geens and Niewold, 2010), present a specially high adherence capability to ETEC F4 fimbriated strains (Koh *et al*., 2008) and it has been successfully used for the study of a *Lactobacillus sobrius* protection effects against an ETEC K88 infection (Roselli *et al*., 2007).

The criteria to select the 5 feedstuffs that composed trial IV was the cost and commercial availability, scientific evidence of its mode of action and effectiveness; and its physical-chemical characteristics. Thus, WB and LB were chosen mainly because they are raw materials with low cost and higher local availability; MOS and CGMP due to its strong scientific evidence of mode of action and AO due to its commercial availability and oligosaccharide composition.

9.3. The molecular basis of the adherence process, the immune response and the interference of dietary components.

The applications of *in vitro* techniques which simulate the gastrointestinal environment are a useful scientific tool to design experiments and to test a large number of treatments. With the use of this cell line culture, we designed a series of *in vitro* trials that compound trial IV to evaluate in one hand the interference of some selected feedstuffs in the adhesion process and on the other hand to measure the inflammatory response of the cell line to the challenge of pathogenic bacteria. In this experiment it was identified that all feedstuffs studied were able to reduce (in different grades) the adhesion of pathogenic bacteria to the cell culture. Other researchers also reported the inhibition effect on the adhesion of pathogenic bacteria for WB (Molist *et al*., 2011); CGMP (Nakajima *et al*., 2005); MOS (Becker and Galletti, 2008); and LB (Zinger-Yosovich and Gilboa-Garber, 2009). For AO, to our knowledge, this is the first experiment that evaluates the capability of this product as an anti-adhesive agent.

Regarding the inflammatory response, some ingredients such as WB and CGMP were able to reduce the gene expression of some inflammatory cytokines and chemokines, likely due to the inhibition on the adhesion of the ETEC studied. However the AO caused by itself stimulation on that response, which it is possible due to galactomannans also presented in the membrane of some pathogenic strains of *Aspergillus* (Verweij *et al*., 1998; Levitz, 2004).

In the trial IV, the methodology employed made possible to demonstrate the ability of some feed ingredients to interfere in the pathogenic process of the ETEC K88, particularly reducing the adhesion of the pathogen to the epithelium. From this study we decided to confirm some of these results on an *in vivo* challenge study. We chose the CGMP for further studies (trial V) where it was used early weaned piglets, challenged by the ETEC K88, as a likely strategy to reduce diarrhea.

9.4 The anti-adhesive therapy to enterotoxigenic E. coli using the piglet as a model

The ETEC K88 is one of the most dangerous pathogen microbes to piglets (Fairbrother *et al*., 2005). Some evidences suggest that the mechanisms that could be involved on the CGMP-ETEC interaction is the highly *O*-linked glycosylation of CGMP (Boutrou *et al*., 2008), conferring a combination of sugars and glycans, such as *N*-acetylgalactosamine, galactose and N-acetylneuraminic (sialic) acids (Fernando and Woonton., 2010). This composition could be critical for recognition by carbohydrate-binding molecules (Kooyk and Rabinovich, 2008) and presents a similar glycan composition as the F4 intestinal receptor (Grange *et al*., 1998 and 2002).

In trial V it was employed fluorescent *in situ* hybridization techniques to evidence the inhibition of the ETEC K88 adhesion on the ileum mucosa in animals pre-treated with diets containing 1.5% of CGMP and challenged by the ETEC K88. This effect was confirmed by a reduction on the percentage of ileal villi with *E. coli* adhered to its mucosa. Moreover, the CGMP inclusion increased the lactobacilli numbers in ileum and colon digesta and reduced enterobacteria in the ileal digesta of challenged pigs.

However to be effectively active on the intestinal tract, the CGMP might reach distal parts of the gut intact; passing through the digestive process with no changes on its conformation and composition. Other researchers reported that this milk glycoprotein is less digested along the gastrointestinal tract than their unglycosylated homologs. This is likely due to their large number of *O*-glycosidic linkages which confer a unique conformation that reduces the digestion by the endopeptidases (Boutrou *et al*., 2008). Moreover, its amino acid sequence, which is free from aromatic amino acids and other essential amino acids (arginine, cysteine, histidine, tryptophan and tyrosine) (Brody, 2000), could make difficult the activity of endogenous proteases such as trypsin, chymotrypsin and pepsin, that preferably cleave protein by these amino acids, (Erickson and Kim, 1990). This special chemical conformation could explain the lower plasmatic urea concentration and the higher concentrations of nitrogen and isoacids on the proximal colon digesta observed in the animals from the CGMP treatment compared to the control group.

In this trial we observed the potential of a natural source glycoprotein in the inhibition effect of CGMP on the *E. coli* attachment to the intestinal mucosa *in vivo*, and the ability of CGMP to reduce the overgrowth of enterobacteria in the digestive tract of piglets after an ETEC K88 oral challenge.

The use of natural feeding strategies aiming for the reduction of intestinal disorders and facilitating the adaptation of young animals to weaning is an open field of study with many possibilities. Some feed ingredients can promote health and productivity, but other management strategies must accompany the dietary interventions, such as a careful animal management, improved environmental conditions of the farms and an appropriate preventive program to control the occurrence of diseases.

In this thesis we have pointed out the importance of minimal requirements of dietary fiber in the young animals in order to assure their gut function and growth. Moreover, the use of a combined approach of *in vitro* and *in vivo* studies may help us to improve our knowledge about the complex number of interactions between the diet, the intestinal microbiota and the immune response in health-challenging situations.

The recently proposed anti-adhesive therapy open new ways to likely design diets with less impact on intestinal inflammatory response, a higher resistance to intestinal pathogens, and a lower risk of chemical residues in the animal products.

9.5. Literature cited

Abbeele PV, Grootaert C, Possemiers S, *et al.* (2009) *In vitro* model to study the modulation of the mucin-adhered bacterial community. *Appl Microbiol Biotechnol* **83**, 349–359

Anderson JW, Baird P, Davis Jr. RH, *et al.* (2009) Health benefits of dietary fiber. *Nutr Rev* **67**, 188-205.

Awati A, Williams BA, Bosch MW, *et al*. (2006) Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in feces of weanling piglets. *J Anim Sci* **84**, 2133-2140.

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

Bikker P, Dirkzwager A, Fledderus J, *et al*. (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J Anim Sci* **84**, 3337-3345.

Boutrou R, Jardin J, Blais A, *et al.* (2008) Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J Agric Food Chem* **56**, 8166-8173.

Champagne CP, Gardner NJ, Lacroix C (2007) Fermentation technologies for the production of exopolysaccharidesynthesizing *Lactobacillus rhamnosus* concentrated cultures. *Electron J Biotechnol* **10**, 211-221.

Choi BY, Sohn YS, Choi C, *et al.* (2003) Lectin histochemistry for glycoconjugates in the small intestine of piglets naturally infected with *Isospora suis*. *J Vet Med Sci* **65**, 389-382

Danielsen V and Vestergaard EM (2001) Dietary fibre for pregnant sows: effect on performance and behavior. *Anim Feed Sci Technol* **90**, 71-80.

Fairbrother JM and Gyles CL (2006) *Escherichia coli* infections. In: *Diseases of Swine*, pp.639-674 [Straw BE, Zimmerman JJ, D'Allaire S *et al.*, editors]. Ames, Iowa, USA: Blackwell Publishing Professional.

Fernando SF, and Woonton BW (2010) Quantitation of N-acetylneuraminic (sialic) acid in bovine glycomacropeptide (GMP). *J Food Composit Anal* **23**, 359-366.

Freire JPB, Peiniau J, Cunha LF, *et al*. (1998) Comparative effects of dietary fat and fiber in alentejano and large white piglets: Digestibility, digestive enzymes and metabolic data. *Livest Prod Sci* **53**, 37-47.

Ghazarian H, Idoni B, Oppenheimer SB (2011) A glycobiology review: Carbohydrates, lectins and implications in cancer therapeutics. *Acta Histochem* **113**, 236-247.

Geens MM and Niewold TA (2010) Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia coli*, *Escherichia coli*, and *E. coli* lipopolysaccharide. *Comp Funct Genom* **2010**, 1-11.

Güll I, Wirth M, Gabor F (2007) Development of a sensitive and reliable ELISA for quantification of wheat germ agglutinin. *J Immunol Met* **318**, 20–29

Grange PA, Erickson AK, Anderson TJ, *et al.* (1998) Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of *Escherichia coli*. *Infect Immun* **66**, 1613-1621.

Grange PA, Mouricout MA, Levery SB *et al.* (2002) Evaluation of receptor binding specificity of *Escherichia coli* K88 (F4) fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. *Infect Immun* **70**, 2336-2343.

Hermes RG, Molist F, Ywazaki M, *et al*. (2009) Effect of dietary level of protein and fiber on the productive performance and health status of piglets. *J Anim Sci* **87**, 3569-3577.

Hermes RG, Molist F, Ywazaki M, *et al*. (2010) Effects of type of cereal and fibre level on growth and parameters of the gastrointestinal tract in young pigs. *Livest Sci* **133**, 225-228.

Kim JC, Mullan BP, Hampson DJ, *et al*. (2008) Addition of oat hulls to an extruded rice-based diet for weaner pigs ameliorates the incidence of diarrhoea and reduces indices of protein fermentation in the gastrointestinal tract. *Br J Nutr* **99**, 1217-1225.

Koh SY, George S, Brözel V, *et al.* (2008) Porcine intestinal epithelial cell lines as a new *in vitro* model for studying adherence and pathogenesis of enterotoxigenic *Escherichia coli*. *Vet Microbiol* **130**, 191-197.

Konstantinov SR, Favier CF, Zhu WY, *et al.* (2004) Microbial diversity studies of the porcine gastrointestinal ecosystem during weaning transition. *Anim Res* **53**, 317-324.

Kooyk Y and Rabinovich GA (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. *Nat Immunol* **9**, 593-601.

Lallès JP, Bosi P, Smidt H, *et al*. (2007) Weaning — A challenge to gut physiologists. *Livest Sci* **108**, 82-93.

Lane JA, Mehra RK, Carrington SD, *et al.* (2010) The food glycome: A source of protection against pathogen colonization in the gastrointestinal tract. *Int J Food Microbiol* **142**, 1-13.

Lange CFM, Pluske J, Gong J, *et al.* (2010) Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livest Sci* **134**, 124-134.

Levitz SM (2004) Interactions of toll-like receptors with fungi. *Microb Infect* **65**, 1351-1355.

Marquardt RR, Jin LZ, Kim JW, *et al*. (1999) Passive protective e¡ect of egg-yolk antibodies against enterotoxigenic *Escherichia coli* K88+ infection in neonatal and early-weaned piglets. *FEMS Immunol Med Microbiol* **23**, 283-288.

Marth JD and Grewal PK (2008) Mammalian glycosylation in immunity. *Nat Rev Immunol* **8**, 874-887.

Mateos GG, Martin M, Latorre MA, *et al*. (2006) Inclusion of oat hull in diets for young pigs based on cooked maize or cooked rice. *Anim Sci* **82**, 57-63.

Martín-Sosa S, Martín MJ and Hueso P (2002) The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic *Escherichia coli* human strains. *J Nutr* **132**, 3067-3072.

Molist F, de Segura AG, Gasa J, *et al*. (2009) Effects of the insoluble and soluble dietary fibre on the physicochemical properties of digesta and the microbial activity in early weaned piglets. *Anim Feed Sci Technol* **149**, 346-353.

Molist F (2010) Wheat bran modifies the microbial population and enhances gut fermentation in the gastrointestinal tract of post-weaning piglets. Doctoral thesis. Universitat Autònoma de Barcelona. 190 pages.

Molist F, Gómez de Segura, Pérez JF, *et al*. (2010a) Effect of wheat bran on the health and performance of weaned pigs challenged with *Escherichia coli* K88+. *Livest Sci* **133**, 214-217.

Molist FM, Ywazaki M, Gómez de Segura A, *et al*. (2010b) Administration of loperamide and addition of wheat bran to the diets of weaner pigs decrease the incidence of diarrhoea and enhance their gut maturation. *Br J Nutr* **103**, 879-885.

Molist F, Hermes RG, Gómez de Segura A, *et al*. (2011) Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *Br J Nut* **105**, 1592–1600.

Nakajima, K., Tamura N, Kobayashi-Hattori K, *et al*. (2005). Prevention of intestinal infection by glycomacropeptide. *Biosci Biotechnol Biochem* **69**, 2294-2301.

Newburg DS (1997) Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria? *J Nutr* **127**, 980S-984S

Nollet H, Deprez P, Van Driessche E, *et al*. (1999) Protection of just weaned pigs against infection with F18+ *Escherichia coli* by non-immune plasma powder. *Vet Microbiol* **65**, 37-45.

Piva A, Galvano F, Biagi G, *et al.* (2006) Chapter 1 intestinal fermentation: Dietary and microbial interactions. In: *Biology of Growing Animals*, pp.3-31 [R. Mosenthin, editor]. Elsevier.

Pluske JR, Pethick DW, Hopwood DE, *et al*. (2002) Nutritional influences on some major enteric bacterial diseases of pigs. *Nut Res Rev* **15**, 333-371.

Pluske JR (2006) New thoughts on nutrition of newly weaned pigs, London Swine Conference, Canada. 63-73.

Rhoades JR, Gibson GR, Formentin K, *et al.* (2005) Caseinoglycomacropeptide inhibits adhesion of pathogenic *Escherichia coli* strains to human cells in culture. *J Dairy Sci* **88**, 3455-3459.

Roselli M, Britti MS, Le Huërou-Luron I, *et al.* (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic *Escherichia coli* K88 in pig intestinal cells. *Toxicol in Vitro* **21**, 224-229.

Sarabia-Sainz A, Ramos-Clamont G, Candia-Plata MM, *et al*. (2009) Biorecognition of *Escherichia coli* K88 adhesin for glycated porcine albumin. *Int J Biol Macromol* **44**, 175-181.

Schierack, P, Nordhoff M, Pollmann M, *et al*. (2006) Characterization of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in swine. Histochem. *Cell Biol* **125**, 293-305.

Sharon N (2009) Lectins. In: *Encyclopedia of Life Sciences,* pp- 1-11. Chichester, UK: John Wiley & Sons, Ltd.

Shoaf-Sweeney KD and Hutkins RW (2009) Adherence, Anti-adherence, and oligosaccharides: Preventing pathogens from sticking to the host. In: *Advances in Food and Nutrition Research*, pp.101-161 [Steve L. Taylor, editor]. Elsevier, Academic Press.

Solà-Oriol D, Roura E, Torrallardona D (2009) Feed preference in pigs: Effect of cereal sources at different inclusion rates. *J Anim Sci* **87**, 562-570.

Tokach MD, Dritz SS, Goodlad RA *et al*. (2003) Nutritional requirements of the weaned pig. In: *Weaning the Pig: Concepts and Consequences*, pp.259-299 [Pluske JR, Le Dividich J, Verstegen MWA, editors]. Wageningen, The Netherlands: Wageningen Academic Publishers.

Vandamme K, Melkebeek V, Cox E, *et al.* (2011) Adjuvant effect of Gantrez®AN nanoparticles during oral vaccination of piglets against F4+enterotoxigenic *Escherichia coli*. *Vet Immunol Immunopathol* **139**, 148–155

Verweij PE, Poulain D, Obayashi T, *et al*. (1998) Current trends in the detection of antigenemia, metabolites and cell wall markers for the diagnosis and therapeutic monitoring of fungal infections. *Med Mycol* **36**, 146-155,

Walter F, Scholl I, Untersmayr E, *et al*. (2004) Functionalisation of allergen-loaded microspheres with wheat germ agglutinin for targeting enterocytes. *Biochem Biophys Res Commun* **315**, 281–287.

Wang Y, Ganzle MG, Schwab C (2010) Exopolysaccharide synthesized by *Lactobacillus reuteri* decreases the ability of enterotoxigenic *Escherichia coli* to bind to porcine erythrocytes. *Appl Environ Microbiol* **76**, 4863-4866.

Wellock IJ, Fortomaris PD, Houdijk JGM, *et al.* (2008) The consequences of non-starch polysaccharide solubility and inclusion level on the health and performance of weaned pigs challenged with enterotoxigenic *Escherichia coli*. *Br J Nutr* **99**, 520-530.

Williams BA, Verstegen MWA, Tamminga S (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutr Res Rev* **14**, 207-227.

Wirth M, Hamilton G, Gabor F. (1998) Lectin-mediated drug targeting: quantification of binding and internalization of Wheat germ agglutinin and *Solanum tuberosum* lectin using Caco-2 and HT-29 cells. *J Drug Target* **6**, 95–104.

Chapter 10

Conclusions

The results obtained in this thesis allow us to conclude that in our experimental conditions:

1) A moderate dietary supplementation with 4% of wheat bran and 2% of sugar beet pulp in highly digestible diets for young pigs does not reduce the feed intake and the animal performance. Moreover, it presented a positive effect on the gastrointestinal function indicated by the higher development of the large intestine, lower protein fermentation and better shift on the microbiota composition, when compared to non-supplemented diets.

2) The incorporation of WB and SBP in the *in vivo* experiments promoted major changes on the intestinal fermentation and positive impact on intestinal microbial populations, specially on high protein or rice-based diets.

3) The *in vitro* screening experiment performed to identify dietary anti-adhesive agents showed that soluble extracts of wheat bran, casein glycomacropeptide and exopolysaccharides from lactobacilli are able to adhere ETEC K88 and therefore could interfere with the adhesion of the bacteria to the intestine. These ingredients therefore should be considered as promising candidates to be used as an anti-adhesive preventive strategy against ETEC K88 in early weaned piglets.

4) The soluble extracts of wheat bran, mannaoligosaccharides, locust bean, bovine casein glycomacropeptide and an *Aspergillus oryzae* fermentation extract are able to reduce adhesion of ETEC to monolayer cultures of porcine intestinal epithelial cells.

5) All these extracts are also able to modify direct or indirectly the innate immune response of monolayer culture of porcine intestinal epithelial cells when challenged by an ETEC K88 strain, being the wheat bran and bovine casein glycomacropeptide those that better down-regulate the inflammatory response. In contrast, *Aspergillus oryzae* fermentation extract, shows a specific pro-inflammatory response in the cell culture, in both challenged and non-challenged cells.

6) The bovine casein glycomacropeptide can decrease the adhesion of ETEC K88 to ileum mucosa in "*in situ*" experimental conditions. Furthermore, its dietary supplementation of 1.5% can decrease the adhesion of the *E. coli* attachment to the ileum mucosa and reduce the overgrowth of enterobacteria in the digestive tract of piglets after an ETEC oral challenge. From these expected capabilities it can be considered to be used as an anti-adhesive therapy against ETEC K88 infection.