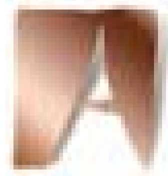


UNIVERSITAT AUTONÒMA DE BARCELONA

Development of gut microbiota in the pig: modulation of bacterial communities by different feeding strategies.

MEMÒRIA PRESENTADA PER MARIA SOLEDAD CASTILLO GÓMEZ

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

BELLATERRA, MARÇ DE 2006

FACULTAT DE VETERINÀRIA DE BARCELONA

Susana M. Martín-Orúe, investigadora del departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

certifica:

Que la memòria titulada **"Development of gut microbiota in the pig: modulation of bacterial communities by different feeding strategies"**, presentada per Maria Soledad Castillo Gómez per optar al grau de Doctor en Veterinària, ha estat realitzada sota la seva direcció i, considerant-la acabada, autoritza la seva presentació per que sigui jutjada per la comissió corresponent.

I per que consti als efectes oportuns, signa la present a Bellaterra, 20 de Març de 2006.

Dra. Susana M. Martín-Orúe.

The author was in receipt of a grant from the Departament d'Universitats, Recerca i Societat de la informació (DURSI) of the Generalitat de Catalunya for this study.

Sin duda, en la elaboración de una tesis y en el periodo de formación predoctoral participan muchas personas además del doctorando, por ello, quiero en primer lugar, agradecer el apoyo recibido durante estos años.

En primer lugar, al equipo docente e investigador del grup de Nutrició, en especial a Susana, gracias por tu apoyo y ayuda durante estos años. Josep, Francisco, Mariola, AnaCris y Roser, gracias por brindarme la oportunidad de formar parte del equipo de Nutrición, por vuestra colaboración, consejos y cafés que hemos compartido. Y, sobre todo a Olga, muchísimas gracias por tu apoyo.

A todos los becarios, aquellos que me brindaron su ayuda y consejos cuando empezaba (Joaquin, Jaume, Dani, Lucía), y a todos los demás con los que he compartido estos años: Montse, Eva, Alba, Núria, Edgar, Ceci, Arantza, Marta, Gabri, Walkiria, Juan Carlos, José, Sandra, Carol, Muzzafer, Francesc, gràcies!!!, A todos con los que he compartido despacho, Aina, Luciano, Glaubert, Feliu, Vincent…També als altres becaris amb els que he compartit l'aventura del doctorat: Mercè, Miquel, Maribel, Montse, Anna, Laura, gràcies!

Also, I would like to thank to the Gut microbiology and Immunology division of the Rowett Research Institute, in particular to Harry Flint, Sylvia Duncan and Gail Skene, for their collaboration and support; as well to the rest of the staff and students who shared with me the adventure of the FISH, thanks a lot!!

With special regards to Justin and Vanessa (and the little Maria Isabella), because they were my american family in Aberdeen, Scotland.

Guillem, Pablo, David, Àlex, Joan, Beti, Anna i Laura, per ser els de sempre; Javier, por estar siempre, gracias.

Muy especialmente a mi familia por su ayuda y apoyo incondicional, sin vosotros esta tesis no hubiera sido posible. Y, por supuesto a todos aquellos que en algún momento han sido cómplices en la elaboración de este trabajo, gracias.

PARA MIS PADRES,

RESUMEN

El objetivo de esta tesis fue el estudio de la microbiota gastrointestinal porcina, para mejorar el conocimiento existente de este complejo ecosistema y así, ayudar de alguna forma en el desarrollo de nuevas estrategias alimentarias para sustituir los antibióticos promotores del crecimiento recientemente prohibidos en la Unión Europea.

Para alcanzar este objetivo, se diseñaron diferentes pruebas experimentales (capítulo 4-9). En la **Prueba I**, se desarrolló la técnica de PCR cuantitativa para cuantificar bacterias totales, lactobacilli y enterobacteria en muestras de contenido digestivo. Con el fin de validar su utilidad, los resultados obtenidos se compararon con los que se obtuvieron con métodos tradicionales (cultivo en medio selectivo para lactobacilli y enterobacteria, y microscopía directa para bacterias totales). La PCR mostró valores superiores, en términos de copias del gen 16S rRNA que la microscopía directa y los cultivos. Sin embargo, a pesar de la diferencia, la ratio lactobacilli:enterobacteria fue similar entre métodos. Diferentes motivos pueden estar detrás de la diferencia entre métodos, tanto una sobreestimación con la PCR como una subestimación con los métodos tradicionales. No obstante, los contajes para el total de bacterias y lactobacilli mostraron una correlación significativa. Por ello, este método se consideró como válido para cuantificar cambios bacterianos en el tracto gastrointestinal del cerdo.

Con el fin de estudiar el establecimiento de la microbiota en el cerdo tras el destete, se diseñó la **Prueba II**. En ésta, 12 lechones (20 ± 2 días) de 6 camadas diferentes fueron divididos en un grupo control, el cual permaneció con la madre, y un grupo experimental el cual fue destetado y alimentado con una dieta pre-starter commercial. Tras una semana, los animales fueron sacrificados y se recogieron muestras de contenido de ciego. Para estudiar el cambio en la microbiota, se cuantificó el total de bacterias, lactobacilli y enterobacterias mediante PCR a tiempo real. Además, para obtener una imagen global del cambio producido por el destete, se utilizó la técnica del t-RFLP (“Terminal restriction fragment length polymorphism”). La población total bacteriana, así como la biodiversidad, medida como número de bandas obtenidas por t-RFLP, fue similar entre grupos, pero hubo un descenso importante en el ratio lactobacilli:enterobacteria. Además, el análisis de similaridad de los perfiles obtenidos

por t-RFLP, mostró una agrupación separada de los grupos experimentales. Infiriendo con los fragmentos teóricos se observaron diferencias entre grupos. Los cerdos lactantes mostraron una mayor diversidad de fragmentos compatibles con bacterias ácido lácticas y se observó la presencia de algunos picos compatibles con *Clostridium coccoides*, *C. butyricum* y *Lactobacillus delbruekii* que no se encontraron en los animales destetados. Estos resultados confirman el destete como un punto crítico en el establecimiento de la microbiota gastrointestinal.

En la **Prueba III**, se utilizaron cerdos en crecimiento para estudiar la microbiota gastrointestinal y a su vez, el potencial de la fibra para modificar este ecosistema. Para ello, 32 cerdos (15 ± 0.38 kg de peso vivo) se distribuyeron en 4 tratamientos: una dieta control, una dieta rica en almidón resistente por la inclusion de maíz con un mayor tamaño de particula, una dieta rica en polisacáridos no amiláceos solubles por la inclusion de pulpa de remolacha, y una cuarta dieta rica en polisacáridos no amiláceos insolubles por la inclusion de salvado de trigo. Tras seis semanas de alimentación *ad líbitum*, los animales fueron sacrificados y el contenido digestivo fue muestreado. La técnica del FISH ("Fluorescent *in situ* hybridization") se utilizó con el fin de describir los grupos bacterianos mayoritarios a lo largo del tracto gastrointestinal. Se utilizaron diferentes sondas para cuantificar bacterias pertenecientes al *Bacteroides/Prevotella* grupo, *Ruminococcus flavefaciens*, *Ruminococcus bromii*, clostridia cluster IV, clostridia cluster IX, *Streptococcus/Lactococcus* y *Lactobacillus/Enterococcus* sp. en estómago, yeyuno distal, colon proximal y recto. Los resultados obtenidos revelaron marcadas diferencias en la composición de estos grupos a lo largo del tracto, que no fueron marcadamente afectados por la dieta. En estómago, streptococci y lactobacilli fueron los grupos predominanates, mientras que en intestino grueso, el grupo de *Bacteroides/Prevotella*, clostridial cluster XIVa, IV, y ruminococci fueron los más abundantes. Los resultados obtenidos por RFLP ("restriction fragment length polymorphism") mostraron cambios en el perfil bacteriano dependiendo de la dieta administrada. Los animales que recibieron salvado de trigo mostraron una menor biodiversidad con unos perfiles más similares entre animales. Además, se hallaron cambios en la fermentación mediante la determinación de ácidos grasos volatiles; Las dietas ricas en polisacáridos no amiláceos mostraron una menor concentración de ácidos grasos ramificados y valerico.

En las pruebas IV y V, se estudiaron diferentes aditivos comerciales como posibles alternativas a los antibióticos promotores del crecimiento, con especial interés en sus efectos en la microbiota gastrointestinal. En concreto, en la **Prueba IV**, se testaron 3 aditivos: avilamicina (como control positivo), butirato sódico y un extracto de plantas (carvacrol, cinamaldehido y capsicum). Un total de 40 (18-22 días) cerdos se distribuyeron en cuatro tratamientos: una dieta control, ésta con 0.04% de avilamicina, con 0.3% de butirato sódico o con 0.03% de extracto de plantas. Después de dos semanas los animales fueron sacrificados y el contenido digestivo fue muestreado. Como en las pruebas anteriores, la PCR a tiempo real se utilizó para estudiar los cambios en la microbiota. No se encontraron diferencias en el total de bacterias a lo largo del tracto gastrointestinal con ninguna de las dietas, aunque la ratio lactobacilli:enterobacteria en ciego fue superior para los animales que recibieron el extracto de plantas. La técnica del RFLP mostró diferencias en el perfil bacteriano, agrupando los animales en función de la dieta administrada. La actividad bacteriana total medida como bases púricas también mostró diferencias entre dietas. Estos resultados podrían indicar que el efecto de los diferentes aditivos testados no se debería a una reducción en el total de bacterias sinó a modificaciones en la composición y actividad de la microbiota.

Finalmente, en la **Prueba V,** una fuente comercial de mananoligosacáridos y de zinc orgánico, administrados por separado o conjuntamente fueron testados para mejorar los indices productivos, microbiota gastrointestinal y respuesta inmune. En este caso, 128 cerdos (18-22 días de vida) se distribuyeron en cuatro tratamientos: una dieta control, ésta dieta con 0.2% de mananoligosacáridos, con 0.08% de zinc orgánico o con ambos aditivos. Las dietas fueron administradas durante cinco semanas. Tras dos semanas, 32 animales fueron sacrificados y el contenido digestivo fue muestreado. Se observó una mejora en el índice de conversion para todo el periodo experimental cuando los dos aditivos se añadieron conjuntamente. Los mananoligosacáridos redujeron la enterobacterias en yeyuno. La adición de zinc orgánico, tendió a incrementar el peso en vacío del ileon, que fue considerado como el segmento de intestino delgado con placa de Peyer continua. Estos resultados sugieren diferentes mecanismos de acción de los aditivos, mientras que los mananoligosacáridos podrían estar actuando modulando el ecosistema bacteriano, mediante la inhibición de algunos grupos, el incremento en el peso vacío del ileon podría sugerir un efecto inmunológico. Además, el efecto positivo

en la ratio vellosidad:cripta cuando ambos aditivos se incluyeron conjuntamente podría indicar acciones complementarias.

Los resultados obtenidos en la presente tesis demuestran la validez de diferentes métodos moleculares para el studio de la microbiota gastrointestinal del cerdo. Ecosistema muy instable durante las primeras edades, con un cambio drástico al destete pero que consigue una estabilización de los grupos mayoritarios en el animal adulto. Por otra parte, los efectos promotores de las diferentes alternativas testadas parecen estar relacionados con sutiles cambios en la microbiota gastrointestinal más que con drásticos efectos antimicrobianos. No obstante, en algunos casos (butirato sódico, zinc) otros efectos diferentes al microbiano podrían estar implicados.

SUMMARY

The main objective of this thesis was to study pig gut bacteria to improve our knowledge of this complex ecosystem as this could help in the development of new feed strategies to substitute antibiotics as growth promoters.

To achieve this main objective, a set of five trials were designed (chapter 4-8). In **Trial I**, real-time PCR was developed to quantify total bacteria, lactobacilli and enterobacteria in digesta samples. To validate its usefulness, results obtained were compared to those obtained by traditional methods (selective culture for lactobacilli and enterobacteria, and direct microscopy for total bacteria). Real time PCR showed higher values in terms of 16S rRNA gene copies than direct microscopy counts or CFU. Despite the differences, the lactobacilli:enterobacteria ratio was similar between methods. Differences between methods might caused by an overestimation with PCR by quantification of dead bacteria or free DNA, and also an underestimation with conventional methods. Values obtained by PCR and traditional methods showed a significant correlation for lactobacilli and total bacteria. Therefore, real-time PCR was considered a valid method to quantify microbial shifts in the gastrointestinal tract.

To study pig gut microbiota establishment in the young pig after weaning, **trial II** was designed. Twelve pigs (20 ± 2 days) from 6 different litters were divided into a control group that remained with the sow and an experimental group that was weaned and fed a commercial post-weaning diet. After one week, the animals were sacrificed and samples from cecal digesta were taken. To assess microbial shift, total bacteria, lactobacilli and enterobacteria were quantified using real-time PCR. To achieve an overall picture of the change in the global microbial profile, terminal restriction fragment length polymorphism of the PCR amplified 16S rRNA gene was applied. Total bacteria and biodiversity of the microbial ecosystem were similar between both experimental groups, although there was a decrease in the lactobacilli:enterobacteria ratio. Also, cluster analysis grouped animals in two different clusters. Considering theoretical restriction fragment lengths, differences in compatible bacterial groups were observed between groups. Suckling pigs showed a higher lactic acid bacteria diversity. Also peaks compatible with *Lactobacillus delbruekii*, *Clostridium coccoides* and *C.*

butyricum were also mostly present in suckling pigs. Results therefore confirm weaning as a challenging point on the indigenous microbiota establishment.

In **Trial III**, growing pigs were used to study pig gut microbiota and the potential of fiber to modify this ecosystem. A total of 32 pigs (15 ± 0.38 kg of body weight) were distributed into four experimental diets: a control diet, a diet enriched in resistant starch by inclusion of coarse-ground corn, a diet enriched in soluble fiber by addition of 8 % sugar beet pulp and a diet rich in insoluble fiber by inclusion of 10 % wheat bran. After six weeks of feeding ad libitum, animals were sacrificed and samples of digesta content were taken. Fluorescent *in situ* hybridization (FISH) was applied to describe main bacterial groups along the gastrointestinal tract and to detect changes related to the diets. Probes to detect changes in total bacteria, *Bacteroides/Prevotella* group, *Ruminococcus flavefaciens*, *Ruminococcus bromii*, clostridia cluster IV, clostridia cluster IX, *Streptococcus/Lactococcus* and *Lactobacillus/Enterococcus* sp. were used in samples from the stomach, distal jejunum, proximal colon and rectum. FISH revealed marked differences in the composition of the microbiota throughout the gastrointestinal tract, which were relatively unaffected by changes in the diet. Streptococci and lactobacilli were predominant in the stomach whereas *Bacteroides/Prevotella*, clostridial cluster XIVa, IV, and ruminococci were predominant in the lower tract. Restriction fragment length polymorphism (RFLP) profiles showed changes in the bacterial profile related to diet, with pigs fed a wheat bran showing the lowest biodiversity and also having the most similar patterns. Moreover, changes in fermentation activity were detected when short-chain fatty acids were measured. Diets rich in non-starch polysaccharides (wheat bran and sugar beet pulp) showed lower molar percentages of branched chain fatty acids and valeric acid.

In trials IV and V, different commercial additives were studied as potential alternatives to antibiotic growth promoters, paying special attention to their effects on gut microbiota. In particular, in **Trial IV**, three different additives were tested: avilamycin (as a positive control), sodium butyrate and a commercial plant extract (carvarol, cinnamaldehyde and capsicum). Forty early-weaned (18 to 22 d) pigs were distributed into four dietary treatments: a control diet, a diet with 0.04% avilamycin, a diet with 0.3% sodium butyrate or with 0.03% plant extract mixture. After two weeks, the animals were sacrificed and samples from digesta were taken. As in the previous trials, real-time PCR was used to assess microbial shifts. The total microbial load did not show differences between diets, although, there was an increase in the

lactobacilli:enterobacteria ratio in the cecum of piglets fed with plant extracts. RFLP also showed differences in microbial profile in jejunum digesta samples, with an increase in biodiversity with the different additives compared to control diet. Total microbial activity measured as purine bases also showed differences between diets. In the light of these results, the effect of the different additives would not be related to a reduction in the toal bacterial load, but rather to changes in the ecological structure and metabolic activity of the microbial community.

Finally, in **Trial V**, a commercial source of mannan-oligosaccharides and organic zinc, offered alone or in combination, were evaluated to enhance performance, gastrointestinal health and immune response. A total of 128 early-weaned pigs (18 to 22 d) were distributed into four dietary groups. For five weeks, animals received either a control diet, a diet with 0.2 % mannan-oligosaccharides, a diet with 0.08 % zinc-chelate or a diet with both additives together. Two weeks after weaning, 32 animals were sacrificed and digesta samples were taken to study the effect of the additives on gut health and immunity. An improvement in feed:efficiency was observed with both additives for the whole period. Mannan-oligosaccharides reduced enterobacteria counts in jejunum. The addition of organic zinc tended to increase empty ileal weight, defined as the segment including the continuous Peyer's patch, and crypt depths were lower in the animals offered both additives together. These results suggest different modes of action of the additives tested; whilst mannan-oligosaccharides might be acting by modulation of intestinal microbiota through inhibition of certain microbial groups, the observed increase in ileal weight with zinc suggests a possible immunological effect. In addition, the response observed in gut architecture may be behind complementary actions when both additives were added together.

Results obtained show the usefulness of different molecular methods for studying pig gut microbiota quantitatively and qualitatively. This ecosystem, as confirmed, is specially unstable during the first weeks of life with marked changes at weaning. However, colonization progresses, resulting in a relatively stable composition in the main bacterial groups in the adult pig. The effect of the different additives tested might be related to subtle changes in microbiota composition more than drastic antimicrobial effects. Moreover, other effects not directly related with microbiota might be involved.

ABBREVIATIONS USED

A: adenine
AB: diet containing 0.04% avilamycin (Trial IV)
AC: diet containing 0.3% sodium butyrate (Trial IV)
ADFI: average daily feed intake
ADG: average daily gain
AGP: antibiotic growth promoter
BCFA: branched chain fatty acids
BD: below detection
BM: diet containing 0.2% mannan-oligosaccharides (Trial V)
BMP: diet containing 0.2% mannan-oligosaccharides plus 0.08% organic zinc (Trial V)
bp: base pair
BP: diet enriched in soluble fiber (Trial III)
BP’: diet containing 0.08% organic zinc (Trial V)
BSA: bovine serum albumin
BW: body weight
C: cytosine
CD: crypt depth
CECT: colección española de cultivos tipo
CFB: cytophaga-flexibacter-bacteroides phylum
CFU: colony forming unit
CP: crude protein
CT: control diet
DAPI: 4’,6’-diamino-2-phenylindole
DGGE: denaturant gradient gel electrophoresis
DM: dry matter
DNA: deoxy nucleic acid
dNTP: deoxy-nucleotide-triphosphate
E:L: ratio enterobacteria:lactobacilli
ENT: enterobacteria
F-ent: forward primer for enterobacteria
FISH: fluorescent *in situ* hybridization
F-lac: forward primer for lactobacilli
FM: fresh matter
F-tot: forward primer for total bacteria
G: guanine
G:F: gain feed ratio
GALT: gut associated lymphoid tissue
GC: diet enriched in resistant starch (Trial III)
GIT: gastrointestinal tract
HPLC: high performance liquid cromatography
IEL: intraepithelia limphocyte
iNSP: insoluble non-starch polysaccharides
L:E: ratio lactobacilli:enterobacteria
LACT: lactobacilli
MAC: microflora associated characteristic
NOD: nucleotide-binding oligomerization domain
NSP: non-starch polysaccharides
OM: organic matter
P: *P*-value
PAMP: pathogen-associated molecular pattern
PAS: periodic acid Schiff reaction
PB: purine bases concentration

PBS: posphate buffered saline
PCR: polymerase chain reaction
PRR: pattern recognition receptor
qPCR: quantitative polymerase chain reaction
R-ent: reverse primer for enterobacteria
RFLP: restriction fragment length polymorphism
R-lac: reverse primer for lactobacilli
RNA: riboncleic acid
RS: resistant starch
R-tot: reverse primer for total bacteria
S: suckling pigs
SCFA: short chain fatty acids
SD: standard desviation
SEM: standard error of the mean
sNSP: soluble non-starch polysaccharides
T: tymine
TGGE: temperature gradient gel electrophoresis
TLR: toll-like receptor
TRF: terminal restriction fragment
t-RFLP: terminal restriction fragment length polymorphism
W: weaned pigs
WB: diet enriched in insoluble fiber (Trial III)
XT: diet containing 0.03% plant extract mixture (Trial IV)
16S rRNA: ribosomal small sub-unit

INDEX

Chapter 1. General introduction p. 1

Chapter 2. Literature review p. 4

2.1. Development of the intestinal microbiota after birth p. 6

2.1.1.First colonizers p. 6

2.1.2.Weaning: the adaptation to dry food p. 9

2.1.3.Autochtonous microbiota in the adult pig p. 12

2.2. Main functions of the indigenous microbiota in the gut p. 16

2.2.1.Effects of indigenous bacteria on gut maturation and development p. 16

2.2.2.Establishment of the gut barrier and colonization resistance p. 18

2.2.2.1.Glycoconjugates of the mucosa as specific attachment site p. 20

2.2.2.2.Molecules involved in bacterial adhesion p. 22

2.2.3.Effects of indigenous microbiota on immune response p. 23

2.2.3.1.Commensal bacteria tolerance-ignorance p. 24

2.2.4.The role of microbiota on digestion and absorption of nutrients p. 26

2.2.4.1.Carbohydrate utilization by indigenous bacteria p. 28

2.2.4.2.Protein utilization by indigenous bacteria p. 29

2.2.4.3.Lipid utilization by indigenous bacteria p. 30

2.3. Modulation of intestinal equilibrium through the feed p. 32

2.3.1.Macro-indredients p. 32

2.3.1.1.The role of dietary fiber p. 33

2.3.1.2.Fermented liquid feed p. 37

2.3.2.Micro-ingredients and in-feed additives p. 39

2.3.2.1.Prebiotics p. 39

2.3.2.2.Probiotics p. 40

2.3.2.3.Symbiotics p. 42

2.3.2.4.Acidifiers p. 42

2.3.2.5.Minerals: zinc and copper p. 44

2.3.2.6.Plant extracts p. 45

2.3.2.7.Other additives p. 46

2.4. New tools for the analysis of the gastrointestinal microbiota p. 48

2.4.1.Quantitative techniques p. 49

2.4.1.1.Quantitative Polymerase Chain Reaction p. 49
2.4.1.2.Fluorescent In Situ Hybridization p. 52
2.4.2.Fingerprinting techniques p. 56
2.4.2.1.Denaturant/Temperature Gradient Gel Electrophoresis p. 56
2.4.2.2.Terminal Restriction Fragment Length Polymorfism p. 59

Chapter 3. Objectives p. 62

Chapter 4. Trial I. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR p. 66
4.1. Introduction p. 68
4.2. Material and methods p. 68
4.2.1.Sample preparation p. 68
4.2.2.Bacteria quantification by traditional methods p. 69
4.2.3.Bacteria quantification by real-time PCR p. 69
4.3. Results and discussion p. 71
4.4. Conclusion p. 75

Chapter 5. Trial II. Influence of weaning on caecal microbiota of pigs: use of real-time PCR and t-RFLP p. 76
5.1. Introduction p. 78
5.2. Material and methods p. 79
5.2.1.Animals and housing p. 79
5.2.2.Sacrifice and sampling p. 79
5.2.3.Statistical analysis p. 83
5.3. Results and discussion p. 83
5.3.1.Bacterial quantitative change measured by real-time PCR p. 83
5.3.2.Ecological bacterial changes, t-RFLP results p. 85
5.4. Conclusions p. 91

Chapter 6. Trial III. Molecular analysis of bacterial communities along the pig gastrointestinal tract p. 92
6.1. Introduction p. 94
6.2. Material and methods p. 95
6.2.1.Animals and diets p. 95
6.2.2.Sample collection and processing p. 95
6.2.3.Statistical analysis p. 99

6.3. Results p. 99
6.3.1.Microflora structure along the gastrointestinal tract as analyzed by FISH p. 99
6.3.2.Effects of fibre on microbial composition as estimated by RFLP and fermentation profiles p. 100
6.4. Discussion p. 103
6.5. Conclusions p. 106

Chapter 7. Trial IV. The response of gastrointestinal microbiota to the use of avilamycin, butyrate and plant extracts in early-weaned pigs p. 108
7.1. Introduction p. 110
7.2. Material and methods p. 110
7.2.1.Animals and housing p. 111
7.2.2.Dietary treatments and feeding regime p. 111
7.2.3.Collection procedures and measurements p. 113
7.2.4.Statistical analysis p. 116
7.3. Results p. 118
7.3.1.Chages in the total microbial counts p. 118
7.3.2.Changes in the microbial ecosystem p. 120
7.3.3.Changes in metabolic bacterial activity p. 122
7.4. Discussion p. 124
7.5. Implications p. 128

Chapter 8. Trial V. Use of mannan-oligosaccharides and zinc chelate as growth promoters and weaning preventative in weaning pigs: effects on microbiota and gut function p. 129
8.1. Introduction p. 131
8.2. Material and methods p. 132
8.2.1.Animals and diets p. 132
8.2.2.Performance and collection procedures p. 132
8.2.3.Analytical methods p. 133
8.2.4.Statistical analysis p. 134
8.3. Results p. 136
8.3.1.Growth performance p. 136
8.3.2.Faecal consistency p. 137
8.3.3.Organ weights and small intestine length p. 138
8.3.4.Short chain fatty acids p. 138

8.3.5.Quantitative changes in microbial population p. 140
8.3.6.Immune proteins and intestinal morphology p. 141
8.4. Discussion p. 141
8.4.1.Changes on microbial ecosystem p. 142
8.4.2.Effect on gut function p. 145
8.5. Implications p. 147

Chapter 9. General discussion p. 150
9.1. Usefulness of quantitative PCR, FISH and t-RFLP to study the intestinal microbiota p. 152
9.2. Weaning: a critical stage in the indigenous pig microbiota establishment p. 158
9.2.1.Establishment of adult gut bacteria p. 161
9.3. Are antibiotics-growth promoters a model to copy? p. 162
9.3.1.Mode of action of antibiotics: quantitative or qualitative effects on gut microbiota? p. 162
9.3.2.Other in-feed additives with antimicrobial properties p. 163
9.3.3.Effects on microbiota by other mechanisms p. 165
9.3.4.Other strategies to improve health and promote growth p. 167
9.4. Summary p. 168

Chapter 10. Conclusions p. 171

Chapter 11. Literature cited p. 174

Figures index

Chapter 2

Fig. 2.1. (A) Evolution of aerobic and anaerobic bacteria in piglet feces from birth to 120 days of life p. 8

Fig. 2.1. (B) Total bacteria counts and percentage of coliforms, Bacteroides and Clostridium in piglet feces (Swords et al., 1993) p. 8

Fig. 2.2. Review diagram of piglets post-weaning challenge p. 10

Fig. 2.3. (A) Diversity of the intestinal microbiota in piglets from birth to 14 days afer weaning. p. 11
(B) Dendogram based on TGGE profiles of one piglet (Inoue et al., 2005) p. 11

Fig. 2.4. Intestinal immune geography of responses to commensal bacteria (Macpherson et al., 2005) p. 27

Fig. 2.5. Representation of real-time PCR with TaqMan (A) and SYBR Green (B) p. 51

Fig. 2.6. (A) Epifluorescent image of mixed culture of seven Bifidobacterium species by multi color FISH p. 55
(B) Identification of Bifidobacterium species in human fecal samples using multi color FISH (Takada et al., 2004) p. 55

Fig. 2.7. PCR-DGGE profile generated from fecal samples obtained from a piglet over a 20-day experimental period using primers specific for the V3-16S rDNA (Simpson et al., 2000) p. 58

Fig. 2.8. An example of fragments and visualization of the electropherogram obtained after an enzymatic restriction p. 60

Fig. 2.9. T- RFLP profiles obtained from cecum digesta in pigs receiving different doses of zinc oxide and copper sulphate (Höjberg et al., 2005) p. 61

Chapter 4

Fig. 4.1. Bacterial loads in jejunum dgesta of pigs as total bacteria, lactobacilli or enterobacteria measured by qPCR, DAPI staining or selective culture technique p. 73

Fig. 4.2. Correlation between the number of total bacteria measured by qPCR or by DAPI in jejunum digesta samples p. 74

Chapter 5

Fig. 5.1. Bacterial loads in the caecum of suckling and weaned pigs measured by quantitative PCR p. 84

Fig. 5.2. Dendogram illustrating the effect of weaning in t-RFLP banding patterns p. 87

Fig. 5.3. Electropherogram produced from Hha I digestion of 16S rRNA PCR products from one suckling and one weaned piglet p. 87

Chapter 6

Fig. 6.1. Ecological changes in microbial population measured by RFLP. Dendogram illustrating the percentage of similarity of PCR -RFLP banding patterns in samples of proximal colon digesta p. 101

Fig. 6.2. Fermentation patterns (pH, SCFA and purine bases concentration) in the proximal colon digesta from experimetal pigs p. 101

Chapter 7

Fig. 7.1. Quantitative PCR for total bacteria. (A) The amplification plot of the standards used to quantify total bacteria p. 118

(B) DNA concentrations plotted vs. Thereshold cycle value to construct the standard calibration curve p. 119

(C) Bacterial loads in the stomach, jejunum, cecum, distal colon colon digesta and in the jejunum mucous layer measured by quantitative PCR in early-weaned pigs p. 119

Fig. 7.2. Ecological changes in microbial population of jejunum digesta measured by RFLP. (A) Example of gel electrophoresis of the PCR amplifiedV3, V4 and V5 regions of the 16S rDNA restricted with the enzyme Hha I p. 121

(B)Dendogram illustrating the correlation between experimental diets in PCR-RFLP banding patterns p. 122

Fig. 7.3. Purine bases concentration in samples from the ileum, cecum,

proximal colon, distal colon and rectum in weaned pigs receiving A control diet (CT) or the same diet with avilamycin (AB), butyric acid (AC) or a plant extract mixture (XT) p. 123

Chapter 8.

Fig. 8.1. Voluntary feed intake of pigs receiving a control diet (CT), the same diet supplemented with Bio-Mos (BM), Bioplex-Zn (BP) or both additives (BMP) the first week post-weaning p. 136

Fig. 8.2. Faecal consistency in pigs receiving a control diet (CT), or the same diet supplemented with Bio-Mos (BM) Bioplex-Zn (BP) or both additives during three weeks after weaning p. 138

Chapter 9

Fig. 9.1. Melting curve oftained after the PCR reaction for total (A), enterobacteria (B) and lactobacilli (C) p. 154

Fig. 9.2. Pie chart with the major 5'-terminal fragments expressed as the mean of the percentage of the toal area in suckling (S) and weaned (W)group p. 160

Fig. 9.3. Total bacteria, Bacteroides/Prevotella group, clostridia cluster XIVa, *F. prausnitzii, R. flavefaciens* and *R. bromii,* clostridia cluster IX, *Streptococcus/Lactococcus* spp. and *Lactobacillus/Enterococcus* spp. measured by FISH p. 162

Tables index

Chapter 2
Table 2.1. Main bacteria traditionally cultured from the pig gut (adapted from Stewart et al., 1999) p. 13
Table 2.2. Major phylogenetic lineages to which the phylotypes from the pig gastrointestinal tract were affiliated (Leser et al., 2002) p. 14
Table 2.3. Number of cellulolytic bacteria from fecal samples of sows fed diets containing various levels of fiber (Varel and Pond,, 1985) p. 35
Table 2.4. Selected bacterial counts (CFU/g wet weight) in the colon of weaned pigs fed diets containing different sources of starch (MacFarland, 1998, reviewed by Hillman, 2001) p. 36
Table 2.5. Some probes used actually to quantify different gastrointestinal Bacteria p. 54

Chapter 5
Table 5.1. Control diet composition (as fed basis) administered to pigs p. 79
Table 5.2. Theroretical restriction 5' fragment length predicted for the major pig gut bacteria.

Chapter 6
Table 6.1. Sequences of oligonucleotides used in the study p. 82
Table 6.2. (A) Fermentation parameters including pH, and purine bases Concentration in samples of proximal colon contents from pigs receiving experimental diets p. 98
(B) SCFA profile in digesta of proximal colon p. 101
Table 6.3. Proportions of specific bacterial groupings in different regions of the porcine digestive tract estimated by FISH p. 101

Chapter 7
Table 7.1. Control diet composition, as fed basis p. 112
Table 7.2. Material and conditions for the quantification of total bacteria, enterobacteria and lactobacilli in digesta simples p. 116
Table 7.3. Bacterial populations, size of lactobacilli, and enterobacteria in the distal jejunum and cecum measured by qPCR in early-weaned pigs p. 120
Table 7.4. Bacterial enzymatic activity in samples of the cecum and colon

contents from early-weaned pigs p. 124

Chapter 8

Table 8.1. Composition as fed basis of pre-starter and starter control diets of phase 1 and phase 2 p. 135

Table 8.2. Initial and final pig body weight (kg), voluntary feed intake (kg/day) average daily gain (kg/day) and feed efficiency in weaned pigs p. 137

Table 8.3. Weight (g/kg BW) and length (m) of different parts of the gut from early-weaned pigs sacrificed two weeks post-weaning p. 139

Table 8.4. pH, SCFA and lactic acid contentration in the stomach, ileum, and caecum of pigs sacrificed two weeks post-weaning p. 140

Table 8.5. Purine bases concentration in the ileum, caecum and rectum, and bacterial populations (lactobacilli and enterobacteria) from the jejunum measured by real-time PCR in ileum digesta in pigs p. 143

Table 8.6. Plasma and ileal immunoglobulin (IgA, IgM, IgG) concentration and jejunum histological parameters in weaned pigs sacrificed two weeks post-weaning p. 144

Chapter 9

Table 9.1 Results of lactobacilli:enterobacteria ratio and its relation with performance from pigs included in trial II, IV and V p. 155

Table 9.2. Summary of the main effects found in the different additives tested in the trials included in the thesis p. 169

Chapter 1

GENERAL INTRODUCTION

Recent concern regarding cross-resistance of pathogens in humans have became into the total ban of antibiotics as growth promotants in livestock in the European Union on January 2006. Since the first restrictive measures were taken, and due to the begining of the negative consequences of the ban, great efforts have been done to look for alternatives or replacement strategies to maintain pig growth performance and controlling enteric bacterial diseases. One of the ways that probably could help to maintain productive indexes without the use of antibiotics would be those related with the maintenance of a robust indigenous intestinal microbiota that helped the animal to resist invasion by potentially disease-causing pathogenic bacteria.

Gastrointestinal microbiota is a complex and dynamic ecosystem that inhabits the pig gut since birth, and have an important influence on the animal health: gut bacteria provides essential products to the host, forms a key barrier against pathogens and also plays important roles in gut morphology, immunity development, digestion and even modulating gene host expression. 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, although at present it is recongnized that these methods misregard an important percentage of bacteria due to failure of many of them to grow in a given culture medium. In this regard, the development in the last years of high resolution molecular techniques based on 16S ribosomal DNA gene has revolutioned our knowledge of complex microbial populations such as the pig gut microbiota, since viability and later growth of cells is not necessary to their study. Those studies have showed that the complexity of microbial community is much greater than previosly thought. Among the diversity of methods, quantitative PCR and some fingerprinting techniques like DGGE and t-RFLP have been extensively used to study pig gut bacteria.

Recently numerous products have appeared in the market with the aim to maintain production indexes of the antibiotics “age”. In this sense, different alternatives to antibiotics growth promoters have been tested with promising results, although still not comparable with those obtained with antibiotics. Among these new alternatives, it may be remarcked the use of prebiotics, probiotics, organic acids, minerals at pharmacological doses and plant extract mixtures. Most of them are thought to act throught an effect on gut bacteria, by shifting the microbial equilibrium, whilst others might be acting by other different mechanisms in the improvement of health and

performance. In this sense, further research is necessary to improve knowledge regarding the mechanism of action of these compounds that undoubtly will help to improve their proper use in field conditions. The use of molecular methods in conjunction with traditional ones will be a key role in further studies regarding pig gut microbiota.

Chapter 2

LITERATURE REVIEW

2.1. Development of the intestinal microbiota after birth

Introduction

Similarly to other microbial ecosystems, the establishment of the pig gut microbiota is a complex process that involves a first colonizing phase during which the gut of newborns is rapidly invaded by bacteria, followed by different successional steps where diverse dominant groups become predominant. This process continues as the pig matures, resulting finally in a characteristic and dynamic bacterial community population for each individual (Rolfe et al., 1996; Zoetendal et al., 2001). More than 500 different bacterial species of indigenous micro-organisms are usually described in the lower tract of the adult pig (van Kessel et al., 2004).

To colonize the gastrointestinal tract, bacterial population need to be stable in size and occurrence over time, by multiplying at a rate that equals or exceeds their rate of washout or elimination at an intestinal niche, or if not, by attachment to the gut wall to maintain a permanent colonization (Mackie et al., 1999). It is influenced also by several factors of both bacterial and host origin. The main factors affecting the colonization process are immune reactivity, the presence of gut receptors, nutrient availability and composition, the flow of digesta, pH, molecular oxygen and oxidation/reduction potential (Stewart et al., 1993). Accordingly, pig microbiota differs quantitatively and qualitatively throughout the gastrointestinal tract (Berg, 1996; Simpson et al., 1999), with the highest counts in the caecum and the colon. There is also a horizontal stratification in the lumen, mucus lining and crypt spaces, with characteristic population in each section (Lee et al., 1984; Simpson et al., 1999).

This chapter will focus on the pig gastrointestinal tract colonization from birth to the adult age, paying special attention to the most recent microbiological works.

2.1.1. First colonizers

At birth, the piglet gastrointestinal tract is sterile. However, from the moment the 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). Recently, comparisons of bacteria metabolic fingerprintings determined by Katouli and co-workers (1997) demonstrated that there was a high similarity among the flora of piglets and their dams during the early stages of the animals life, confirming therefore that sows were the initial source of the gut flora for piglets. In particular, the mother's feces might be a key factor in this adquisition and future microbiota development, as it is confirmed that piglets can consume up to 85 g 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).

The first bacteria detected in the piglet digestive tract are very diverse, reflecting the miscellany of the microbial populations associated with the mother and the environment (Ewing and Cole, 1994). However, in the following days, simplified microbiota profiles have been characterized, which will become more complex with time, increasing its diversity as the animal grows (Conway, 1994; Favier et al., 2002; Inoue et al., 2005). In this regard, may be remarked a comprehensive work by Swords and co-workers (1993; Figure 2.1) who studied 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.

In this first phase, aerobes and facultative anaerobes from the sow and the environment become the predominant bacterial groups, comprising 80% of the total flora by three hours after birth. The gut colonization is extremely fast; only twelve hours after birth, total bacteria in distal colon reaches counts of 10^9 CFU/g colonic content (Swords et al., 1993; Jensen et al., 1998).

First colonizers modify the gastrointestinal environment (by consumption of molecular oxygen and reduction of the redox potential), making it more favorable for the following colonization by anaerobes. Although not only the change in gut environment is involved in the substitution of these first bacteria. Calostrum immunoglobulins also act excluding antigens from entering the gut (Brandtzaeg, 2002). As a result, aerotolerant bacteria are gradually supplanted by strict anaerobes,

and 48h after birth, piglets already show 90% of anaerobic bacteria (Swords et al., 1993; Figure 2.1(A)).

Figure 2.1 (A). Evolution of aerobic and anaerobic bacteria in piglet feces from birth to 120 days of life (adapted from Swords et al., 1993).

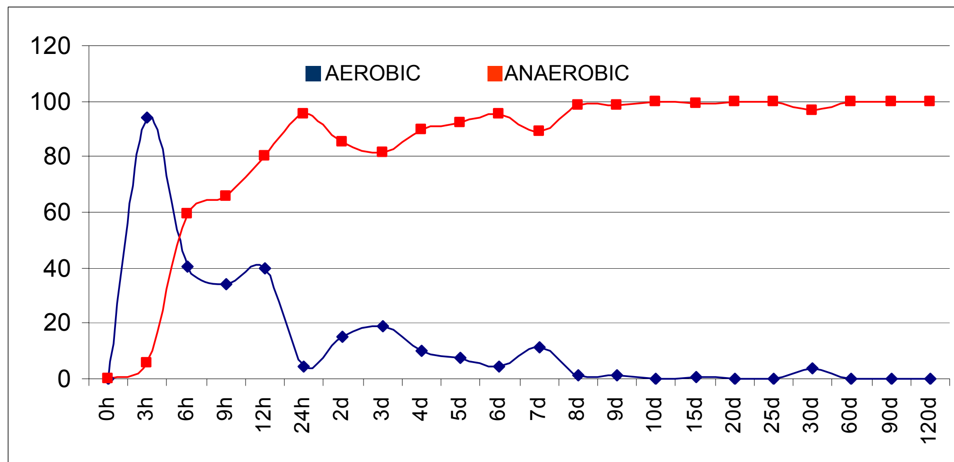
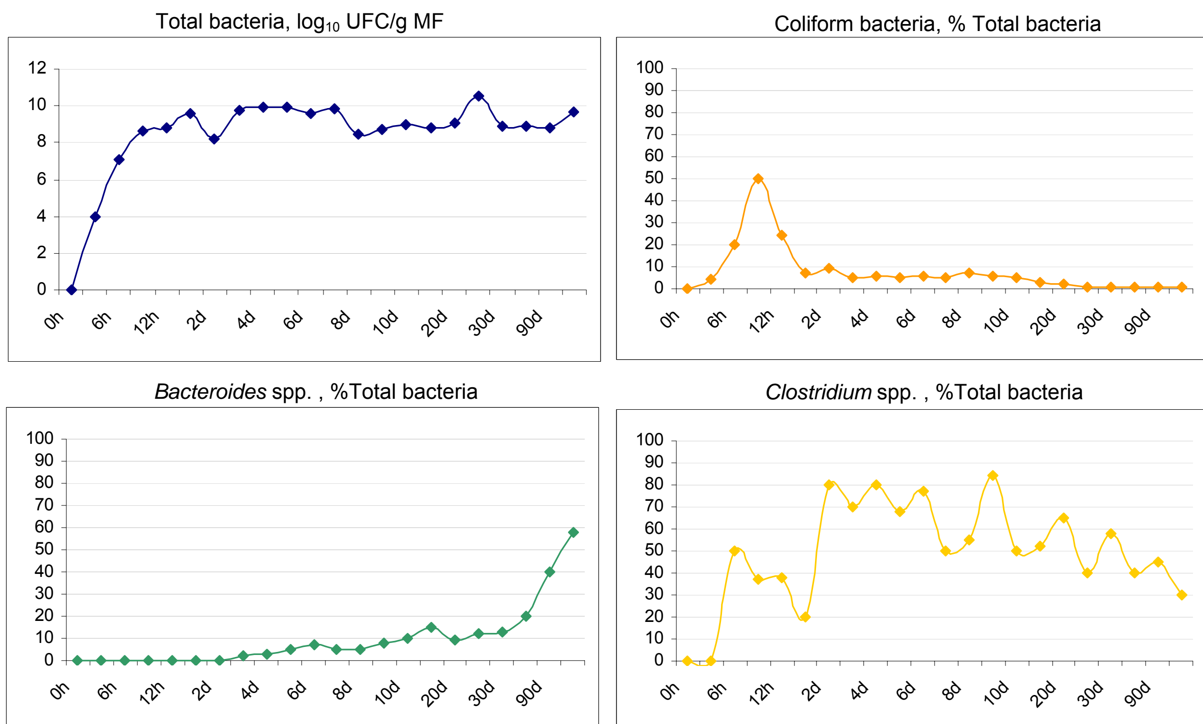


Figure 2.1 (B). Total bacteria counts and percentage of coliforms, *Bacteroides* spp. and *Clostridium* spp. in piglet feces from birth to 120 days of age (adapted from Swords et al., 1993).



Of these bacterial groups, lactobacilli and streptococci become the dominant bacteria at the end of 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; Ewing and Cole, 1994).

Microbiota remains fairly stable in terms of species composition during the second phase when the piglets receive milk from their mother (Drasar and Barrow, 1985; Mathew et al., 1998). The diversity of anaerobic bacteria increases in this period (Inoue et al., 2005) and supplantation of aerobic and facultative anerobic bacteria by anerobic bacteria becomes almost completed in this phase. As has been mentioned before, lactobacilli and streptococci continue being dominant bacteria, which are well adapted to utilize substrate from the milk diet. *Clostridium*, *Bacteroides*, *bifidobacteria*, and low densities of *Eubacterium*, *Fusobacterium*, *Propionibacterium* and *Streptococcus* spp. are also usually found in this second phase (Radecki and Yokohama, 1991; Swords et al., 1993).

2.1.2. Weaning: the adaptation to dry food

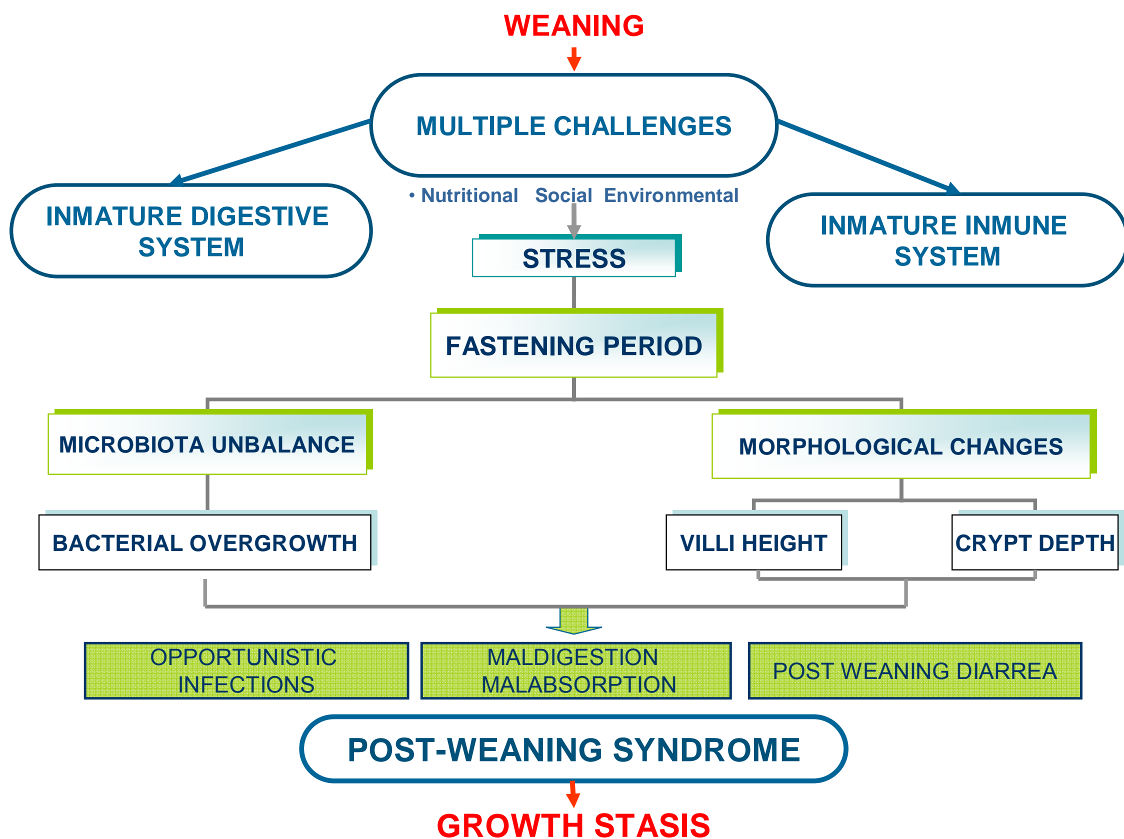
Modern pig production involves very early and suddenly weaning, usually at three or four weeks of life. At this moment, the piglet is subjected to complex social changes, including separation from its mother, separation from litter-mates and exposure to unfamiliar counterparts, environmental and nutritional changes (Fraser et al., 1998).

As a result, weaned piglets refrain from eating (Le Dividich and Herpin, 1994) and concurrently, profound changes in intestinal structure with associated disrupted functional capacity take place (Hampson, 1986; Pluske et al., 1997) which lead to growth stasis (McCracken et al., 1995, 1999; Figure 2.2).

In particular, anorexia leads to rapid changes in the microbiota as substrate available for microbial fermentation depletes. As a consequence, during the first week postweaning the microbiota becomes especially unstable, with a marked decrease in biodiversity (Wallgren and Melin, 2001) which will be restored after a reestablishment period of two or three weeks (Jensen et al., 1998). In this regard, increases in biodiversity have been reported 24 days after weaning (Inoue et al., 2005; Figure 2.3).

Swords and co-workers (1993) defined weaning as the start of the third phase in pig gut colonization process with the introduction of solid food with carbohydrates as the main energy source instead of lipids, and more complex chemical composition as the key factor in the microbiota change; major quantitative and qualitative changes are described immediately after piglets are weaned (Mathew et al., 1996; Jensen, 1998; Konstantinov et al., 2004a).

Figure 2.2. Review diagram of piglets post-weaning challenge.

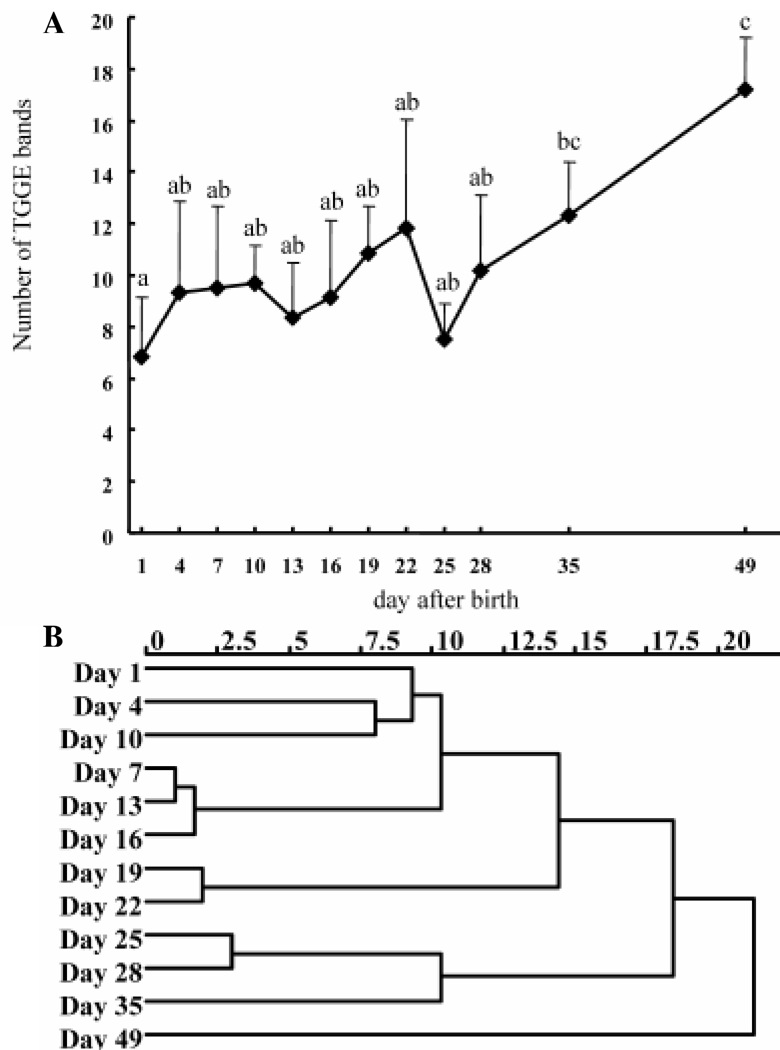


There is a decrease in total culturable bacteria after weaning (Franklin et al., 2002), with marked changes in some characteristics groups. Also, the third phase in pig gut colonization is characterized by the supplantation of the gram-positive anaerobes by members of the gram-negative genus *Bacteroides* which will represent one of the main bacteria populations in the adult pig (Swords et al., 1993). This agrees with Jensen (1998) 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 (Mathew et al., 1993, 1996; Jensen et al., 1998; Franklin et al., 2002). In fact, abrupt weaning has been associated with a 100-fold drop in the numbers of lactobacilli in the intestine, and a 50-fold increase in the numbers of *Escherichia coli* (Huis in't Veld and Havennar, 1993).

The main result of this microbiota disruption in the period immediately following weaning is that piglets become more susceptible to overgrowth with potentially disease-causing pathogenic bacteria (Hopwood and Hampson, 2003; Pluske et al., 2003).

Figure 2.3. (A) Diversity, expressed as number of bands obtained by Temperature Gradient Gel Electrophoresis (TGGE), of the intestinal microbiota in piglets from birth to 14 days after weaning. **(B)** Dendogram based on TGEE profiles of one piglet. Weaning takes places on day 25 (Inoue et al., 2005).



2.1.3. Autochthonous microbiota in the adult pig

After weaning, the normal adult flora develops and, in the healthy adult animal, it became stable and characteristic for each individual (Zoetendal et al., 1998; Simpson et al., 2000). This adult microbial "climax" is influenced by environmental factors as well as by host genotype with an increasing gradient of indigenous microbes from the stomach to the cecum (Ewing and Cole, 1994).

The stomach and small intestine contain relatively low numbers of bacteria compared with the lower gastrointestinal tract (10^7-10^9 CFU/g fresh matter, in Jensen and Jorgensen, 1994). The acidic conditions, the rapid flow of digesta and the rate of bacterial washout restrict the bacterial population in these sections. However, the ability of lactic acid bacteria to associate with the stratified squamous epithelial surface of the stomach (pars oesophagea) allows their colonization, and this is probably the reason why these bacteria become the predominant group in the upper gastrointestinal tract (mainly lactobacilli and streptococci; Jensen, 2001). Beside lactic acid bacteria, other groups like enterobacteria, *Clostridium*, *Eubacterium* and *Bifidobacterium* are also found (Melin, 2001; Conway, 1994).

In the distal small intestine, the environmental conditions slightly differ form the upper sections. The slower passage rate, the greater amount of digesta and a higher pH result in an increased density and diversity of bacteria. This section of the gastrointestinal tract is considered a transition zone preceding the large intestine (Jensen and Jorgensen, 1994). *Lactobacillus, Streptoccoci, Clostridium,* Enterobacteria*, Bacillus and Bacteroides* spp. are the most important culturable bacteria described (Conway, 1994; Jensen, 2001; Hill et al., 2005).

The cecum and colon are the major sites for bacterial fermentation in the pig gut, characterized by a high diverse population. The high amount of substrate, the slow digesta flow, the neutral pH and the low redox potential constitute the perfect environment for the development of a diverse and stable microbiota (Fonti and Gouet, 1989). Several hundred anaerobic bacterial species coexist (Pryde et al., 1999; Gaskins, 2003) with total counts of more than 10^{11}-10^{12} CFU /g digesta (Ewing and Cole, 1994). The majority of the culturable bacteria described in the pig cecum and colon are gram-positive anaerobes: streptococci, lactobacilli, eubacteria, clostridia and peptostreptococci. The gram-negative bacteria cover only about 10% of total

culturable bacteria, most isolates belonging to the *Bacteroides* and *Prevotella* groups (Russell, 1979; Salanitro et al., 1979; Moore et al., 1987; Table 2.1).

Table 2.1. Main bacteria traditionally cultured from the pig gastrointestinal tract (adapted from Stewart et al., 1999, in alphabetic order).

Bacteria
Bacteroides (Prevotella) ruminicola
Bacteroides fragilis, B. suis, B. uniformis, B. furcosus, B. pyogenes, B. amylophilus
Bifidobacterium adolescentis, B. boum, B. suis, B. therophilum, B. pseudolongum
Butyribibrio sp., B. fibrisolvens
Clostridium sp., C. putrificum, C. welchii, C. perfringens
Enterococcus sp., E. avium, E. faecium, E. faecalis, E. hirae.
Escherichia coli and other members of the *Enterobacteriaceace* family
Eubacterium sp., E. tenue, E. lentum, E. cylindroids, E. rectale
Fibrobacter succionogenes
Fusobacterium prausnitzii, F. necrophorum
Lactobacillus sp., L. acidophilus, L. brevis, L. crispatus, L. fermentum, L. johnsonii, L. agilis, L. amylovorus, L. reuteri, L. plantarum, L. delbrueckii, L. salivarius
Megasphaera elsdenii
Pediococcus halophilus
Peptostreptococcus anerobius
Propionibacterium acnes, P. granulosum
Ruminococcus sp., R. flavefaciens
Streptococcus sp., S. salivarius, S. bovis, S. morbillorum, S. intermedius, S. durans, S. equines, S. intestinalis

Recently, advances in molecular biology have greatly increased our knowledge of this complex ecosystem. In particular, may be remarked an elegant work by Leser and co-workers (2002; Table 2.2), who carried out an experiment where the pig gastrointestinal microbiota was extensively described by 16S rDNA sequencing.

Surprisingly, they found that 83% of the sequences amplified were unknown because had a <97% of similarity to any sequences in the database and therefore may represent yet-uncharacterized bacterial genera or species. This confirms again the high ignorance regarding microbial ecosystems that we still have today.

Despite this high percentage of unknown bacteria, functional groups of bacteria agreed to a great extent with culture results. The major phylotypes found belonged to the low-G+C gram-positive division (81%), and 11.2% were affiliated to the *Bacteroides* and *Prevotella* groups. In Table 2.2, the major phylogenetic lineages to which phylotypes were affiliated are shown.

Table 2.2. Major phylogenetic lineages to which the phylotypes from the porcine GI tracts were affiliated (adated from Leser et al., 2002).

Phylogenetic group[a]	No. of phylotypes detected	Similarity (%)[b]
Eubacterium and relatives	125	93.0
Clostridium and relatives	109	92.2
Bacillus-Lactobacillus-Streptococcus subdivision	46	96.7
Flexibacter-Cytophaga-Bacteroides group	42	87.5
Proteobacteria	20	94.8
Sporomusa and relatives	15	94.7
Mycoplasma and relatives	8	78.6
High-G+C bacteria	4	93.5
Spirochetes and relatives	2	86.4
Clostridium purinolyticum group	1	94.4
Planctomyces and relatives	1	86.0
Flexistipes sinusarabici assemblage	1	85.9
Anaerobaculum thermoterrenum group	1	84.3

[a] Phylogenetic grouping according to the Ribosomal Database Project.

[b] Mean similarity of all the phylotypes affiliated to that group to the most closely related sequences in the RDP alignment version 7.1.

Summary

The establishment of the pig gastrointestinal microbiota is a large and successional process that is influenced by several factors. It starts immediately after birth, when environmental bacteria begin gut colonization. However, commercial weaning, stresses the animal resulting in a disruption in the natural bacterial succession with both quantitative and qualitative changes. In consequence, the pig becomes more susceptible to overgrowth with potentially disease-causing pathogenic

bacteria. After this alteration, the normal colonization continues and in the healthy adult pig becomes a stable and characteristic ecosystem with *Eubacterium*, *Clostridium* and bacteria belonging to the *Bacillus-Lactobacillus-Streptococcus* subdivision and the *Cytophaga-Flexibacter-Bacteroides* group as the main bacteria.

2.2. Main functions of the indigenous microbiota in the gut

Introduction

The mammalian gut harbors a complex, dense, dynamic and spatially diversified community of non-pathogenic micro-organisms. Studies suggest that between 500-1000 different bacterial species colonize the adult intestine (Noverr and Huffnagle, 2004) with approximately 10^{14} bacteria (Pickard et al., 2004), ten fold higher than the total mammalian cells (Van Kessel et al., 2004). This ecosystem is an active metabolic unit that provides essential products to the host, forms a key barrier against pathogens and plays 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). These physiologic contributions are reciprocated by the provision of stable niches in the intestine for the bacteria, making the relationship between the host and its microbiota a true "mutualism" more than a "commensalism" as it has been traditionally described (Darveau et al., 2003).

This chapter focuses on the importance of the interaction between the microbiota and the host, paying special attention to the role of bacteria on gut morphology, and to the establishment of the gut barrier, nutrient digestion and immunity development.

2.2.1. Effects of indigenous bacteria on gut maturation and development

Gut microbiota influences gut structure, function and maturation (Berg et al., 1996; Falk et al., 1998). These effects are directly due to the presence of commensal bacteria and have been largely studied by comparing germ-free animals bred and kept in a sterile environment, with the same specie kept using conventional husbandry; mainly mice and rats. Comparisons have demonstrated several anatomic, physiologic and biochemical changes, attributed to the microbiota, that have been called microflora-associated characteristics (MACs; Midvedt, 1989; Box 2.1).

Morphologic changes found in the absence of microbiota include: a reduction in intestinal mass per unit length, intestinal thickness and length (Wostmann, 1996), and

an enlarged caecum with a thinner mucosa. The reduction in intestinal mass can be explained by a manifestly reduced cellularity of the lamina propia which contains fewer lymphocytes, plasma cells, and mononuclear cells (Van Kessel et al., 2004) which may be related to the lack of microbiota stimuli on immune response. On the other hand, the enlargement of the caecum is due to the accumulation of undegraded mucus (Gustaffson et al., 1970). Carlstedt-Duke (1986) demonstrated that the enlargement can be easily reversed by the monocolonization of germ-free rats with the mucolytic bacteria *Peptostreptococcus micros*.

At a histological level, the absence of microbiota is also related to thinner villi and shorter crypts, and as a consequence villi:crypt ratio increased (Umesaki et al., 1993, 1995; Wostmann, 1996). The shorter crypts are the reflection of a reduced mitotic index and a cell turnover rate in the intestinal epithelium of germ-free animals with a reduction in the number of cells (Alam et al., 1994). However, not all bacteria species exert the same effects on intestinal morphology. Recently, Lafuente and co-workers have demonstrated that whereas some commensal bacteria such as *Lactobacillus* may improve the tightness of the barrier, other commensal-non pathogenic species such as *Escherichia coli* may impair colonic barrier function and increase the colonic permeability to luminal toxins (García-Lafuente et al., 2001). In spite of this, individual effects of isolated bacteria species on the intestinal epithelium are probably not completely representative of the effect of these same bacteria in a complex microbial ecosystem where the function of each individual is modulated by the presence of the others.

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). Similarly to some other characteristics usually described in germ-free animals, it has been seen that after colonizing the animals with the normal caecal contents of a conventionally raised animal, the motility is restored (Huseby et al., 1994). One possible cause of this effect may be related with end-products of microbiota fermentation. Different research groups have studied the effect of short-chain fatty acids on gut motility, including systemic humoral and neural pathways as well as local reflexes and myogenic responses (Yajima, 1985; Cherbut et al., 1996, 1997). 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).

Box 2.1. Characteristics of germfree rodents compared with conventional rodents with an indigenous microbiota (Berg et al., 1996).

MORPHOLOGICAL CHARACTERISTICS

Increased cecum size
Decreased weight of intestinal wall
Decreased surface area
Thinner intestinal villi
Thinner lamina propia
Decreased size of liver, heart, adrenals…
Decreased blood volume

PHYSIOLOGICAL/BIOCHEMICAL CHARACTERISTICS

Decreased intestinal motility
Decreased rate of villus epithelial cell renewal
Altered mucosal enzyme patterns
Increased oxigen levels
Decreased basal metabolic rate
Decreased cardiac output
Decreased regional blood flow
Decreased sintesis of vitamin K and vitamin B complex
No bile acid transformation in intestines
Lack of short-chain fatty acids

IMMUNOLOGICAL CHARACTERISTICS

Decreased lymph node and spleen size
Decreased Peyer's patches size
Decreased serum immunoglobulins levels
Decreased numbers of inmunoglobulin-A-producing lymphocites in lamina propia
Decreased number of intraepithelial T cells
Decreased imflammatory response
Delayed immune response against antigenic challenge

2.2.2. Establishment of the gut barrier and colonization resistance

Besides the indigenous microbiota contribution 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 (Van der Waaij et al., 1989; Rolfe et al., 1996; Hooper et al., 1998). This process involves several different complex interacting mechanisms of both the bacteria and the host.

The host factors involved in colonization resistance are diverse: the peristaltic movement; the secretion of diverse digestive enzymes and electrolytes; the secretion of mucus; epithelial cell desquamation; the gut associated lymphoid tissue; and secretory IgA (Stewart et al., 1993). On the other hand, indigenous microbiota prevents bacterial colonization by competing for epithelium receptors (Blomberg et al., 1993; Bernet et al., 1994) and enteric nutrients (Stewart et al., 1999), producing antimicrobial compounds such as bacteriocines (Brook, 1999) and metabolizing nutrients to create a restrictive environment which is generally unfavorable for the growth of many enteric pathogens (Fons et al., 2000; Lievin et al., 2000).

Moreover, bacterial recognition and adhesion to receptors is not only a prerequisite for colonization, which determines microbiota composition and permanent colonization, especially in the upper gastrointestinal tract (Alander et al., 1999). It also determines antagonistic activity against enteropathogens (Coconnier et al., 1993), modulation of the immune system (Schiffrin et al., 1997) and also the improvement of healing in the damaged gastric mucosa (Elliot et al., 1998).

Several factors are involved in the control of bacterial attachment and thus in the modulation of the indigenous microbiota profile (Freitas et al., 2002). Special interest is nowadays focused on genetic modulation of receptors by the host and the bacteria, as we will see in the following chapters. Bacterial-mucose attachment appears consequently as a key point defining indigenous microbiota composition and different bacterial-mediated functions. Two main components are essential to the recognition between the host and the bacteria: the glycoconjugates on the gut enterocytes and bacterial adhesins.

2.2.2.1. Glycoconjugates of the mucosa as specific attachment site

The gastrointestinal epithelium is covered by a layer of mucus, which forms a barrier between the lumen content and the mucosa against chemical, microbiological and physical injury (Forstner and Forstner, 1994). The presence of this mucus barrier is also essential in the mechanisms of bacterial colonization and therefore in the colonization resistance process (van Dijk, et al., 2002).

Mucus is secreted by specialized epithelial cells called goblet cells and consists of a continuous layer (100-200 µm in thickness, Pullan et al., 1994) overlaying the epithelial surface (Specian and Oliver, 1991). The mucus is the result from non-covalent interactions between large and highly hydrated glycoconjugates that co-exist with other components such as water, peptides and surfactant phospholipids (Kindon et al., 1995; Matsuo et al., 1997). These high molecular weight glycoconjugates or mucins are the key molecules in the bacterial recognition by the enterocytes, and consist of a peptide core with many long side-chains of sugars (Mantle and Stewart, 1989). Different types of carbohydrate are involved: N-acetylglucosamine, galactose, N-acetylgalactosamine, fucose, N-acetylneuraminic acid or sialic acid, mannose, glucose and xylose. These glycoproteins are classified as either N- or O-glycoproteins. In N-glycoproteins the oligosaccharide is attached to the nitrogen atom of the lateral chain of asparagine whereas in O-glycoproteins the oligosaccharide is attached to the oxygen atom of the lateral chain of serine or threonine (Mouricout and Julien, 1987).

The structural diversity of these carbohydrate structures on mucin macromolecules and the different linking ways, becomes in a huge different target recognition sites for adhesion of both commensal and pathogenic bacteria. When indigenous bacteria are recognized by these receptors and occupy those, avoid the attachment of newly incoming bacteria potentially pathogenics, retarding access of microorganisms to mucosal surface (Forstner and Forstner, 1996).

In addition to the colonization resistant effect, the ability to bind to mucin carbohydrates enables some bacterial groups to colonize the mucus layer, favoring its establishment in the gut (Lu and Walker, 2001).

Factors affecting gut glycoconjugates

The epithelial cell receptors are host specific and are strongly affected by several factors: genetics (Falk et al., 1998), cell maturity (Specian and Oliver, 1991), the portion of the digestive tract involved (Barrow et al., 1980), the age of the host (Dean, 1990; Turck et al., 1993), and the diet administered (Kotarski and Savage, 1979; Turck et al., 1993; Sharma and Shumacher, 1995). These factors result in changes in susceptibility to colonization (King, 1995; Stewart et al.,1999).

Different composition has been related to cell maturation. Immature goblet cells produce mucins containing little sialic acid, and as they mature and migrate to the villus tip, the sialic acid residues increase (Specian and Oliver, 1991). Age related changes have also been found. A progressive change from α2,6 sialylation to α1,2 fucosylation of microvillar glycoconjugates occurs during postnatal development in pigs (Kelly and King, 1991; King et al., 1993). Turck and colleagues (1993) found differences in fucose, glucosamine and sulphate contents of glycoconjugates when comparing suckling with artificially fed piglets.

In recent years, special attention has been focused on the ability of microbiota to modulate the expression of glycoconjugates by the host.

Recent studies suggest that the host epithelial cell can express specific glycoconjugates in response to the presence of bacteria (DDai, unpublished observations, 2000; in Lu and Walker, 2001). Therefore, the gut microflora appears to be the most responsible for: a) initiating production of host cellular glycoconjugates needed for particular genera to join an intestinal niche (Umesaki et al., 1995; Freitas et al., 2002), and b) to modulate the gut glycosylation pattern, both quantitatively and qualitatively by changing distribution of glycans (Freitas et al., 2002) and consequently modifying potential sites for attachment.

This phenomena forms part of the “cross-talk process” that take place between the host and its indigenous microbiota (Hooper and Gordon, 2001). Sharma and Shumacher (1995) found that the presence of a determined microflora influences the relative proportions of sulphated and sialylated types of mucins, and similarly some recently investigations have demonstrated an exchange of biochemical signals, in the form of soluble molecules, between *Bacteroides thetaiotaomicron* and the mice enterocytes. This factor could cause alterations in fucosylated glycoconjugate production through the induction of a host α1,2 fucosyltranseferase (Bry et al., 1996).

These host-induced mucin modifications may potentially modify colonization of different bacteria, and also provide cellular fucosylated glycoconjugates that may be used as an energy source by this bacteria. This could be a selective advantage when competing with other bacteria for a niche with limited resources (Salyers et al., 1982). In addition, it seems that bacteria have the ability to decide the best moment to start host-induced modifications; Hooper and co-workers (1998) demonstrated that a regulatory mechanism would allow different bacteria to achieve a consensus on whether the intestinal environment has been adequately colonized before making the energetic investment required for the initiation of this complex metabolic response that involves modification of host properties.

Besides modification of genetic expression glycoconjugates, mucins may also be altered by bacterial endo- and exo- glycosidases. It has been described that some strains of *Ruminococcus*, *Bifidobacterium*, *Clostridium* and *Peptostreptococcus* hold a high quantity of glycoside hydrolases, that as a result, may degrade the oligosaccharide chain of mucins. This leads to the creation or abolition of specific adhesion sites that may modify potential colonization, and also produces smaller sugars that become available for other bacteria that are unable to digest the glycoconjugates by themselves (Hoskins et al., 1992; MacFarlane et al., 1999).

2.2.2.2. Molecules involved in bacterial adhesion

As described above, for the permanent colonization of the gut, the attachment of indigenous bacteria to glycoconjugates is essential. However, although much effort has been done to dilucidate the mechanisms of attachment to the intestinal wall, the adherence of indigenous bacteria to the intestinal mucosa is not entirely known. The interaction can be both specific by recognition of glycoproteins or glycoconjugates and inespecific involving complex mechanisms including bacterial motility, chemotactic attraction, and non-specific attachment to the mucus gel (Kelly et al., 2005).

Different bacterial surface elements are involved in the attachment of bacteria to the mucopolysaccharides. These elements include molecules of protein type, such as outer membrane proteins and fimbriae (pili) which are described in the major part of gram negative bacteria (Costerton et al., 1981). Other fimbrial structures and

fimbriosomes have been described in some hyperadhesive strains (Abraham et al., 1985). Specifically, mannose sensitive fimbriae, also called type-1 fimbriae, which are associated with several bacteria from the *Enterobacteriaceae* family, including *Esherichia*, *Klebsiella*, *Shigella* and *Salmonella* spp. (Knutton et al., 1985, 1987; Clegg and Gerlach, 1987). Other fibrilar structures have also been described in enterotoxigenic *E. coli* K88 (Bijlsma and Bouw, 1987).

Some other bacteria present non-protein type adhesines. Most are polysaccharides of the capsule or slime as lipotechoic acids that are mainly present in gram positive bacteria (Sato et al., 1982; Contrepois et al., 1988). The surface of gram-positive bacteria such as bifidobacteria, streptococci and staphylococci presents a linear polyglycer-phosphate anchored to the cytoplasmic membrane which is known as lipoteichoic acid (Poxton and Arbuthnott, 1990; Kelly et al., 1994).

2.2.3. Effects of indigenous microbiota on immune response

It is well known that the resident microbiota also affects immunity in the host, since it is usually described as the major source of antigenic material for the animal. It is especially important in early life when the immune system is still not completely developed, particularly in piglets, that are born with the immune system immature. Newborn piglets depend completely on the passive transfer of maternal antibodies by calostrum and milk and do not develop an active immunity until 4-7 weeks of age (Stokes et al., 1992; Gaskins and Kelley, 1995).

Considering this fact and that commercially reared piglets are early weaned at 3-5 weeks, the knowledge of the host-bacteria cross-talk in relation to the host immune system acquires special importance. This bacterial stimulus is especially important in early life, in order to prime the immune system in the correct way and for the whole life life and to maintain a functional immune system (Kelly and King, 2001).

Studies comparing germ-free and conventional-reared animals have demonstrated that the presence of bacteria in the gastrointestinal tract strongly influences the maturation and development of local and systemic immunity (Cebra et al., 1999). Particularly, commensal bacteria play a key role in the development of the gut associated immune system (Fioramonti et al., 2003). In the absence of microbiota, the animal mucosal-associated lymphoid tissue is underdeveloped, with defects of cell-

mediated immunity (MacDonald and Carter, 1979). The numbers of lymphocytes in the lamina propia are decreased; and intestinal lymphoid aggregates, Peyer patches and lymph nodes also decrease in size (Umesaki et al., 1993; Wostmann, 1996); macrophage chemotaxis and phagocytic activity are inhibited (Starling and Balish, 1981); and the immunoglobulin class profile is also altered, with much lower concentration of IgG and low or no production of IgA (Wostmann et al., 1996).

Despite these marked effects of indigenous bacteria on immune response, the exact mechanisms by which microbial colonization modulates the immune system are still largely unknown (Cebra et al., 1999); even though it is thought that they involve complex events that are probably triggered following the route of antigen uptake and processing (Kelly and King, 1994).

In a similar way, the precise mechanism by which the immune system does not over-react against indigenous bacteria, and becomes “tolerant” is not completely known. It seems clear that the immediate acquirement of microbiota during the post-natal period is essential for the development of tolerance to indigenous bacteria and also to other luminal antigens. The presence of pattern recognition receptors in immune and epithelial gut cells may be behind this adaptative process (Hooper et al., 2001; Shanahan, 2002).

2.2.3.1. Commensal bacteria tolerance-ignorance

Dilucidation of how the commensal bacteria tolerance is established, retaining the capacity to respond to pathogens seems the key to clarify the described effects of resident bacteria on immune system development. Although much effort is focused on this field, it is still not clear the exact mechanisms involved.

There are several host factors in a healthy immune system that control bacterial community within the gut lumen and gut wall: the mucus layer described above; the Peyer patches; organized lymphoid tissues that contain B lymphoid follicles and interfollicular populations of T cells; and the gut lamina propia that presents a broad spectrum of lymphoid cells, especially IgA plasmablasts, T cells, and dendritic cells, and the intraepithelial leukocytes (Cebra et al., 1999; Stokes et al., 2001). The role of the secretory IgA which promotes bacterial niches formation but also limits the expansion and translocation of pathogens is especially important (Bollinger et al.,

2003; Suzuki et al., 2004) as are the antimicrobial peptides produced by Paneth cells in the intestinal crypts (Ayabe et al., 2004).

Among these host factors, recognition of bacteria by immune and epithelial gut cells seems to be the key in the tolerance-establishment process. It relies on a wide repertoire of specific receptors, which recognize highly conserved structures of microorganisms (pathogen-associated molecular patterns; PAMPs) called pattern recognition receptors (PRRs, Kopp and Medzhitov, 1999).

The nucleotide-binding oligomerization domain molecules (NODs) and Toll-like receptors (TLRs) are pattern recognition receptors. There are several examples of TLRs that have been described to respond to bacterial stimulus: lipotheichoic acid and peptidoglycan of gram positive bacteria are recognized by TLR2; lipopolysaccharides of gram negative bacteria are recognized by TLR4; and flagellins and bacterial DNA are recognized by TLR5 and TLR9 respectively. When a pathogen is recognized it results in signaling of immune cells (Akira et al., 2001; Schiffrin and Blum, 2002), and immediately starts the synthesis of antimicrobial peptides, cytokines and chemokines, and dendritic cells are also activated to eliminate it (Netea et al., 2004, Kelly et al., 2005). In this regard, it has been recently demonstrated that TLRs are coupled to signal transduction pathways that control expression of a variety of inducible immune-response genes (Kopp and Medzhintov, 1999).

However, whereas the immune system reacts against potential pathogens recognized by the different TLRs, evidence suggests that when an indigenous bacteria is recognized by a specific receptor, there is a tolerance against commensal microbiota without signaling the immune system to eliminate it. Therefore, it seems that this bacterial recognition by TLRs may be the key in the host ability to discriminate between pathogen and commensal bacteria. Although the exact mechanism is not known yet, different hypothesis have been proposed to clarify the tolerance process. One explains indigenous tolerance by the presence of difference traits (PAMPs) in commensal bacteria that might be absent in pathogens (Schiffrin and Blum, 2002). Matzinger (1998) postulated that in addition to this first recognition by TLRs, that would be common to pathogens and commensal bacteria, a second signal might also be included to initiate the appropriate response to pathogenic bacteria. Other studies suggest that lymphocytes may downregulate pro-imflamatory responses by intestinal epithelial cells to commensal bacteria (Haller et al., 2002) and

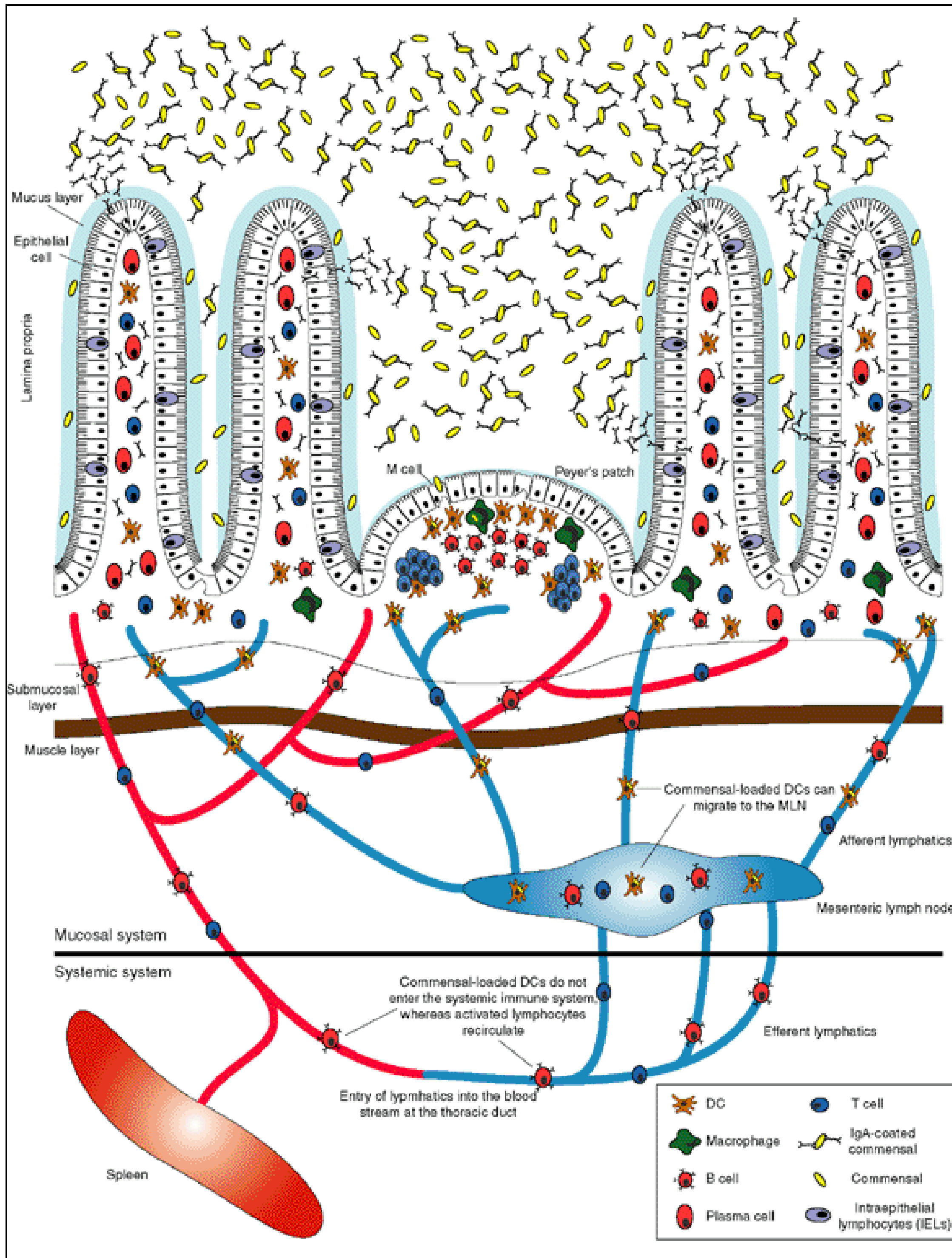
recently, Kelly and co-workers (2005) suggested that the absence of some of the members of the TLRs family on the apical surfaces of epithelial cells might contribute to the tolerance of the gut towards its microbiota.

However, it is important to remark that, in contrast with this local immune tolerance against commensal bacteria described above, Macpherson and co-workers (2005) demonstrated recently that the host systemic immune system remains naïve to resident bacteria. It results in an effective immune response when an indigenous bacteria leaves the gut. They found that pathogen-free mice did not have specific IgG against *Enterobacter cloacae* (a dominant member of its commensal flora) but it was induced after intravenous injection of live micro-organisms. By this way, the host preserves the ability to mount an effective systemic response against commensal epitopes when necessary. It can be achieved by the compartmentalization that the immune system has. When dendritic cells picks up commensal bacteria, they can travel only from Peyer's patches to the mesenteric lymph nodes without re-circulation within the body (Macpherson et al., 2005; Figure 2.4).

2.2.4. The role of microbiota on digestion and absorption of nutrients

The microbiota plays a very important role in the digestion of the dietary compounds that are not degradable by the pig endogenous enzymes, especially in the large intestine, where materials are retained for prolonged periods of time. Therefore, bacterial interaction with the host differs in the upper and lower gastrointestinal tract. Whereas in the proximal gut, bacterial competition for absorbable nutrients could be more detrimental than beneficial to the host, microbial digestion of non-digestible dietary residue that reach the distal gut (mainly carbohydrate polymers) is beneficial to the host because it extracts nutrient value from otherwise poorly utilized dietary substrates. Studies comparing conventional with gnotobiotic animals have proved that in germ-free animals, while utilization of polysaccharides is less complete, the utilization of dietary lipid is more efficient. Also, amino acid absorption and mineral absorption seems to be affected (Fuller and Reeds, 1998, and reviewed by Tannock, 1999).

Figure 2.4. Intestinal immune geography of responses to commensal bacteria. Commensal bacteria are largely restricted from gaining access due to the physical epithelial and mucus barriers (Macpherson et al., 2005).



2.2.4.1. Carbohydrate utilization by indigenous bacteria

Carbohydrates are the main energy substrate for bacteria, as a result of the inability of mammals to produce enzymes capable of degrading dietary fiber (the sum of polysaccharides and lignin and resistant starch which are not digested by the endogenous secretions of the gastrointestinal tract (Trowell et al., 1976; Englyst, 1989). The digestion of those compounds depends totally on the activity of different bacteria that produce saccharolytic enzymes, cellulases, hemicellulases, pectinases and xylanases (Salyers et al., 1977; Varel and Yen, 1997).

Pig microbiota harbors different highly cellulolytic and hemicellulolytic bacterial species as *Fibrobacter succinogenes*, *Fibrobacter intestinalis*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, *Butyrivibrio* spp., and *Prevotella ruminicola* (Varel and Yen, 1997). Other carbohydrate substrates such as B-glucans and pectins are preferently fermented by lactobacilli (Graham et al., 1986) which are specially important in the hindgut (Hill et al., 2005).

The fermentation of carbohydrates in the pig colon results in the production of high SCFA concentrations (70 to 100 mM) , lactic acid and gases (hydrogen, carbon dioxide, methane) (Bach Knudsen et al., 1991) varying in concentration and relative proportions depending of the gastrointestinal section. Whereas lactic acid is the main organic acid in the stomach and small intestine, SCFA 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 et al., 1991).

Short chain fatty acids production is affected by several factors such as: the type and chemical structure of polysaccharides; substrate redox and availability; and gastrointestinal tract transit time and microbiota (Allison and MacFarlane, 1989; Wang et al., 2004).

SCFA are rapidly absorbed from the gut lumen (Argenzio and Southworth, 1974). Acetate reaches the systemic circulation and acts as an energy substrate for muscle tissue (Cummings and Englyst, 1987), and propionate is converted to glucose in the liver (Montagne et al., 2003). Of special interest is butyrate which is the main energy substrate for colonocytes and promotes a normal phenotype in these cells. SCFA production is related to a normal large bowel function and to prevention of pathology, through its action in the lumen and on the colonic musculature and vasculature

(Topping and Clifton, 2001). Their contribution to maintenance energy requirement has been estimated as 15 to 24 % in finishing pigs (Dierick et al., 1989; Yen et al., 1991; McBurney and Sauer, 1993).

Besides the contribution of SCFA to energy pig requirements and colonocytes nutrition, other implications in animal health have been described. Diarrhea is limited as SCFA stimulate the reabsorption of water and sodium (Roediger and Moore, 1981), and because, especially in acidic conditions, high contentrations of SCFA have been inhibit the growth of certain opportunistic pathogens as *Salmonella*, *Clostridium difficile* and *Escherichia coli* growth is inhibited by SCFA (Prohaszka, 1986; May et al., 1994).

2.2.4.2. Protein utilization by indigenous bacteria

Microbiota can use nitrogen from dietary nitrogenous compounds as well as enzymatic secretions of the host, mucin, and sloughed epithelial cells (Yen, 2001). Bacteria also have the ability to utilize N not only in the form of protein but also from other organic or inorganic sources. In particular, urea coming from plasma can be efficiently utilized by bacteria for the synthesis of their own proteins; this is confirmed by the high amount of urea found in the colon of germ-free rats (Moreau et al., 1976; Forsythe and Parker, 1985). Degradation of protein by bacteria in the small intestine seems to be scarce. Salter (1984) found a similar degree of protein digestion at the end of the small intestine of pigs reared in germ-free conditions compared with conventional pigs, although other authors have found some amino acid degradation produced by upper gastrointestinal tract bacteria that would diminish their availability to the pig (Gaskins et al., 2001). In the large intestine proteolytic fermentation is very important in contrast to small intestine. As carbohydrate sources become depleted due to fermentation by bacteria, the fermentation changes and becomes more proteolytic (Piva et al., 1995).

In the large intestine numerous bacterial species may use peptides and aminoacids as a source of carbon, nitrogen and energy. As a result, branched-chain VFAs are formed by the use of branched chain amino acids valine, leucine and isoleucine (MacFarlane et al., 1992).

However, this proteolytic fermentation can also lead to the formation of potentially toxic metabolites such as NH3, amines, phenols and indols (Russell et al., 1983; MacFarlane et al., 1992; Williams et al., 2001).Bacteria belonging to the genera *Bacteroides*, *Clostridium*, *Enterobacterium*, *Lactobacillus* and *Streptococcus* possess the ability to produce amines by decarboxilation of amino acids (MacFarlane and MacFarlane, 1995). Aromatic aminoacids are metabolized into phenols and indols compounds, mainly by bacteria from *Bacteroides*, *Lactobacillus*, *Clostridium* and *Bifidobacterium* genera (MacFarlane and MacFarlane, 1995).

A wide range of intestinal bacteria possess urease activity although studies have not been conducted on the pig. An excess in urease activity compared to the ability of bacteria to synthesize new protein can lead to an increase in ammonia. 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; Nousiainen, 1991).

2.2.4.3. Lipid utilization by indigenous bacteria

Despite the high body of evidence of microbial collaboration in carbohydrate digestion, little attention has been given to the digestion and metabolism of lipids by the commensal bacteria.

Especially important is the metabolism of bile acids produced by the intestinal microbiota. Microbial deconjugation and dehydroxylation of bile acids impair lipid absorption by the host animal (Eyssen, 1973) and produce toxic degradation products, as certain secondary bile acids are citotoxic and potentially carcinogenic (Baron and Hylemon, 1997). It has been found that *E. coli*, *Bacillus cereus*, *Streptococcus faecalis*, *Bacteroides spp*., *Eubacterium* spp., and *Clostridium* spp. have the ability to dehydroxilate bile acids. Studies with germ-free mice have demonstrated that lactobacilli contribute at least 74% of the total conjugated bile acid hydrolase activity (Tannock et al., 1989).

Several bacteria such as *Clostridium* spp, *Eubacterium lentum*, *Peptostreptococcus* spp. and *Ruminococcus* spp. also have different dehydrogenases capable of bile acid transformation. Advantages of metabolising this substrate may rely on the energy obtained from bile acid transformation, but more probably on the

growth inhibition of competing bacteria due to the toxicity of some of the compounds released (Baron and Hylemon, 1997). Deconjugation of bile acids can affect negatively the digestion of dietary fatty acids as they act as emulsifiers, facilitating their process of absorption. In this regard, digestion of lipids in gnotobiotics rats has been found to be higher that in normally reared animals (Fuller and Reeds, 1998, reviewed by Tannock, 1999).

Moreover, microbiota increases biohydrogenation of unsaturated fatty acids, resulting in a relative high proportion of stearic acid that is less well absorbed (Yen, et al., 1991). Cholesterol, dietary sterols, and other lipids are also altered by microbiota in the large intestine (Ratcliffe, 1991). Cholesterol is reduced to coprostanol and coprostanone by the microbiota. Germ free rats excrete unmodified cholesterol whereas conventional reared rats excrete coprostanol and coprostanone in amounts of up to 55% of the total fecal sterols (McNamara et al., 1981). Different bacteria belonging to the *Eubacterium* genus such as *Bacteroides*, *Bifidobacterium* and *Clostridium* possess the ability to metabolize cholesterol to coprostanol (Baron and Hylemon, 1997).

Summary

Mammals have co-habited with gut bacteria during thousand of years. This co-evolution has become a narrow relationship with an established balance between the eukaryotic and prokaryotic cells. Different studies have demonstrated that a continuous cross-talk exists between them. It results in several beneficial effects for both, becoming in a mutualist association. Bacteria achieve steady niches in the gut, with a stable environment and nutrient afford which is reciprocated to mammalian host that obtains several benefits such as protection (immune system development and homeostasis, the barrier effect), trophic (gut evolution and maturation) and nutritional effects.

2.3. Modulation of intestinal equilibrium through the feed

Introduction

Since the 1940's, when antibiotics were first used as growth promoters, commercial pig diets have been regularly fortified with antibiotics in prophylactic doses to prevent gastrointestinal disorders and to improve growth rate and feed efficiency (Cromwell, 2002; Mroz, 2003). Currently it is known that the efficacy of growth promotant antibiotics is mainly due to modification of the microbial ecosystem and to subsequent direct and indirect effects on the host animal. However, antibiotic specificity for microbial populations differ, and neither their effects on specific bacterial populations nor their exact mode of action promoting animal growth are completely defined (Gaskins et al., 2002).

Recent concerns regarding cross-resistance of pathogens in human therapy (Hillman, 2001) have led to the total withdrawal of antibiotics as growth promoters in the European Union. The first consequences of the ban are appearing already, with lower post-weaning daily weight gain and a higher prevalence in post weaning diarrhea (Casewell et al., 2003). In the light of these results, and since the first restrictive actions by the European Union were carried out, huge efforts are being made to seek alternative or replacement strategies for controlling enteric bacterial diseases by the maintenance of the piglet gastrointestinal ecosystem.

This chapter will focus on the major feed strategies that currently are used to manage the pig gastrointestinal ecosystem with special attention to those used in young pigs.

2.3.1. Macro-ingredients

The main source of growth substrate for the gastrointestinal microbiota comes from the diet; thus, the single and most important control for the bacterial gut ecosystem may be the modification of the amount and type of substrate available for its use. This allows a direct and simple control over the process of fermentation in the gastrointestinal tract through pig feed composition (Jensen et al., 2003) that produces changes in microbiota and in the dominant bacteria inhabiting the gastrointestinal

tract (Conway, 1994). Specifically simple sugars are the main growth substrates to bacteria in the upper gastrointestinal tract, whilst in the large intestine, where the major biomass is located, dietary fiber is the major substrate for pig gut microbiota (Bach Knudsen et al., 1991; Hampson et al., 2001).

2.3.1.1. The role of dietary fiber

Dietary fiber was first defined by Trowell et al. (1976) as “the sum of lignin and polysaccharides that are not digested by endogenous secretions of the digestive tract in the man”. The concept is applied also in monogastric animals. It consist of mainly non-starch polysaccharides (cellulose, hemicellulose, xylans, beta-glucans, fructans, mannans, pectins) resistant starch and lignin (Conway, 1994; Bach Knudsen, 2001). Although non-digestible oligosaccharides can also be included in the dietary fiber definition, they will be exposed in the prebiotics chapter as usually are classified in this group (Gibson and Roberfroid, 1995).

Starch and NSP (non-starch polysaccharides) are the main plant polysaccharides. Pig lacks of endogenous enzymes capable of degrading NSP and, although amylose and amylopectine from starch are susceptible to be hydrolysed by pig gastrointestinal enzymes, usually these compounds do not reach complete hydrolysis. The part of this starch that is not digested, named resistant starch, together with NSP reach the lower gastrointestinal tract where are susceptible to bacterial fermentation (Montagne et al., 2003). Starch may be resistant to enzymatic hydrolysis for three reasons that have determined its classification into three main types: RS1 includes resistant starch trapped within whole plant cells and food matrices, thus physically inaccessible to the enzymatic host package; RS2 comprises poorly gelatinised starch granules that are highly resistant to digestion by α-amylase; and RS3 comprises retrograded starch (Englyst et al., 1992).

Different factors influence the response of microbial fermentation to fiber administered in the diet. The most important are the source of dietary fiber (its solubility, degree of lignification and processing) and the level of inclusion in the diet (Bach Knudsen and Hanse, 1991; Macfarlane and Cummings, 1991, Jensen, 1998).

Although the ability of dietary fiber to modulate the gastrointestinal microbiota has been clearly demonstrated, there is still a lack of knowledge about the specific

effect of different types and amounts of fibre on particular microbial groups and about its rational use to promote the establishment of a gut health promoting indigenous microbiota. Different authors have studied the influence of different types and doses of fiber in the pig diet, denoting changes in the composition and metabolic activity of the large intestinal microbiota in pigs.

Several works have shown how increases in fiber content in the diet can modify total microbial load and total bacterial activity throughout the gastrointestinal tract. In this regard, Bach Knudsen and co-workers (1991) in a trial where diets with different sources and levels of wheat and oat dietary fibre were administered, found marked changes in total microbial activity throughout the pig gastrointestinal tract. In agreement, Jensen and Jorgensen (1994), when administering a high–fiber diet (based on barley supplemented with pea fiber and pectin) to adult pigs, found an increase in the amount of total culturable bacteria in the stomach, and a higher total microbial activity in all segments of the hindgut. Modulation of microbiota activity has been confirmed by other authors. Varel and Yen (1997) found that the administration of a high-fiber diet increases total bacteria activity, as demonstrated by the 5 times greater ATP quantity, and 5 to 9 times CO_2 and CH_4 produced in the gastrointestinal tract of pigs. In a similar study from our group (Morales et al., 2002),we found that administration of diets rich in maize or sorghum and acorn produces differences in bacterial enzymatic activities in the lower gastrointestinal tract; once more showing ability of microbiota to adapt to substrates offered. Recently, Martinez-Puig and co-workers (2003) have also found increases in total bacteria activity, measured as purine bases content, when potato starch (highly resistant) was administered to growing pigs, compared to corn starch. Pigs fed the potato starch diet also showed a greater SCFA concentration in the hindgut than pigs fed corn diet.

In addition to changes in total bacteria loads and activity, the ability of microbiota to adapt by changing in species composition has also been shown. The swine gut harbors highly active ruminal cellulolytic and hemicellulolytic bacterial species [*Bacteroides succinogenes, B. intestinalis* (currently re-classified into the *Fibrobacter* genus), *Ruminococcus flavefaciens, R. albus, Butyrivibrio and Prevotella ruminicola*], which indicates the high potential that pigs have to profit from dietary fiber by microbiota utilization (Varel et al., 1982, 1985). In response to an increase in dietary fiber, the microbial ecosystem is able to adapt by increasing total cellulolitic

populations (Varel and Pond, 1985; Table 2.3). Moreover, when adult sows were fed with a high-fiber diet (35% dehydrated alfalfa meal) changes in particular species were found, with increased numbers of *Ruminococcus* and *Bacteroides* compared to animals receiving a low-fiber diet (based on corn and soybeans; Varel et al., 1984).

Table 2.3. Number of cellulolytic bacteria from fecal samples of sows fed diets containing various levels of fiber (Varel and Pond, 1985).

Days on diet	Cellulolytic bacteria (x 10^8 CFU /g dry matter)			
	Control	20% corn	40% alfalfa	96% alfalfa
0	14.7	6.0	10.8	14.1
5	10.1	10.2	34.4	56.5
14	22.4	17.5	18.8	24.2
21	28.4	16.9	41.3	71.0
35	27.8	16.3	105.3	54.9
49	24.6	32.8	43.5	76.3
70	25.0	9.3	56.5	59.3
98	33.3	12.5	50.2	63.7
Overall	23.3^b	15.2^b	45.1^a	52.5^a

$^{a, b}$ Means with different superscripts differ ($p < 0.05$)

The administration of different vegetal sources of starch (more or less resistant) has also been related with specific changes in bacteria species. When potato starch, corn and waxy corn were administered to young pigs, shifts in the gastrointestinal ecosystem were found, as was recently demonstrated by MacFarland (1998; Table 2.4). Animals fed with potato starch had significantly lower coliform and *E. coli* population in relation to the other two starches. Moreover, combinations of these starches produced intermediate effects in comparison with individual starches, suggesting that this form of manipulation should have the potential to accurately control microbial population within the gut.

Direct modification of starches as amylose/amylopectine ratio and retrogradation has also shown potential to modify the gastrointestinal ecosystem composition. Reid and Hillman (1999) found marked decreases in total anaerobic counts when diets were supplemented with a high amylopectine content starch, and increases in

Lactobacillus spp., when animals were fed a diet rich in retrogradation of amylopectine-rich starch. Moreover, retrogradation decreases the coliform population, which was reflected in a high lactobacilli:coliform ratio in distal colon, specially with the high amylopectine starch.

In a similar way, a recent work of Martínez-Puig and co-workers (2006) demonstrated marked different microbial patterns in hingut digesta after feeding growing pigs with potato or maize starch. T-RFLP profiles showed different microbiota depending on the diet administered.

Table 2.4. Selected bacterial counts (CFU / g wet weight) in the proximal colon of weaned piglets fed meal diets containing different source of starch (MacFarland, 1998, reviewed by Hillman, 2001).

	Potato	**Waxy Corn**	**Corn**	**Potato/ Waxy Corn**	**Potato/ Corn**	**Corn/ Waxy Corn**
Total anaerobes	9.82^{a}	8.66^{b}	8.88^{b}	10.05^{a}	9.86^{a}	9.56^{ab}
Coliform bacteria	1.90^{a}	6.93^{b}	7.88^{b}	6.12^{b}	4.24^{ab}	7.12^{b}
Escherichia coli	1.73^{a}	6.62^{bc}	7.39^{c}	5.99^{bc}	3.64^{ab}	6.52^{bc}
Lactobacillus spp.	9.62^{a}	8.62^{b}	8.76^{ab}	9.67^{a}	9.67^{a}	9.17^{ab}
Enterococcus spp.	5.20^{a}	6.44^{ab}	7.43^{b}	6.11^{ab}	4.69^{a}	6.19^{ab}
Bacteroides spp	7.16^{a}	7.43^{ab}	7.16^{a}	8.27^{bc}	8.54^{c}	7.17^{a}

$^{a,\,b}$ Means with different superscripts differ ($p< 0.05$).

Although potential benefits on gastrointestinal microbiota can be related to different types and amount of fiber added into the pig diet, today consensus regarding its inclusion in the diet, particularly of young animals, does not exist. This lack of consensus is due to controversial results regarding inclusion of different sources of dietary fiber in the diet and the occurrence of gastrointestinal disorders such as post-weaning colibacillosis and swine dysentery. Post-weaning colibacillosis (PWC) is the main intestinal disorder in the immediate post-weaning period, and although multifactorial, it is associated with proliferation of enterotoxigenic haemolytic *E. coli* in the small intestine. In growing pigs, swine dysentery is one of the most important diseases. It is caused by *Brachyspira hyodysenteriae*, which produces colitis in the lower gastrointestinal tract (Pluske et al., 2002). A few years ago, it was suggested

that the administration of fiber from oats, wheat, and barley supports protection against proliferation of enteropathogen *E. coli* and the occurrence of PWC in piglets (Thomlinson and Lawrence, 1981) and also the insoluble fiber limited the severity of PWC (Bertschinger and Effenberger, 1978). However, recent studies suggest that diets high in fermentable carbohydrate sources, such as soluble NSP in weaner diets, are detrimental to post-weaning growth and also have a positive correlation post-weaning colibacillosis occurence. McDonald and co-workers (1997, 1999, 2001) reported an increased intestinal proliferation of *E. coli* in piglets infected experimentally when they were fed fiber enriched diets (guar gum and pearl barley), and in non-infected piglets when they were fed carboxymethylcellulose.

Similarly, the effect of dietary fiber on swine dysentery is also controversial. Different works have reported that diets low in dietary fiber and resistant starch prevented pigs from infection with *Brachispira hyodisenteriae*, and thus from swine dysentery disease (Pluske et al., 1996a; Durmic et al., 1998). However, recently works from Kirkwood and co-workers (2000) and Lindecrona and co-workers (2003) did not confirm these results, postulating that inclusion of fiber in the diet did not affect swine dysentery disease development.

Regardless of fiber effect on post-weaning colibacilosis and swine disentery, the main disadvantage of feeding diets with a high content of dietary fiber to pigs is that these materials tend to affect growth performance negatively. However, negative effects depend so much on the age of the animals, type of diet and level of inclusion (Moore et al., 1988; Valencia and Chavez, 1997).

2.3.1.2. Fermented liquid feed, an example of feed strategy

An interesting strategy to improve pig gut health by dietary manipulation is the administration of fermented liquid feed, obtained by mixing dry feed with water and usually, adding bacteria inoculums that act as fermentation starter (Jensen and Mikkelsen, 1999).

It has been demonstrated that administration of fermented liquid feed improves the performance and gut health of pigs, especially weaner piglets (Geary, 1996; Brooks et al., 1996; Scholten et al., 1999). Different hypotheses have been proposed to explain these results. All these hypotheses end from the main characteristics that

this feed have: its low pH and high concentration of lactic acid, and its high numbers of lactobacilli and yeasts (Adams, 2001).

The low pH and high amount of lactic acid in the fermented feed is related to a lower pH of luminal contents of the upper gastrointestinal tract (Ravindran and Kornegay, 1993) and to higher levels of organic acids (van Winsen et al., 2001). This modification in the gut environment may also influence gut microbiota.

In particular, some members of the *Enterobacteriaceae* family that are specially inhibited by acidic conditions are affected (Jensen and Mikkelsen, 1999). There is a reduction of enterobacteria in upper gastrointestinal tract that is also maintained in the lower intestine (vanWinsen et al., 2001), probably due to influences of increased populations of lactobacilli (Urlings, et al., 1993; Van Winsen et al., 2001) and to an improvement of colonization resistance mechanisms (Mulder et al., 1997).

Administration of fermented liquid feed has also been related to a lower total bacteria population in the stomach and small intestine, and to higher lactic acid bacteria (Jensen and Mikkelsen, 1999). A recent study by Moran and co-workers (2000) demonstrated a change in the ratio of lactobacilli:coliform throgought gastrointestinal tract of piglets, with a significant reduction of coliform compared to dry-fed animals. Jensen and co-workers also found a lower concentration of coliform bacteria in the gastrointestinal tract of slaughter pigs fed fermented liquid feed compared with pigs fed dry feed (1998), and changes in microbiota structure in the colon of pigs fed fermented liquid feed have also been shown (Leser et al., 2000). In addition to these results, feeding fermented liquid feed would also affect *Brachyspira hyodisentariae*, showing a lower incidence and severity of the disease when administered to pigs (Lindecrona et al., 2003).

In addition to these effects, it is necessary to take into account that when a lactic acid bacteria is used as starter inoculum to produce feed acidification, live and therefore possibly probiotic lactobacilli are continually fed to the animals, increasing the potential benefits of fermented liquid feed. (Moran et al., 2000; Hillman, 2001).

2.3.2. Micro-ingredients and in feed additives

2.3.2.1. Prebiotics

Prebiotics are defined as “non-digestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995).

To be classified as a prebiotic, a food ingredient must be: 1) neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract; 2) a selective substrate for one or a limited number of potentially beneficial commensal bacteria in the colon, thus stimulating the bacteria to grow or become metabolically activated, or both; and 3) able as a consequence to alter the colonic microflora toward a more healthier composition (Collins and Gibson, 1999).

Agarooligosaccharides, fructooligosaccharides, galactooligosaccharides, mannan-oligosacharides, xylooligosaccharides, arabinoxylans, raffinose, stachyose, glucosyl-sucrose, isomalturose, inulin, isomaltose, lactosucrose, lactulose, and lactose are the main prebiotics used (Patterson and Burkholder, 2003).

Similarly to dietary fiber, prebiotics act by stimulating bacteria fermentation in the lower gastrointestinal tract, but their mode of action is clearer by thorough selective enrichment of specific bacterial populations. According to this definition, mannan-oligosaccharides are not strictly prebiotics as their mode of action seems to be due to the neutralization of binding pathogens to mucus receptors and not acting as specific substrate, however, they have been usually included in this group (Spring et al., 2000).

Results using prebiotics have been promising. Studies in vitro have demonstrated the selective enhancement of the growth of different intestinal bacteria with the supply of oligosaccharides. Fructooligosacharides increased the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. (Jaskari et al., 1998; Sghir et al., 1998). Beta-glucooligomers and xylooligomers also improved *Lactobacillus* spp. and *Bifidobacterium* spp. strains (Jaskari et al., 1998), whereas pathogenic and putrefactive bacteria have reduced abilities to degrade these nutrients (Gibson and Roberfroid, 1995).

In vivo studies have also shown microbial shifts. Farnworth and co-workers (1992) found numerical increases in total anaerobes, total aerobes, bifidobacteria and

coliforms when weanling pigs were fed with inulin. Houdijk et al. (1997) found decreases in total aerobes in the ileum in response to feeding oligofructose, and other studies have also shown improvements in pig growth performance after administration of different prebiotics (Morimoto et al., 1984; Orban et al., 1997; Davis et al., 1999, 2004a; Shim et al., 2005).

Modification of the gastrointestinal ecosystem has also been confirmed by changes in gut chemical environment. Different studies with non-digestible oligosaccharides, oligofructose and transgalactooligosaccharides in the diet of pigs increased short chain fatty acids production and diminished luminal pH (Bolduan et al., 1993; Molis et al., 1996; Houdijk et al., 1997; Mikkelsen et al., 2003). The selective increase of some bacteria groups also promotes production of their metabolism, inhibiting the growth of many other species of bacteria (Russell and Diez-Gonzalez, 1998).

These specifically targeted microbial changes can have different beneficial effects that could explain the growth promoting effect of probiotics. Non-digestible oligosaccharides have been shown to increase resistance to invasion by pathogens in rats (Bovee-Oudenhoven et al., 1997), reduce translocation of pathogens (Berg, 1992), and diminish the availability of some toxins in rats (Zhang and Ohta, 1993).

2.3.2.2. Probiotics (live microbial feed supplements)

Probiotics are defined as living micro-organisms in feed which, when taken at certain levels, provide stability of the intestinal flora, and consequently have a positive effect on the host (Metchinkoff, 1908).

Currently, there are 13 preparations of micro-organisms that are authorized in the EU as livestock feed additives. Basically, three different groups are used: lactic acid bacteria, mainly Enterococci (*Enterococcus faecium*), lactobacilli (*Lactobacillus farciminis* and *Lactobacillus rhamnosus*) and *Pediococcus acidilactici*; bacteria belonging to the genus Bacillus (Bacillus *cereus, Bacillus licheniformis* and *Bacillus subtilis*); and Saccharomyces yeasts (*Sacharomyces cerevisiae*). Probiotic preparations are applied at concentrations of 10^8 to 10^9 CFU /kg of feed, mainly in the form of pelleted mixed feed (Simon et al, 2003).

Recent research has demonstrated positive effects of probiotics for pigs. Different studies have shown positive effects on growth performance using different strains (*Bifidobacterium pseudolongum, Lactobacillus casei, Lactobacillus acidophilus, Enterococcus faecium, Streptococcus thermophillus*, *Bacillus* spp. and *Sacharomyces* spp.; Danek et al., 1991; Kirckgessner et al., 1993; Abe et al., 1995; Kumprecht and Zobac, 1998; Mathew et al., 1998; Zani et al., 1998; Alexopoulus et al., 2004a, 2004b; Taras et al., 2005), that was accompanied in some cases with reductions in coliform bacteria and clostridia and with increases in lactobacilli numbers in the gut (Tortuero et al., 1995; Nemcova et al., 1999). Similarly to these results, Gedek and co-workers (1993) demonstrated that after the administration of *B. cereus* to young pigs, the populations of lactobacilli, bifidobacteria, eubacteria and *Escherichia coli* in the upper gastrointestinal diminish, whereas an increase was detected in ileum, caecum and colon. Despite these promising results with probiotics, it is fair to remark that there are also several works that have not found positive effects on pig performance (Kowarz et al., 1994; Brown et al., 1997; Gardiner et al., 1999).

The usefulness of probiotics in preventing post-weaning diarrhea is also ambiguous. Whilst some authors have demonstrated a diminution in the incidence of diarrhea (Zani et al., 1998; Durst et al., 1998; Kyriakis et al., 1999) others have not seen such an effect (Eidelsburger et al., 1992; Kirchgessner et al., 1993). Controversial results may be partly due to the complex etiologic factors involved in the post-weaning syndrome.

In general, beneficial properties of probiotics have been related to an improvement of the intestinal microbial balance of pigs and to the strength of the indigenous microbiota (Havenaar and Huis In't Veld, 1992). However, today the exact mode of action of probiotics is not entirely clear, and different hypothesis have been postulated.

Probiotics might modulate the intestinal ecosystem by competition with pathogens for epithelial receptors (competitive exclusion), by competition for nutrients, or by the production of antimicrobial compounds such as bacteriocines and organic acids with inhibitory effect for undesirable bacteria. Some probiotic effects have also been related to intestinal immune response stimulation, and also to a passive aggregation to pathogenic bacteria (Doyle, 2001; Adams, 2001; Simon et al., 2003).

It has also been suggested that probiotics affect the permeability of the gut and increase uptake of nutrients (Stewart et al., 1993; Starvic et al., 1995; Lee et al., 1999).

2.3.2.3. Symbiotics

Another way to modify pig microflora is the use of symbiotics, which is the use of probiotics and prebiotics in combination (Gibson and Roberfroid, 1995). The live bacteria must be used with specific substrates for growth. Therefore, the colonization by an exogenous probiotic could be enhanced and extended by simultaneous administration of a prebiotic being specifically used by the probiotic strain as a substrate in the intestinal tract (Rolfe, 2000).

Although works with symbiotics in pigs are still scarce, results are promising. Recently, the administration to weanling pigs of *Lactobacillus paracasei* in addition to oligofructose resulted in higher numbers of total anerobes, total aerobes and lactobacilli, with a decrease in enterobacteria and clostridia (Nemcova et al., 1999). Estrada and co-workers (2001) feeding early-weaned pigs with fructooligosaccharides and *Bifidobacterium longum* found an improvement in feed efficiency. Although resistant starch is not considered as a prebiotic, an interesting result was found by Brown and co-workers (1997) who demonstrated that concurrent feeding of high-amylose corn starch and bifidobacteria to pigs resulted in a higher fecal excretion of bifidobacteria than when the probiotic was administered alone. In addition to the increase of substrate for bacteria, it seems that the effect found, might be due to the bifidobacteria attachment to the surface of the starch granules that might be act as a carrier through the gastrointestinal tract (Crittenden, 1999).

2.3.2.4. Acidifiers

Several organic acids and their salts are recognized as preservatives and modulators of the gut ecology in pigs. Some of the most used are: formic, acetic, propionic, butyric, lactic, fumaric, Ca-formate, Ca-propionate, K-diformate, and Na-benzoate (Mroz, 2003).

Organic acids and their salts appear to be potential alternatives to prophylactic in-feed antibiotics for improving the performance of weaned piglets, fattening pigs and reproductive sows. As with other feed additives however, acidifiers are mainly used in young pigs as a way to prevent the problems associated with early weaning (Maxwell and Carter, 2001). In this regard, the administration of organic acids has also been reported to be helpful in overcoming problems of the post-weaning period in piglets (Partanen and Mroz, 1999; Tsiloyiannis et al., 2001).

Moreover, administration of organic acids, such as formic, acetic, propionic, lactic, citric, fumaric, sorbic, tartaric and malic acid, and some of their salts on growth performance are well defined (Han et al., 1998; Radcliffe et al., 1998; Siljander-Rasi et al., 1998; Øverland et al., 1999; Bosi et al., 1999).

The exact mechanism of action of organic acids remains unclear, although several hypotheses have been postulated. The primary antimicrobial action of organic acids (strain-selective growth inhibition or delay) is through pH depression of the diet, acting as a preservative, inhibiting the growth of many species of bacteria, yeasts and moulds on the feed previously its consumption. The specificity depends on the type of acid used; whilst acetic acid has demonstrated a broad spectrum and inhibits growth of bacteria, yeast and moulds, the action of propionic acid is primarily against moulds, with poor activity against bacteria and none against yeasts (Foegeding and Busta, 1991, Partanen, 2001).

However, more important than preservative action of organic acids, is their action by different direct effects on the animal. One of the first mechanisms proposed was the acidification of the digesta, particularly in the stomach of young pigs which have a limited secretion of HCl in the first stages of life (Maxwell and Carter, 2001). Nevertheless, the evidence suggests that this gut pH reduction is not the main effect of these compounds (Risley et al., 1992, Roth et al., 1992; Partanen and Mroz, 1999) and several trials have failed to demonstrate reductions of digesta pH after the inclusion of different acids in the diet (Risley et al., 1991; Gubert and Sauer, 1995; Franco et al., 2005).

The ability of acids to change from undissociated to dissociated form, depending on the environmental pH, has been recently proposed as the most plausible mechanism of action. This capacity makes organic acid effective antimicrobial agent in the pig gut. Undissociated form become lipophilic and can freely diffuse through

the bacteria membrane into their cytoplasm (Partanen, 2001). Once inside the cell, the acid dissociates and suppresses different bacterial enzymes such as ATPases and enzymes responsible of nutrient uptake (Cherrington et al., 1991; Russell, 1992). Organic acids with higher pKa values are more effective and their antimicrobial efficacy is generally improved as chain length and degree of unsaturation increase (Foegeding and Busta, 1991). Overall, the antimicrobial activity is primarily against yeasts and bacteria belonging to the *Enterobacteriaceae* family (Frank, 1994). Lactic acid bacteria are more resistant to their effects (Lueck, 1980).

Different in vivo studies have demonstrated microbiota shifts when acidifiers are used. Specifically, different types of organic acids are related to marked reductions in pig coliform bacteria (Mathew et al., 1996; Jensen, 1998; Øverland et al., 1999; Øverland et al., 2000). In the upper and lower intestine, micro-organism counts of lactobacilli, bifidobacteria, and eubacteria have also been shown to decrease (Gedek et al., 1992). However, results regarding microbial shifts are not consistent and some authors have not found changes when administering different acidifiers to pigs (Bolduan et al., 1988; Risley et al., 1992).

In addition, organic acids may be used as energetic substrate or as modulator for mucosal development, epithelial cell growth and increasing absorptive capacity, and also as precursors for synthesis on non-essential amino acids, DNA and on lipids required for intestinal growth (Mroz, 2003).

2.3.2.5. Minerals: zinc and copper

Dietary supplementation with high levels of minerals such as copper and zinc has usually been used in piglets to modulate intestinal microbiota and improve gastrointestinal health. Administration of pharmacological doses of ZnO have been related to improvements in post-weaning performance (Hahn and Baker, 1993; Hill et al., 1996; Smith et al., 1997; Mahan et al., 2000; Hill et al., 2000; Case and Carlson, 2002) and preventing the apparition of post-weaning diarrhea (Poulsen, 1998; Jensen Waern et al., 1998).

The effects shown may be due to microbiota modulation. The supplementation of high doses (2500 ppm) of zinc oxide to piglets has been related to increases in the biodiversity of coliforms and increases in the stability of pig microbiota (Katouli et

al., 1999). Recently, the same doses have shown a reduction in the total number of anerobes and in lactic acid bacteria in the stomach and ileum, parallel with an increase in coliform and enterococci throughout the gastrointestinal tract (Höjberg et al., 2005).

Similarly to ZnO, administration of pharmacological doses of copper sulphate has shown improvements in feed efficiency and weight (Cromwell et al., 1989; Dove and Hayden, 1991; Dove, 1995; Hill et al., 2000). Again, the improvement in growth performance was related to an antimicrobial action of copper (Fuller et al., 1960). A reduction in lactic acid bacteria and lactobacilli throughout the gastrointestinal tract and in colonic coliform bacteria (Höjberg et al., 2005) have been shown recently. However, it is possible that beside the potential antimicrobial effects, benefits of copper sulphate and also zinc oxide are due to a systemic effect; Zhou and co-workers (1994a) observed an increase in gain when pigs were injected intravenously with copper.

2.3.2.6. Plant extracts

For thousands of years, herbs and spices containing essential oils have provided distinctive properties to foods, and many have proved to be potent antimicrobial agents. Some of the most common plant products known for their antimicrobial properties belong to the genus *Allium,* and include garlic, onion and leek; others are thyme, oregano, marjoram, basil, cumin and bay. There are also spices, of which cloves, cinnamon, pepper and nutmeg may be remarked.

In vitro studies have demonstrated antibacterial activity of different plant extracts (Sen et al., 1998; Dorman and Deans, 2000; Friedman et al., 2002), and in recent years, the inclusion of these products in the pig diet has been proposed as a means to prevent intestinal disorders, especially at weaning, and to promote growth.

However, effects on growth response are not consistent and depend so much on the plant used. Different studies have added garlic to weanling pig diets without positive influence on performance (Horton et al., 1991; Holden et al., 1998; Holden and McKean, 2000), although a promising effect reducing post-weaning mortality has been found (Peet-Schwering et al., 2000). Similarly, a positive effect on growth performance of weanling pigs was also shown by Cromwell and co-workers (1985),

after administration of yucca plant extract, and herbal mixtures (great nettle, garlic and wheat grass; Grela et al., 1998).

Effects on performance are generally attributed to an effect of plant extracts on intestinal microbiota. In this regard, Tedesco and co-workers (2005) recently found an improvement in weaning pigs performance and also marked changes in microbiota composition (lower total bacteria, *E. coli*, total anerobic and *Enterococcus* spp. in feces) when different herbal additives were added to the feed (*Lycium barbarum*, *Taeonia lactiflora*, *Olea europea* and *Portulaca oleracea*.

2.3.2.7. Other additives

There are several other additives that are being used in pig feed, of which enzymes are among the most important. Many beneficial effects have been reported as a result of the inclusion of enzymes in animal diet (Bedford and Schulze, 1998). The main effect of using enzymes is a high availability of dietary nutrients for the animal (Verstegen and Williams, 2002), which help digestion and the breaking down of substrates that could provoke excessive microbial fermentation and disturb microbial equilibrium (McCartney, 2005). Although there is no known evidence regarding microbial shifts after enzymes addition in pigs, evidence in poultry suggests that an effect may be expected. The addition of xylanase to broiler diets reduced total ileal bacteria numbers by 60% and also reduced the proportion of bacteria with lower guanidine:cytosine content (Apajalahti et al., 2001).

Summary

Concerns regarding resistance in human bacterial pathogens have led to the total ban of antibiotics as growth promoters in animal feed on January 2006. As a consequence important efforts have been made to look for alternatives or replacement strategies to improve growth performance and to control enteric bacterial diseases by the maintenance of the gastrointestinal ecosystem in pigs. The use of different strategies has been proposed with positive results, among them may be remarked different sources of fiber, prebiotics, probiotics, organic acids, mineral at pharmacological doses and plant extract mixtures. A better understanding of the

modes of action of these products will allow in the near future a more rational design of non-antibiotic growth promoters.

2.4. New tools for the analysis of the gastrointestinal microbiota

Introduction

Traditionally, gut microbiota has been studied by classical selective-culture methods based on phenotytic characterisation. However, the inadequacy of these classical methods to detect all gut community bacteria has been established by different studies: low sensitivities, inability to detect non-cultivatable bacteria and unknowns species, time-consuming aspects and low levels of reproducibility (Fuhrman et al., 1992; Dutta et al., 2001). Recent studies have demonstrated that only 10-40% of total gut bacteria are culturable (Zoetendal et al., 1998; Suau et al., 1999) and that the classical taxonomy based on physiological/biochemical analysis of bacteria isolates is often inadequate (Leser et al., 2002).

In order to overcome the problems mentioned above, higher resolution molecular techniques based on 16S ribosomal DNA genes have been developed in recent years (Amann et al., 1995). The introduction of these methods in gastrointestinal microbiology has greatly enhanced our knowledge of complex microbial populations such as the pig gut microbiota (Vaughan et al., 2000). Nowadays, the 16S rRNA gene is the key bacterial marker due to its genetic stability, its domain characteristic composition with highly conserved and variable regions, and its high copy number in bacterial cell (Woese, 1987; Amann et al., 1990a). In addition, the growth of the genes sequence data bank (www.ncbi.nlm.nih.gov/entrez/, rdp.cme.msu.edu/index.jsp) allows easy comparison between sequences from across the world. The use of these new methods has been especially focused on the description of the different species inhabiting the gastrointestinal tract and also on the detection of bacterial shifts related to parameters such as age, diet or illness.

This chapter will focus on different quantitative and qualitative molecular methods habitually applied in gut microbiology, paying special attention to recent works involving molecular methods for studying pig gut microbiota.

2.4.1. *Quantitative techniques*

Several different techniques may be used to quantify bacteria in the gastrointestinal tract. These methods can be classified basically into two groups: quantitative PCR-based methods such as real time PCR, and other methods not dependent on this previous amplification but on the use of labelled probes, such as fluorescent *in situ* hybridization, dot blot and microarrays. Of these, we will look closely at two methods in this chapter due to their relatively more frequent use in gastrointestinal microbiology in the last few years: real time PCR and fluorencent in situ hybridization.

2.4.1.1. Quantitative Polymerase Chain Reaction (qPCR)

Real-time PCR is a method based on the polymerase chain reaction with on-line measurement of the amplification reaction. Data are automatically collected throughout the entire PCR process, rather than at the end of the PCR as conventional reaction was traditionally performed.

Real-time PCR system is based on the detection and quantification of a fluorescent reporter (Lee et al., 1993; Livak et al., , 1995) that increases its signal in direct proportion to the amount of PCR product in the reaction. Quantification of the product takes place in the exponential phase of PCR, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of target DNA, the sooner a significant increase in fluorescence is detected. Absolute quantitation can be achieved by interpolating unknown samples from a standard curve constructed with a known amount of the target gene.

There are two different methods of real-time PCR (Figure 2.5). The one that first appeared was the TaqMan assay (Holland et al., 1991). This method is based on the use of a fluorogenic labelled probe in addition to both primers that are essential in the PCR reaction. TaqMan probes are oligonucleotides that contain a fluorescent dye usually on the 5’end, and a quenching dye (usually TAMRA) on the 3’ end, and it is designed to anneal to an internal region of the PCR product. When this probe is irradiated, the excited fluorescent dye transfers energy to the quenching dye molecule rather than fluorescing (Hiyoshi and Hosoi, 1994). When the target sequence is

present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of the Taq polymerase as the primer is extended. This probe cleavage produces a separation from the two dyes that mark the probe and the reporter dye starts to emit a signal that increases in each cycle proportional to the rate of probe cleavage. At the same time, Taq polymerase removes the probe from the target, allowing extension to end the template strand. With each PCR cycle, additional reporter dye molecules are cleaved from their respective probes resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced.

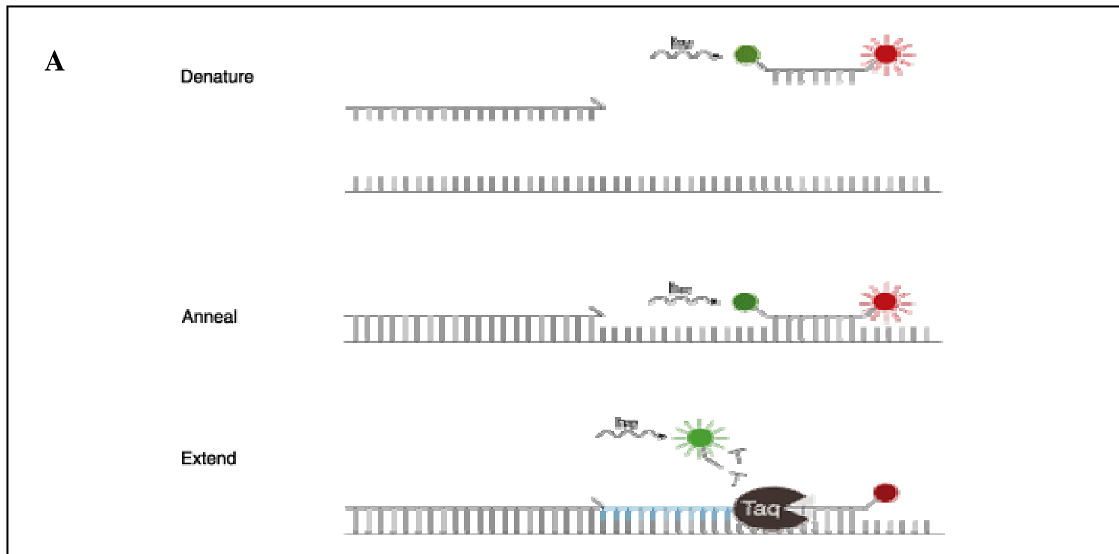
The second real-time PCR system is the SYBR Green dye. This method uses a non-sequence specific fluorescent intercalating agent (SYBR Green) that only emits when bound to double stranded DNA. SYBR Green dye is a fluorogenic minor groove binding dye that shows little fluorescence when in solution, but emits a strong signal upon binding to double-stranded DNA (Morrison et al., 1998). During the PCR, the polymerase amplifies the target sequence, creating new PCR products. Then, the SYBR Green dye binds to each new copy of double-stranded DNA. As the PCR progresses, more amplicons are created, increasing the intensity of fluorescence detected with each new amplification cycle.

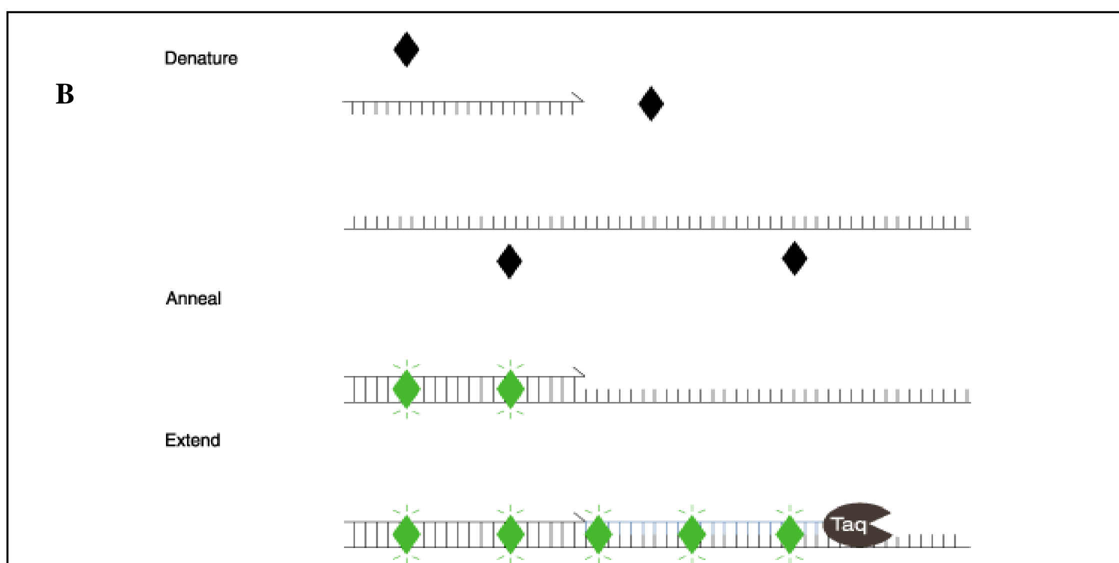
The main difference between both methods is that SYBR Green chemistry detects all double-stranded DNA, including non-specific reaction products, and primer-dimer, making it therefore more important to have a well-optimized reaction so as not to obtain unspecific amplification that may generate false positive signals. This problem is especially important with low quantity template (Hein et al., 2001), although it can be minimised since non-specific amplification can be easily discarded by analysis of melting or dissociation curve of the product amplified (Ririe, 1997). On the other hand, the main disadvantage of TaqMan chemistry is that the synthesis of different probes is required for each different sequence that wants to be detected. This increases the assay set-up and running costs.

Real-time PCR methodology has different advantages in bacteria quantification compared to traditional culture: higher sensitivity, rapidity and reproducibility (Bustin et al., 2000). The possibility of storing the samples until their analysis, avoiding the need to work in fresh, is undoubtedly a remarkable advantage of this method compared to traditional ones. However, this method tends to overestimate bacterial

populations ((Nadkarni et al., 2002; Huijsdens et al.,2002) and also has a relatively high cost.

Figure 2.5. Representation of real-time PCR with TaqMan primers (A) and SYBR Green (B). (**A**). In the intact TaqMan probe, energy is transferred from the short-wavelength fluorophore (green circle) to the long-wavelength fluorophore (red circle), quenching the short-wavelength fluorescence. After hybridization, the probe is susceptible to degradation by the endonuclease activity of a Taq polymerase. Upon degradation, quenching is interrupted, modifying the fluorescence detected. (**B**). SYBR Green I dye (black diamonds), present in the PCR mixture, becomes fluorescent (green diamonds) upon binding to all double-stranded DNA, providing a direct method for quantifying PCR products in real time (Invitrogen PCR Handbook).





In recent years, real-time PCR has been widely used to quantify selective bacteria from the gastrointestinal tract of humans (Huijsdens et al., 2002; Matsuki et al., 2003; Ott et al., 2004; Penders et al., 2005), pigs (Collier et al., 2003; Hill et al., 2005), chickens (Selim et al., 2005; Wise and Siragusa, 2005) and ruminants (Tajima et al., 2001), and also to detect pathogen bacteria in different environments such as water, feces and soil (Smythe et al., 2002; Ibvekwe and Grieve, 2003; Fukushima et al., 2003; Wu et al., 2005).

Particularly in pigs, real time PCR is being used to quantify total bacteria and some specific bacterial groups. Collier and co-workers (2003) used real-time PCR with SYBR Green dye chemistry to quantify total bacteria and *Lactobacillus* spp. in the ileal and colonic contents of growing barrows fed with different experimental diets. Real time PCR allows the detection of significant differences in total bacteria and lactobacilli bacteria in pigs fed the experimental diet. More recently, Hill and co-workers (2005) quantified different pig gut bacteria belonging to the Bacillales, *Clostridium* spp., *Streptococcus alactolyticus*, and *Lactobacillus amylovorus* using real time PCR and also SYBR Green chemistry in weanling pigs fed different diets. In this case, Chaperonin-60 gene was used as a targeted gene instead of the 16S rDNA gene. This study demonstrated the usefulness of real time PCR for detecting changes in specific pig gut bacteria and the potential of Chaperonin-60 gene as an alternative to the use of 16S rDNA gene in microbial ecology.

2.4.1.2. Fluorescent *In Situ* Hybridization (FISH)

Fluorescent *In Situ* Hybridization (FISH) is a quantitative molecular method with an increasing interest in gut microbiology. It was first used in bacteriology by Giovannoni and co-workers (1988) with radioactively labelled oligonuclotide probes. Although the basis of the method has not changed, fluorescent probes have now replaced radioactive ones. FISH method detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary target sequence within the intact bacterial cell (Moter and Göbel, 2000). Depending on the specificity of the probe used, different specific bacterial groups can be counted; if universal probes are used, total bacteria can be quantified. To date, several probes have been standarized and are actually being used to quantify the main gut bacteria (Table 2.5).

FISH procedure is relatively easy though laborious. Bacterial cell is chemically treated to allow cell fixation and permeabilization. Once fixed, bacterial cells are immobilized on a pre-treated glass slide or kept in suspension depending on the method of quantification that will be used afterwards. Then, hybridization under stringent conditions allows proper annealing of the selected probe to the target sequence. Generally, probes are 15-30 nucleotides in length and covalently labelled at the 5' end with a fluorescent dye. Common fluorophors include fluorescein, etramethylrhodamine, Texas red, and carbocyanine dyes such as Cy3 and Cy5 (Southwick et al., 1990). Nowadays, two different methods are used to quantify stained cells. The most common method used is via epifluorescence microscopy though it is laborious and subjective (Wagner et al., 2003). Alternatively, flux cytometry appears as a potential method with high-resolution to bacteria counts.

One of the main advantages of FISH is that being a molecular method it does not depend on purification or amplification steps, avoiding biases that are typically described on PCR based methods (Wintzingerode et al., 1997).

Another advantage, when microscope slides are used, is that counts can be made using a confocal laser scanning microscope, obtaining an accurate image of the spatial distribution of microbial communities as well as information about morphology (Moter and Göbel, 2000; Daims et al., 2001). Moreover, when the technique is standarized, counting can be automatized, thus avoiding the biases that manual counts can produce (Jansen et al., 1999). In addition, recently a multi-color fluorescence *in situ* hybridization method has been developed, which detects, in a single reaction, seven species of Bifidobacterium (*B. adolescentes*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, and *B. longum*; Takada et al., 2004, Figure 2.6). This approach may be an interesting alternative to quantify different groups of bacteria at a time in digesta samples.

Especially interesting is the recent application in gastrointestinal microbiology of flow citometry to FISH signal detection, which allows a relatively faster and more sensitive quantification than traditional microscopy (Wallner et al., 1997).

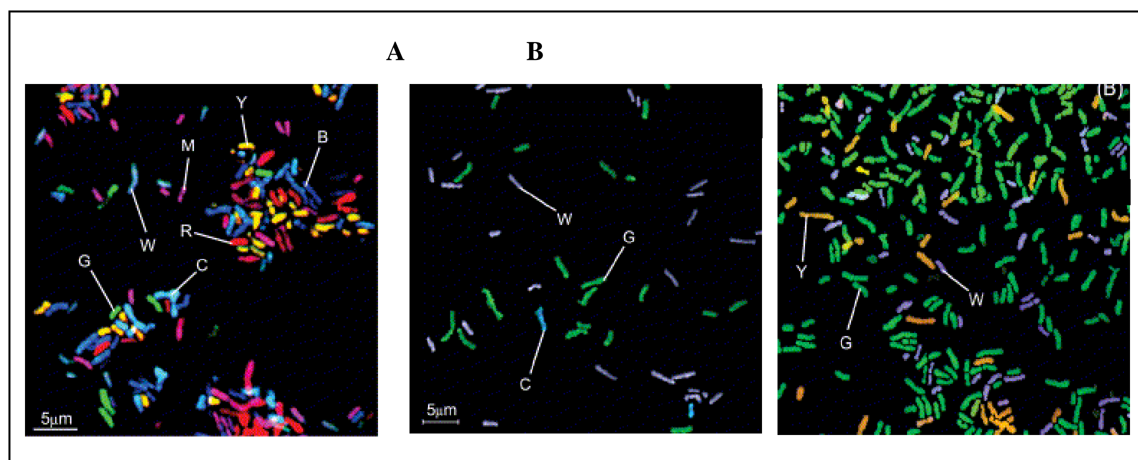
Table 2.5. Some probes currently used to quantify different gastrointestinal bacteria.

Probe name	Target group	Probe sequence(5'-3')	Reference
S-D-Bact-0338-a-A-18	Bacteria	GCTGCCTCCCGTAGGAGT	Amann et al., 1990b
S-*-Bacto-0303-a-a17	CFB phylum	CCAATGTGGGGGACCTT	Manz et al., 1996
S-S F.suc-0650-a-A-20	*F. succinogenes*	TGCCCCTGAACTATCCCAAGA	Amann et al., 1990a
S-S-F.int-0136-a-A-20	*F. intestinalis*	CGGTTGTTCCGGAATGCGGG	Lin et al., 1994
S-*-F.prau 0645-a-A-23	*F. prausnitzii*	CCTCTGCACTACTCAAGAAAAC	Suau et al., 2001
S-*-Erec-0482-a-A-19	*C. coccoides* cluster	GCTTCTTAGTCAGGTACCG	Franks et al., 1998
S-*-Elgc-01-a-A-19	*C. leptum* cluster	GGGACGTTGTTTCTGAGT	Franks et al., 1998
S-*-Chis-0150-a-A-23	*C. histolyticum*	TTATGCGGTATTAATCTYCCTTT	Franks et al., 1998
S-*-Bdis-0656-a-A-18	*Bacteroides distansonis*	CCGCCTGCCTCAAACATA	Franks et al., 1998
S-*-Bfra-0602-a-A-19	*Bacteroides fragilis*	GAGCCGCAAACTTTCACAA	Franks et al., 1998
S-S-Bvulg1017-a-A-21	*Bacteroides vulgatus*	AGATGCCTTGCGGCTTACGGC	Rigottier-Gois et al., 2003
S-*-Bacto-1080-a-A-18	*Bacteroides* spp.	GCACTTTAAGCCGACACCT	Doré et al., 1998
S-G-Bif-0164-a-A-18	*Bifidobacterium* spp.	CATCCGGCATTACCACCC	Langedink et al., 1995
S-*-Rfla-729-a-A-18	*Ruminococcus albus* and *R. flavefaciens*	AAAGCCCAGTAAGCCGCC	Harmsen et al., 2002
S-*-Rbro-730-a-A-18	*C. sporosphaeroides, R. bromii*, *C. leptum*	TAAAGCCCAGYAGGCCGC	Harmsen et al., 2002
S-*-Ehal-1469-a-A-18	*Eubacterium halii* group	CCAGTTACCGGCTCCACC	Harmsen et al., 2002
S-G-Lab0158-a-A-21	*Lactobacillus-Enterococcus* spp.	GGTATTAGCAYCTGTTTCCA	Harmsen et al., 2002
L-S-E.coli-1531-a-A-21	*Escherichia coli*	CACCGTAGTGCCTCGTCATCA	Krogfelt et al., 1993
S-*-Enter-1432-a-A-15	Enterobacteriaceae	CTTTTGCAACCCACT	Sghir et al., 2000

[a]Probe names have been standarized as follows: S or L for Large or Small subunit rDNA as the target; D for Domain, O for Order, F for Family, G for Genus, S for Species and Ss for Subspecies. ; letters designating the target group of the oligonucleotide probe; nucleotide position in E. coli gene; letter designating the version of the probe; S or A for Sense or Antisense direction; number indicating the length in nucleotides of the probe (Alm et al., 1996). An asterisk shows that this probe has not been standarized.

However, the FISH methods have also some limitations. One of them is the different penetration of probes in bacteria with various cell wall types. The high complexity of gram-postitive bacteria wall hinders its permeabilization and probe hybridization which can result in an underestimation of these bacteria (Langendijk et al., 1995; Jansen et al., 1999).

Figure 2.6. (A) Epifluorescent image of mixed culture of seven different Bifidobacterium species by multi color FISH. In the images, *B. adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium* and *B. longum* are shown in G (green), R (red), C (cyan), B (blue), Y (yellow), M (magenta) and W (blue-ish white), respectively. **(B)** Identification of Bifidobacterium species in human fecal samples using multi color FISH (Takada et al., 2004).



FISH methods have been used in different microbiological studies. FISH has been used extensively to identify and count bacteria, specially from marine environments (Ramsing et al., 1996; Glöckner et al., 1996; Alfreider et al., 1996; Lemke et al., 1997; Jürgens et al., 1999), and also to study microbial diversity in wastewater treatment (Amann et al., 1996; Snaidr et al., 1997; Bond et al., 1999). Moreover FISH has been used in the study of different complex bacterial ecosystems in the human body such as those of the oral cavity (Moter et al., 1998a, 1998b) and the gastrointestinal tract (Langedijk et al., 1995; Franks et al., 1998; Harmsen et al., 1999; Jansen et al., 1999; Harmsen et al., 2000a, 2000b, 2002; Zoetendal et al., 2002a; Hold et al., 2003). Finally, FISH has been also applied in the detection of

pathogens in tissue samples (Boye et al., 1998; Trebesius et al., 1998; Jensen et al., 2000) and in feces (Waar et al., 2005).

Although FISH has been extensively used to study human intestinal microbiota, only a few published works can be found on the study of pig gut bacteria. Konstantinov and co-workers (2004b) used a universal probe to quantify total bacteria (Bac338), and a probe to quantify Lactobacillus-enterococcus group (Lab158) and developed and evaluated a specific probe to quantify bacteria belonging to the genera *Lactobacillus amylovorus* and *L. reuteri*-like in weanling pigs that were receiving diets rich in fermentable carbohydrates. Utilization of FISH allowed determination of a higher prevalence of *L. reuteri* and *L. amylovorus*-like populations in the ileum and colon of pigs fed diets rich in fermentable carbohydrates.

2.4.2. Fingerprinting techniques: DGGE, t-RFLP

Fingerprinting techniques are based on the existence of polymorphisms in the 16S rDNA gene within different bacteria, which provide specific patterns or profiles for each microbial community depending on the bacteria harbouring it. Genetic fingerprinting techniques are actually being used to elucidate the complexity, dynamics and diversity of complex bacterial populations. Terminal restriction fragment length polymorfism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) are one of the most used.

T-RFLP is based on comparison of banding patterns obtained from DNA restriction with an endonuclease that recognize specific sequences within the gene (Charteris et al., 1997; Hozapfel et al., 2001), and DGGE is based on the separation of an amplified fragment of the 16S rDNA in a denaturing electrophoresis depending on its sequence (Muyzer et al., 1993).

2.4.2.1. Denaturant/Temperature Gradient Gel Electrophoresis (DGGE/TGGE)

Denaturant/Temperature Gradient Gel Electrophoresis allows separation of objective DNA molecules based on variability of its sequence in the variable regions and thus in its chemical stability of 16S rDNA. First introduced in microbial ecology by Muyzer and co-workers (1993) it is widely used currently.

In these techniques, PCR-amplified 16S rDNA products are separated by applying a temperature gradient or denaturing gradient in an electrophoresis system. A temperature or chemical gradient is established in a polyacrylamide gel in parallel to the electric field. The DNA samples migrate through the gradient from low to high temperature, or low to high chemical gradient. At the point in the gradient where partial denaturation of the double-stranded DNA happens, the migration of the DNA fragment is drastically retarded and sequences of the same size, but of different thermal or chemical stability (by its sequence), are separated (Reisner et al., 1992). Separation is therefore based on the melting of the DNA fragments. Sequence variation causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel (Muyzer and Smalla, 1998).

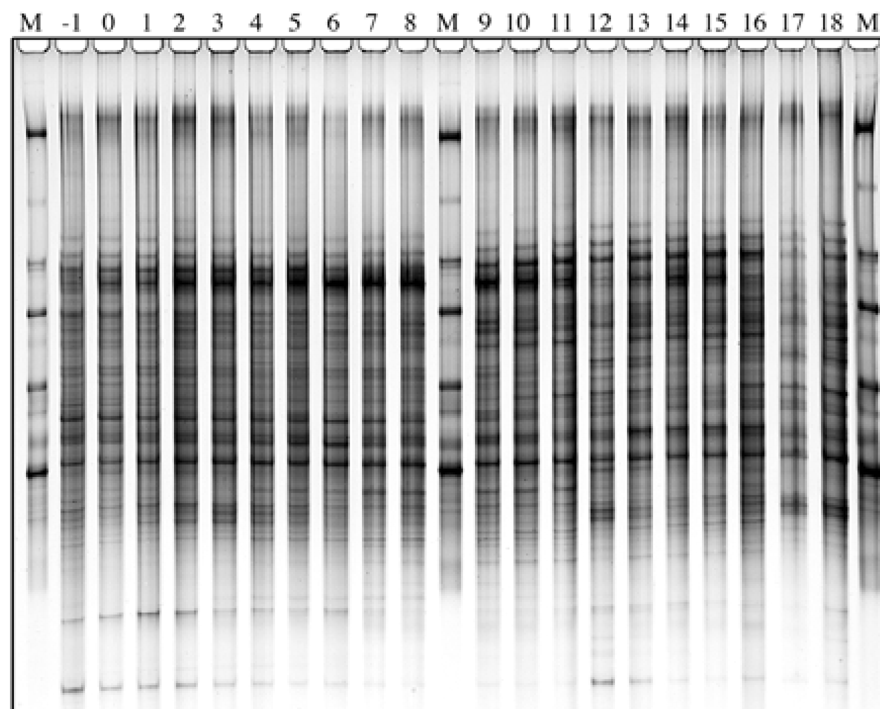
DNA bands are thereafter visualised using ethidium bromide, silver staining or SYBR Green I. The PCR banding pattern is indicative of the number of bacterial species that are present and thus allows visualization of the genetic diversity of microbial populations (Simpson et al., 1999). Subsequent identification of specific bacterial groups or species in the sample can be achieved by cloning and sequencing the excised bands from the gel, or by hybridization of the profile using phylogenetic probes (Muyzer and Smalla, 1998).

Both methodologies have been successfully used in gut microbial studies due to the fact that these techniques are reliable, rapid, comparatively inexpensive and with good reproducibility (Ampe et al., 2001; Schmalenberger et al. 2001; McCartney, 2002). These techniques have been used in human samples (Zoetendal et al., 1998; Zoetendal et al., 2002b; Favier et al., 2002; Malinen et al., 2003; Gueimonde et al., 2004), in pig gastrointestinal samples (Simpson et al., 1999; Simpson et al., 2000; Collier et al., 2003; Konstantinov et al., 2003; Konstantinov et al., 2004b; Inoue et al., 2005), in "in vitro" modification of pig microbiota after substrates inoculums (Zhu et al.., 2003) and to study bacterial biofilms (Muyzer et al., 1993; Muyzer and de Waall, 1994).

Different groups have been using DGGE to study pig gut microbiota. The first work using this technique was done by Simpson and co-workers (1999, 2000; Figure 2.7), with the aim of determine if DGGE could be effectively applied to measure changes in bacterial populations in the gastrointestinal tract, based upon age, diet, or

anatomic compartment. The authors concluded suitability of the method as DGGE analysis revealed diverse and stable individual bacterial populations between pigs of different ages and among individual gut compartments. Difference in patterns observed have also been elucidated after administration of different fermentable carbohydrates to weanling pigs (Konstantinov et al., 2003, 2004b), showing an increase in microbial stability and higher diversity. Similarly, Zhu and co-workers (2003) found differences in band patterns in an in vitro study with feces of weaning piglets as inoculum of sugar beet pulp fermentation. Collier and co-workers (2003) have also been using this technique to determine differences in pig colonic and ileal microbiota after an antibiotic growth promoter administration. Differences in band patterns demonstrated the effect of the antibiotic compared to control pigs. Recently, an interesting study have found changes in piglet microbial profiles during the first weeks of life using DGGE, detecting remarkable changes in microbiota diversity and composition (Inoue et al., 2005).

Figure 2.7. PCR-DGGE profile generated from fecal samples obtained from an individual piglet over a 20-day experimental period using primers specific for the V3-16S rDNA. Bacterial standard marker lanes are denoted as M (Simpson et al., 2000).



2.4.2.2. Terminal Restriction Fragment Length Polymorfism

Terminal restriction fragment length polymorphism is a very useful tool for comparing microbial communities (Kitts, 2001) that allows the fingerprinting of a community by analyzing the polymorphism of the 16S rDNA. It is a high-throughput, reproducible method that allows a qualitative analysis of the diversity of bacteria in an ecosystem.

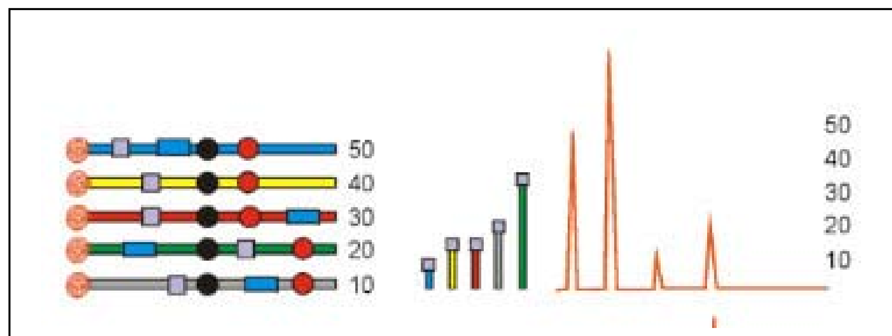
Firstly, DNA from the sample is extracted and by the use of universal primers, total bacterial DNA is amplified by conventional PCR, similarly to DGGE with the difference that one of the primers used is labelled fluorescently at the 5' end. The amplified DNA is then digested with a restriction enzyme, which is an endonuclease that recognizes one determined specific sequence into the amplicon. Once the restriction is obtained, fragments are separated by capillary electrophoresis. Generally, a DNA sequencer with a fluorescence detector is used to separate fragments, thus, T-RFLP gives only one band per species as only the fragment containing the fluorescently labelled primer site will be detected (Figure 2.8). The samples are run on long sequencing gels that give high resolution and sensitive detection. Once electropherogram is obtained, inference of potential bacteria present in the sample can be achieved by comparison of fragments obtained in the samples with *in silico* restriction with the primers and enzyme used, using the analysis function TAP-tRFLP from the Ribosomal Database Project II software (Cole et al., 2003).

T-RFLP has appeared recently as an attractive tool for studying pig gut microbiota (Leser et al., 2000; Khan et al., 2001; Hogberg et al., 2004), chicken microbiota (Gong et al., 2002), human microbiota (Gong et al., 2003; Nagashima et al., 2003; Ott et al., 2004; Wang et al., 2004), rats microbiota (Kaplan et al., 2001), and characterization of bacteria from environmental samples (Liu et al., 1997; Clement et al., 1998; Dunbar et al., 2000; Blackwood et al., 2003).

Particularly in pigs, different studies have used t-RFLP to study gut microbiota. Leser and co-workers (2000) compared bacterial communities in the colon of pigs fed different experimental diets based on either modified standard feed or cooked rice supplemented with dietary fibers. After feeding animals with the experimental diets, differences in bacterial community structure were detected as different patterns were obtained. Similarly, Högberg and co-workers (2004) studied the effect of different

cereal non-starch polysaccharides on the gut microbiota in growing pigs. The authors observed a particular pattern depending on the diet administered. Recently, Höjberg and co-workers (2005) also found differences in microbial cecal profiles in pigs receiving different doses of zinc oxide and copper sulphate (Figure 2.9).

Figure 2.8. An example of fragments and visualization of the electropherogram obtained after an enzymatic restriction. Bars represent different sequences, red spirals indicate fluorescent label; and circles, squares and rectangles indicate different restriction enzyme sites and their location in each sequence. The fragment analysis peaks would look like the graph on the right.



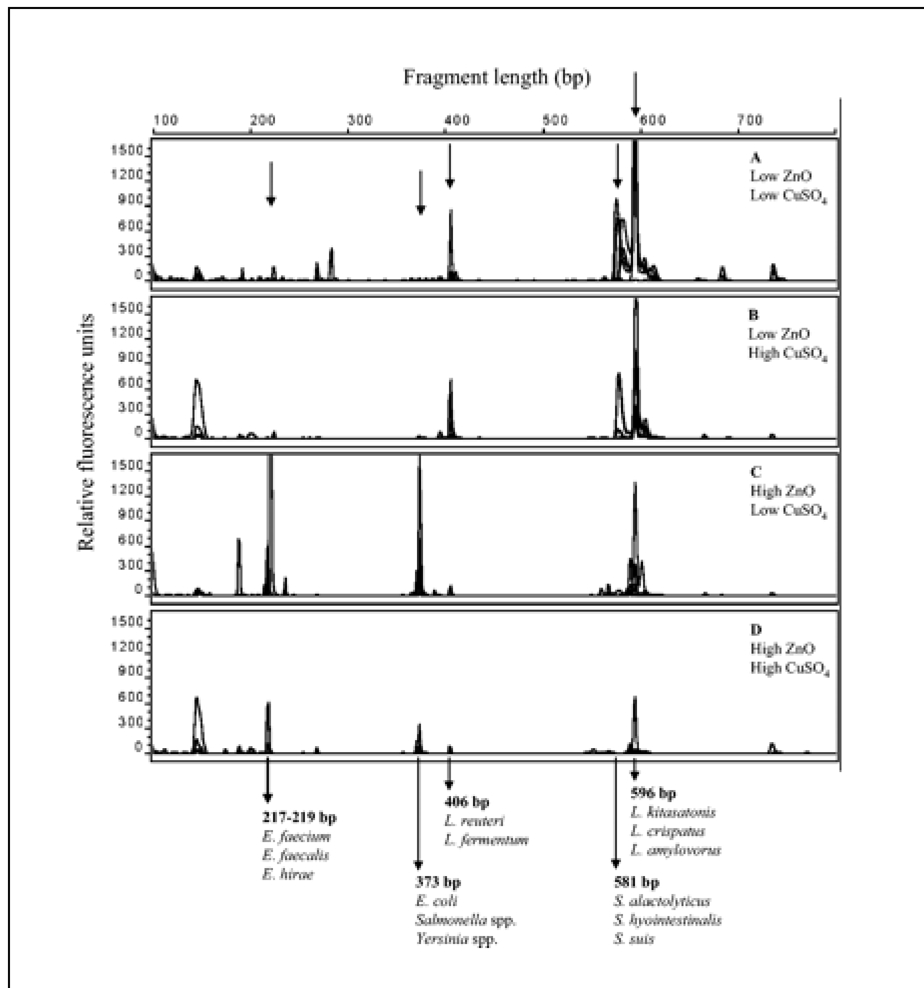
As is with other PCR based methods, both these fingerprinting techniques (t-RLP and DGGE) are highly determined by the pair of primers chosen for PCR amplification. Primers chosen will limit the number of targeted DNA and thus may bias the profile obtained, being especially important to select universal primers when an overall description of an ecosystem wants to be achieved. Specifically with t-RFLP, the selection of primers and restriction enzyme is particularly important. An inappropiate selection can make that many bacteria species share the same length of fragment, avoiding therefore the precise recognition of all the microbial diversity (Liu et al., 1997; Marsh, 1999; Kaplan et al., 2001).

Summary

In recent years, the increasing concern on gut health has rekindled the interest for gut bacteria. This fact with the inadequacy of classical culture-dependent methods to accurately describe all microorganisms, has overcome the development of different

molecular methods to study gastrointestinal microbiota. As a consequence, nowadays there is a current plethora of genetic techniques for quantification, identification and community characterisation with a huge amount of information regarding the ecosystem and how it changes with age, illness and dietary modification. Although the majority of works are still concentrating on human microbiota, important efforts are being made to apply these methods on pig gut; this is significantly increasing our microbiota knowledge and in the near future will provide important information regarding the key role of gut bacteria for animal health.

Figure 2.9. T-RFLP profiles obtained from cecum digesta in pigs receiving different doses of zinc oxide and copper sulphate (Höjberg et al., 2005).



Chapter 3

OBJECTIVES

The main objective of this thesis was to improve our knowledge of pig gut microbiota, bearing in mind the development of new feeding strategies to substitute antibiotics as growth promoters. To achieve this, three secondary objectives were considered:

1. To develop and/or evaluate molecular methods to study pig gut microbiota: real time PCR and fluorescence *in situ* hybridization (FISH) to quantify particular microbial groups, and terminal-restriction fragment length polymorfism (t-RFLP) to determine global changes in the community profile.

2. To study microbiota establishment and changes produced in the piglet by weaning, and the quantitative importance of major bacterial groups along the gastrointestinal tract in the growing animal.

3. To study potential modification of this ecosystem by the inclusion of different commercial in-feed additives or fibrous ingredients in the diet of weaned or growing pigs.

To assess these three objectives, five different trials were designed. Results will be included in chapters 4-8.

In **Trial I,** real-time PCR was assessed as an alternative method to quantify total bacterial load, lactobacilli and enterobacteria in pig digesta samples. Results were compared with those obtained by traditional methods as reference values (selective culture for lactobacilli and enterobacteria, and direct microscopy for total bacteria).

Trial II was designed with the aim of studying microbial establishment process after weaning. Cecal digesta from weaned and suckling pigs was collected and real-time PCR was used to study specific bacterial shifts in lactobacilli and enterobacteria populations. Also, a fingerprinting method (t-RFLP) was evaluated as a useful method for studying global changes in cecal bacterial profile.

Trial III was designed with two different objectives. Firstly, to describe the main bacteria groups throughout the gastrointestinal tract of the growing pig using FISH as a method of study; and secondly, to study the effect of different types of dietary fibre (resistant starch and different non-starch polysaccharides) on microbiota using FISH

and RFLP. Changes in the fermentation pattern were also studied by measuring short-chain fatty acid concentration in the colon.

Trial IV was designed to study the effect of commercial additives on pig gut microbiota of weaned pigs. Avilamycin was used as a positive control and sodium butyrate and a commercial plant extract mixture were tested as alternatives. Real-time PCR was used to study changes in total bacteria, lactobacilli and enterobacteria along the gastrointestinal tract, and RFLP was used to assess changes in bacterial profile. Microbial activity was also measured by purine bases content, and some specific bacterial enzymatic activities.

Finally, **Trial V** was designed to evaluate the effect of a commercial mannan-oligosaccharide and organic zinc, administered alone or in combination, on growth performance, gut microbiota, gut histology and immune response of weaned pigs. Real-time PCR was applied to quantify lactobacilli/enterobacteria ratio, and purine bases and short-chain fatty acids, to measure microbial activity. To evaluate the immune response, immunoglobulin concentration in plasma and digesta, and the development of continuous Peyer's Patch were determined.

Chapter 4

QUANTIFICATION OF TOTAL BACTERIA, ENTEROBACTERIA AND LACTOBACILLI POPULATIONS IN PIG DIGESTA BY REAL-TIME PCR

4.1. Introduction

Recently, molecular methods have shown that the complexity of microbial communities is much greater than previously thought and that the majority of gut bacteria are still unknown (Pryde et al., 1999; Leser et al., 2002). This lack of knowledge is mostly attributed to the failure of many bacteria to grow in a given culture medium (Langendijk, et al., 1995; Huijsdens et al.,2002). Quantitative molecular methods could be more sensitive and selective than traditional methods taking into account that they do not rely on the ability of bacteria to grow. Moreover, DNA-based methods offer the option of storing samples until their analysis, which could be an important advantage in field conditions.

Considering the high complexity of gut microbiota, some authors have tried to find particular microbial groups that could serve as an index of a health-promoting microbiota. Conventionally, the ratio lactobacilli:enterobacteria has been used as a simple index and an increase in this ratio is related with a higher resistance to intestinal disorders (Muralidhara, et al., 1977; Reid and Hillman, 1999). Specifically in the weaning pig, lactobacilli could have a predominant role in controlling colibacillosis, which is one of the most common intestinal disorders during the first months of life (Tortuero et al., 1995; Nemcova et al., 1999).

The objective of this work was to evaluate the use of real time PCR to quantify total bacteria, lactobacilli and enterobacteria in pig digesta samples.

4.2. Material and methods

4.2.1. Sample preparation

Samples of jejunum digesta were obtained from healthy early weaned (20 ± 2 days) pigs of approximately 40 days old. Animals received commercial diets and were sacrificed with an intravenous injection of sodium pentobarbitone (200mg/kg body weight). For comparison of qPCR, selective culture and DAPI staining, 32 animals from the same herd were sampled. To study the effect of pre-treatment of samples on microbial counts, 18 animals from a second herd were used. The management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

For microbiological culture procedures and for DAPI staining a fragment of 10 cm from the distal jejunum was tied, cut-off and kept in ice for further dilution. For qPCR counts, one gram of digesta was kept in tubes that contained 3 ml of ethanol as preservative. Samples were gently mixed with the ethanol and stored at 4 ºC until analysis. To assess the effect of pre-treatment of the sample on the total bacteria qPCR counts, approximately 5 g from jejunum digesta were sampled and frozen until analysis.

4.2.2. Bacteria quantification by traditional methods

For selective culture, digesta samples were serially diluted (wt/vol) in sterile PBS and plated in selective media. Enterobacteria were enumerated using MacConkey agar at 37 ºC (24h) (CM-115, Oxoid, Madrid, Spain) and lactobacilli in Rogosa agar at 37ºC in a 5% CO2 atmosphere (48h) (CM-627, Oxoid).

Direct quantification of total bacteria was carried out by epifluorescent direct count method (Hobbie et al. 1977) using 4',6-diamidino-2-phenylindole (DAPI) staining. One gram of sample was diluted ten times with sterile PBS, and 0.5 ml of this suspension was fixed with 4.5 ml of 2 % formaldehyde. Samples were stained with DAPI (10 min, 1 µg/ml) and filtered through polycarbonate membrane filters (0.22 µm, Whatman International, Kent, UK). Bacteria were enumerated using an ocular graticule and ten random fields per sample were counted. (Olympus NCWHK 10x, Olympus, Barcelona, Spain).

4.2.3. Bacteria quantification by real-time PCR (qPCR)

DNA extraction. The equivalent volume to 400mg of digesta samples preserved in ethanol was precipitated by centrifugation (13000g, 5 min). The DNA from the precipitate was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The lysis temperature was increased to 90 ºC and an incubation with lysozyme was added (10 mg/mL, 37 ºC, 30 min) to improve the bacterial cell rupture. The DNA obtained was stored at -80º C.

To evaluate possible disregard of bacteria attached to particulate material during pre-treatment of the samples for culturing and DAPI staining, DNA extraction was also performed after a previous 1/10 dilution of the samples. One gram of each

sample was diluted ten times with sterile PBS and homogenized 1 minute with a vortex mixer. Diluted samples were let to stand on the bench during another minute and 4 ml of the liquid phase were centrifugated (20,000 x g, 20 min). The DNA was extracted and purified from the pellet using the same commercial QIAamp DNA Stool Mini Kit and procedures described above.

The DNA from pure cultures of Lactobacillus acidophilus (CECT 903NT) and Escherichia coli (CECT 515NT) was harvested from the bacterial pellet obtained by centrifugation of 6 ml of culture using the same Qiagen Kit. Pig genomic DNA was obtained from blood samples that were collected aseptically using the Mammalian Genomic DNA extraction kit (CAMGEN, Cambridge Molecular Technologies Ltd., Cambridge, UK).

Quantitative PCR. To quantify total bacteria, lactobacilli and enterobacteria different primers were used: F-tot (forward) 5'GCAGGCCTAACACATGCAAGTC3' (adapted from Marchesi et al. (1998) and R-tot (reverse) 5'CTGCTGCCTCCCGTAGGAGT 3' (adapted from Amann et al. (1995) for total bacteria. For lactobacilli: F-lac 5'GCAGCAGTAGGGAATCTTCCA3', R-lac 5'GCATTYCACCGCTACACATG3' (adapted from Walter el al. (2001)) and for enterobacteria F-ent 5'ATGGCTGTCGTCAGCTCGT3' (adapted from Leser et al. (2002)) and R-ent 5'CCTACTTCTTTTGCAACCCACTC3' (adapted from Sghir et al. (2000)). The oligonucleotides were adapted from published specific primers or probes using the Primer Express Software to qPCR recommendations (Applied Byosistems, CA, USA). The different primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST (Altschul et al., 1990) and the absence of amplification of porcine DNA was tested empirically by PCR using the DNA extracted from pig blood.

Standard curves were constructed using PCR product of the 16S rRNA gene of *E. coli* and *L. acidophilus*. Primers and PCR conditions were those published by Leser et al. (2002). The PCR product was purified with the commercial kit DNA purification system (Promega Biotech Ibérica, Spain) and the concentration measured at 260 nm (Biophotometer, Eppendorf Ibérica S.L., Spain). Products obtained were also sequenced (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) to

confirm them, and number of copies calculated. Serial dilutions were performed and 102, 103, 104 and 105 copies of the gene per reaction were used for calibration. Amplicons from *E .coli* were used for quantification of the total bacteria and enterobacteria and amplicons from *L. acidophilus* for quantification of lactobacilli. The functions describing the relationship between Ct (threshold cycle) and x (log copy number) for the different assays were: Ct = -3.19 x + 53.66; R2 = 0.99 for total bacteria; Ct = - 2.60 x + 46.82; R2 = 0.99 for lactobacilli; and Ct = - 2.32 x + 43.88; R2 = 0.99 for enterobacteria.

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 (diluted 1/10). The reaction conditions for amplification of DNA were 50 ºC for 2 min, 95 ºC for 10 min, 40 cycles of 95 ºC 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.

4.3. Results and discussion

Minimum levels of detection for the different PCR reactions ranged from 105-106 gene copies/g fresh matter (FM) and were conditioned by the minimum dilution of sample DNA that did not inhibit the PCR reaction, and by the presence of contaminating E. coli DNA in the commercially supplied reagents. Dilution 1/10 was found not to affect the efficiency of amplification, giving equivalent values to 1/100 and 1/1000 dilutions. On the other hand the degree of contamination of the reagents was variable but ranged between 10 and 200 copies / reaction. Similar contamination has been previously described (Suzuki et al., 2000; Nadkarni et al., 2002).

Results for total bacteria, lactobacilli and enterobacteria in jejunum samples using qPCR and traditional methods are shown in Figure 4.1. The values obtained, by qPCR and traditional methods respectively, were 11.1 ± 0.88 log gene copies / g FM and 7.8 ± 0.37 log bacteria /g FM for total bacteria; 10.8 ± 1.66 log gene copies / g FM and 7.9 ± 0.79 log bacteria /g FM for lactobacilli and 8.4 ± 0.56 log gene copies /

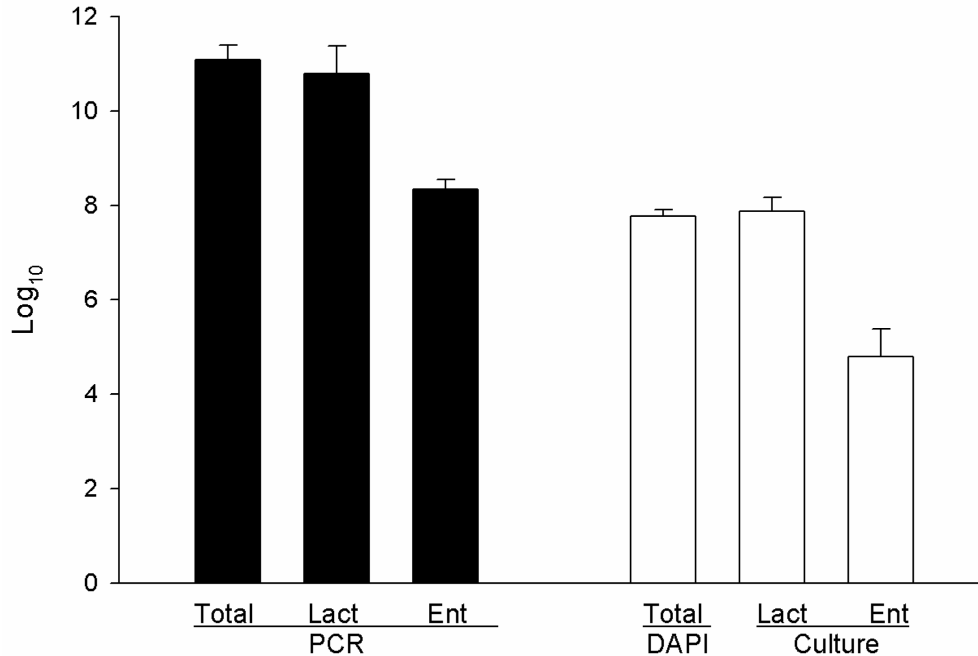
g FM and 4.8 ± 1.68 log bacteria /g FM for enterobacteria. It should be noted that regardless of the method used, lactobacilli counts were close to total bacteria counts, confirming Lactobacillus spp. as one of the major groups in upper gastrointestinal tract of pigs (Khaddour et al., 1998; Reid and Hillman, 1999). In all cases, quantification by qPCR gave higher values in terms of 16S rDNA than DAPI counts or CFU (3.4 ± 0.71, 2.9 ± 1.73 and 3.6 ± 1.72 log units higher for total bacteria, lactobacilli and enterobacteria respectively). However, lactobacilli:enterobacteria ratio (expressed as the difference of logarithms) was similar between methodologies (2.5 ± 0.58 for PCR and 3.1 ± 0.71 for selective culture, $P = 0.39$). Similar discrepancies between PCR and culturing have been found by other authors (Nadkarni et al., 2002; Huijsdens et al.,2002) and they have been related to the multiplicity of 16S rRNA gene copies (Fogel et al., 1999), to the presence of non viable, or viable but not culturable bacterial cells, and to free DNA. In that sense, recently, Apajalahti et al. (2003) found that between 17-34% of bacteria in fecal samples were dead and thus, permanently beyond any culture method.

The use of real-time PCR with SYBR® Green dye could also lead to overestimation due to formation of non-specific amplicons (Hein et al., 2001). However, the dissociation curve obtained at the end of each PCR was checked and always had a similar melting point to the standard samples, without any additional peak, indicating the absence of non-desired PCR products.

Another reason to the overestimation registered, are differences in the pre-treatment of the digesta. The presence of a quantitative important bacterial community attached to the coarse particulate material could have been discharged somehow with culturing and DAPI methods but not with qPCR. In this study DNA samples were directly extracted from the original material without any previous isolation of the bacterial pellet, whereas for culture or DAPI, a previous 1/10 dilution was performed with a subsequent sub-sampling that generally overlooks most of the coarse digestive material that persists in the bottom of the tubes. To validate this hypothesis we compared qPCR results for total bacteria using DNA extracted directly from digesta samples or from pre-diluted samples. Results confirmed a reduction in numbers when subjecting samples to a previous dilution. Mean values were 11.1 ± 0.60 for directly extracted and 10.3 ± 0.51 log units for diluted samples (n = 18). This would suggest that a high percentage of microbial population remains attached to the coarse particulate material. Previous works have described a high percentage of

microbes attached to the solid phase (over 70 % in the rumen, Yang et al., 2001). Moreover, for fecal and digesta samples, DNA extraction protocols are diverse (Anderson et al., 2003), some authors extract DNA directly from the samples, while others isolate previously the bacterial pellet. This previous isolation could affect results quantitatively and also compromise the representativity of the species composition taking into account ecological differences between free bacteria and attached populations (Michalet-Doreau et al., 2001). Results obtained indicate the importance of previous treatment of samples whatever the method of microbial quantification we use.

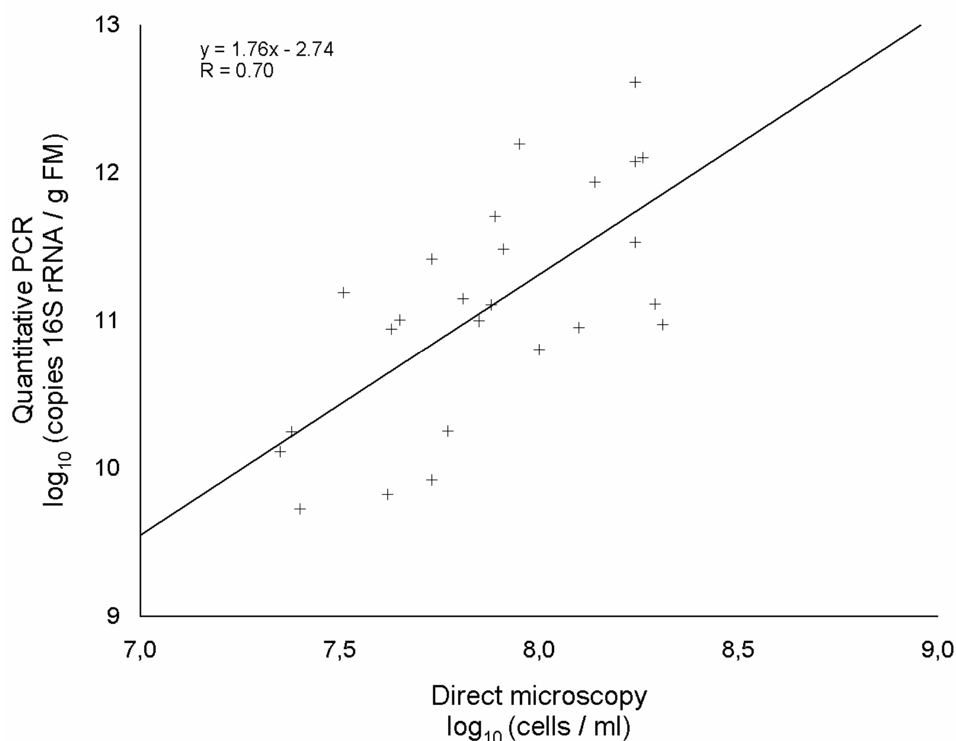
Figure 4.1. Bacterial loads in jejunum digesta of pigs (n = 32) as total bacteria, lactobacilli or enterobacteria measured by qPCR (log 16S rRNA gene copy number/g fresh matter (FM)), DAPI staining (log cells/g FM, for total bacteria) or selective culture technique (log CFU/g FM, for lactobacilli and enterobacteria). Graph shows means and standard error of the means.



In spite of PCR overestimation of microbial counts, values obtained by qPCR and DAPI for total bacteria showed a significant correlation despite (r = 0.7; P < 0.001)

(Fig 2). It is interesting to point out that qPCR overestimation was higher with the highest counts than with the lowest counts. It could be due to an increase in the amount of cellular debris and free bacterial DNA with the highest counts or also to an increase in the percentage of bacteria attached to particulate material that had been somehow discarded with the DAPI method as we have mentioned before. Another possible reason to consider is a change in the number of 16S rRNA copies related to changes in bacterial species and in metabolic activity of bacteria (Fogel et al. 1999). Similarly to total bacteria, PCR and culture counts for lactobacilli showed a significant correlation (r = 0.48; $P < 0.01$) as did the lactobacilli:enterobacteria ratio (r = 0.51; $P < 0.01$). However results obtained for enterobacteria did not show significant correlation. It could be due to differences in the bacteria species considered by the two methodologies following a phenotypic (culture) or a genotypic (qPCR) criterion.

Figure 4.2. Correlation between the number of total bacteria measured by qPCR as log 16S rRNA gene copy number/g FM or by DAPI staining as log cell/g FM in jejunum digesta samples collected from jejunum samples of pigs.



4.4. Conclusion

The results obtained suggest that real-time PCR may well be a practical method for studying quantitative shifts in pig gut bacteria although numerical values are higher than for traditional methods. Differences in absolute values could be related to the amplification of DNA from dead cells with qPCR and to the loss of some particle-attached bacteria with DAPI and selective culture. Relative values between groups such as the lactobacilli:enterobacteria ratio could be used as an index of the gut health status of pigs. The ease and rapidity of qPCR (once implemented) compared with traditional culture, and the possibility of storing samples until analysis, could turn qPCR into the preferred method for quantifying gut bacterial shifts in the near future.

Chapter 5

INFLUENCE OF WEANING ON CAECAL MICROBIOTA OF PIGS: USE OF REAL-TIME PCR AND T-RFLP

5.1. Introduction

At weaning, the piglet is subjected to countless of stressors due to complex social, nutritional and environmental changes which can also enhance piglet susceptibility to diseases and reduce growth (Pluske et al., 1997; Jensen, 1998). In fact, one of the most important problems in pig production nowadays is the post-weaning syndrome that appears at weaning and involves diarrhoea, growth stasis and consequently economical loses (McCracken et al., 1995; 1999). In order to enhance growth, and to control the activity of the gut microbiota at weaning, antibiotics growth promoters have been traditionally fed to pigs. However, since their recent total ban in the European Union (January 2006), new feed strategies and/ or feed additives to maintain piglet gut health are required.

It is it generally recognised that the establishment of a diverse bacterial microbiota, characteristic and dynamic for each individual (Simpson et al., 2000), plays a key role in the maintenance of the gastrointestinal health avoiding the colonization by pathogens (Van Kessel et al., 2004). It may be of extreme importance especially at stressful periods such as weaning when sow's milk withdrawal involves the removal of immunoglobulin A and lysozyme, among other products, such as lactoferrin, that prevent the growth of opportunistic bacteria (Edwards and Parret, 2002). All this, together with the fact that piglets are firstly exposed to many different complex carbohydrates, causes substantial quantitative and qualitative changes in the bacterial community (Katouli et al., 1995, 1999; Melin et al., 1997, 2000) , becoming the piglet more susceptible to microbial disbiosis with potential overgrowth of opportunistic disease-causing pathogenic bacteria (Mathew et al., 1996). However, little is known about the specific changes of microbial ecosystem during this critical phase. In that sense, a more exhaustive knowledge of the bacterial shifts that takes place at weaning would be of great help to follow the proper strategy to replace antibiotics growth promoters in weaned pigs.

Bearing this in mind, an experiment was designed to study microbial shift in pigs at weaning using t-RFLP and real-time PCR.

5.2. Material and methods

5.2.1. Animals and housing

A total of 12 piglets (4.4 ± 0.36 kg; 20 ± 2 days, mixed males and females) were selected from six commercial litters, taking initial body weight into account. One piglet from each litter was weaned and fed a high quality commercial post-weaning diet for one week (Table 5.1) whereas the other piglet remained during this week in the original commercial farm with the dam and the rest of littermates. Weaned pigs were allocated in a box in the Universitat Autònoma de Barcelona facilities. The management, housing, husbandry and slaughtering conditions conformed to the European Union Guidelines.

Table 5.1. Control diet composition (as fed basis).

Ingredient	%
Corn	46.70
Full fat extruded soybeans	17.00
Lactose	15.00
Soybean meal,	10.00
Potato protein	3.77
Whey powder	1.52
L-Lysine HCl (78)	0.17
DL-Methionine	0.10
L-Threonine	0.01
Bicalcium phosphate	3.04
Salt	0.80
Calcium carbonate	0.44
Vit-Mineral premix	0.25
Sepiolite	1.20

5.2.2. Sacrifice and sampling

On day 28 of life, the animals were euthanized in the corresponding farm with an intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Spain;

200 mg/kg BW). Animals were bled, the abdomen was immediately opened and samples (1 g) of the caecum content were taken and kept in tubes with 3 mL of ethanol (96 %) as a preservative.

DNA extraction. The equivalent volume to 400 mg of digesta samples preserved in ethanol was precipitated by centrifugation (13000g x 5 min) and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90 ºC and a posterior incubation step with lysozyme was added (10 mg/mL, 37 ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was stored at -80º C until analysis.

Real-time PCR (qPCR). Total bacteria, lactobacilli and enterobacteria were quantified using real-time PCR following procedures and primers described by Castillo et al. (2006). The oligonucleotides used were based on regions of identity within 16S rDNA gene and were adapted from published specific primers or probes using the Primer Express Software (Applied Byosistems, CA, USA). For total bacteria, primers used were: F-tot (forward) 5'GCAGGCCTAACACATGCAAGTC3' (adapted from Marchesi et al. (1998) and R-tot (reverse) 5'CTGCTGCCTCCCGTAGGAGT 3' (adapted from Amann et al. (1995). For lactobacilli: F-lac 5'GCAGCAGTAGGGAATCTTCCA3' and R-lac 5'GCATTYCACCGCTACACATG3' (adapted from Walter el al. (2001)) and for enterobacteria F-ent 5'ATGGCTGTCGTCAGCTCGT3' (adapted from Leser et al. (2002)) and R-ent 5'CCTACTTCTTTTGCAACCCACTC3' (adapted from Sghir et al. (2000)). Amplification and detection of DNA by quantitative real-time PCR was performed with the ABI 7900 HT Sequence Detection System using optical grade 96-well plates and SYBR Green dye (PE Biosystems, Warrington, UK). For absolute quantification, PCR products obtained from the amplification of the whole 16S rDNA of *Escherichia coli* (CECT 515NT) and *Lactobacillus acidophilus* (CECT 903NT) were used to construct the standard curves. The PCR conditions corresponded to those published by Leser et al. (2002). The amplified gene from *E. coli* was used for absolute quantification of the total bacteria and enterobacteria and the amplified gene from *L. acidophilus* for quantification of the lactobacilli.

Terminal-Restriction Fragment Length Polymorfism (t-RFLP). T-RFLP analysis of bacteria community was performed following the procedure described by Höjberg et al., (2005). Briefly, a 1,497 bp fragment of the 16S rDNA gene was amplified using a 6-carboxy-fluorescein-labeled forward primer: S-D-Bact-0008-a-S-20 (5'-6-FAM-AGAGTTTGATCMTGGCTCAG-3') and reverse primer PH1552 (5'-AAGGAGGTGATCCAGCCGCA-3'). Duplicate PCR were made for each sample. The fluorescently labeled PCR products were purified on QIAquick PCR purification kit columns (Qiagen, West Sussex, UK,) and eluted in a final volume of 30 µL of milli-Q water. After that, the resultant PCR product was submitted to a restriction with *Hha* I (20,000 U/µl) (Biolabs Inc. New England, USA). The fluorescently labeled terminal restriction fragments (TRF) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with 25-U detection threshold. Determinations of the sizes of TRFs in the range of 50 to 700 base pairs were performed with the size standard GS-1000-ROX (PE Biosystems).

Treatment of t-RFLP data. Sample data consisted of size (base pairs) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all TRF peak areas in the pattern was used to normalize peak detection threshold in each sample. Following Kitts (2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 detection threshold in the sample with the smallest total peak area). For each sample, peaks with lower area were deleted from the data set. New total area was obtained by the sum of all the remained peak areas in each pattern.

Diversity was considered as the number of peaks in each sample once standardized. For pairwise comparisons of the profiles, Dyce coeficient was calculated and dendograms constructed using Fingerprinting II (Informatix, Bio-Rad, Ca, USA) software and unweighted pair group method with averaging algorithm (UPGMA).

In order to infer the potential bacterial composition in the samples, *in silico* restriction for the major pig gut bacteria with the primers and the enzyme used were obtained using the analysis function TAP-tRFLP from the Ribosomal Database Project II software (Cole et al., 2003; Table 5.2).

Table 5.2. Theoretical restriction 5'- fragment length predicted for the major pig gut bacteria. Results were obtained from the TAP-RFLP tool of the Ribosomal Database II Project software.

Bacteria groups	Compatible bacteria [a]	*In silico* restriction [b]	Real restriction [c]	Frequency [d]	
				Suckling	Weaned
Lactic Acid Bacteria	*L. acidophillus*, *L. brevis*, *L. bifermentum*, *rhamnosum, casei*	597, 598, 599	597, 599	1 (1.45)	2 (0.54)
	L. delbruekii sp. *Delbruekii*	254	254	4 (1.96)	1 (0.98)
	L. delbruekii sp. *Lactis*	223	221-223	5 (4.59)	0
	L. fructivorans	68	68	3 (0.88)	3 (0.59)
	Lactococcus lactis, Lactobacillus vaginalis	61	62	6 (14.22)	5 (19.42)
	Enterococcus sp.	216, 218, 220	214	5 (2.18)	2(1.35)
Bacteroides and relatives	*Cytophaga*	92, 94, 96, 100	89-104	6 (4.06)	5 (5.17)
	Flexibacter	82, 84, 90, 94, 96, 97			
	Bacteroides	95, 96, 98, 101, 102, 104			
Fibrobacter	Fibrobacter succinogenes	139, 141, 145	138, 140, 142-145	6 (1.80)	4 (2.70)
	Fibrobacter intestinales	148, 152	148-152	6 (3.46)	5 (0.69)
Clostridium and relatives	*Clostridium coccoides*	66	66	3 (1.67)	0
	Clostridium butyricum	544	544	5 (0.86)	0
	Eubacterium	188, 190, 192, 194, 203	188-193	2 (1.19)	4 (0.63)
	Ruminococcus	189			
	Clostridium clostridiforme, C. Symbiosum	190			
	Roseburia	192			
	Butyrivibrio	193			
	Other Clostridium spp.	229, 231, 233, 237	229-232, 237	6 (1.30)	5 (1.36)
Proteobacteria	*Escherichia* sp	371, 372, 373, 374	376-377	3 (0.51)	1 (0.55)
	Other enteric bacteria (*Salmonella*, *Citrobacter*, *Klebsiella*)	367, 370, 371, 372, 373,			

[a] Major pig gut bacteria with a potential compatible fragment found in at least three animals. Other peaks with 58, 59, 69, 111-120, 123, 133, 162, 211, 278 and 279 did not correspond with any 16S rDNA sequences in the database from the Ribosomal Database Project 8.1 software.
[b] *In silico* restriction was performed using the tap-tRFLP tool from the Ribosomal Database project II.
[c] Terminal fragment length obtained after PCR product restriction with *Hha I.*
[d] Number of animals that showed the peak in each experimental group. In braquets abundance of the peak expressed as % of total area. Mean value is calculated only considering the animals showing the peak.

5.2.3. Statistical Analysis

The effect of weaning on total bacteria, lactobacilli, enterobacteria and biodiversity was tested with an ANOVA using the GLM procedures of a SAS statistic package (SAS Inst., Inc. 8.1, Cary, NC). The individual pig was used as the experimental unit. Statistical significance was accepted at $P \leq 0.05$.

5.3. Results and discussion

The animals remained in good health throughout the experiment. Diarrhea was not detected in any of the pigs, although there was one case of yellowish liquid faeces (W group). Initial live weight was similar for both groups, with 4.4 ± 0.16 kg for S and 4.4 ± 0.15 kg for W, and, as expected, at the end of the experimental period body weight (BW) was higher for piglets that remain with the sow (6.1 ± 0.25 kg and 5.05 ± 0.27 kg for S and W respectively, $P < 0.001$). Expressed as average daily gain (ADG), growth rate was higher for suckling than for weaned pigs (0.25 ± 0.02 and 0.10 ± 0.02 kg for S and W respectively, $P < 0.001$).

5.3.1. Bacterial quantitative change measured by real-time PCR

The total microbial population, lactobacilli and enterobacteria were quantified in caecum digesta using qPCR (Figure 5.1).

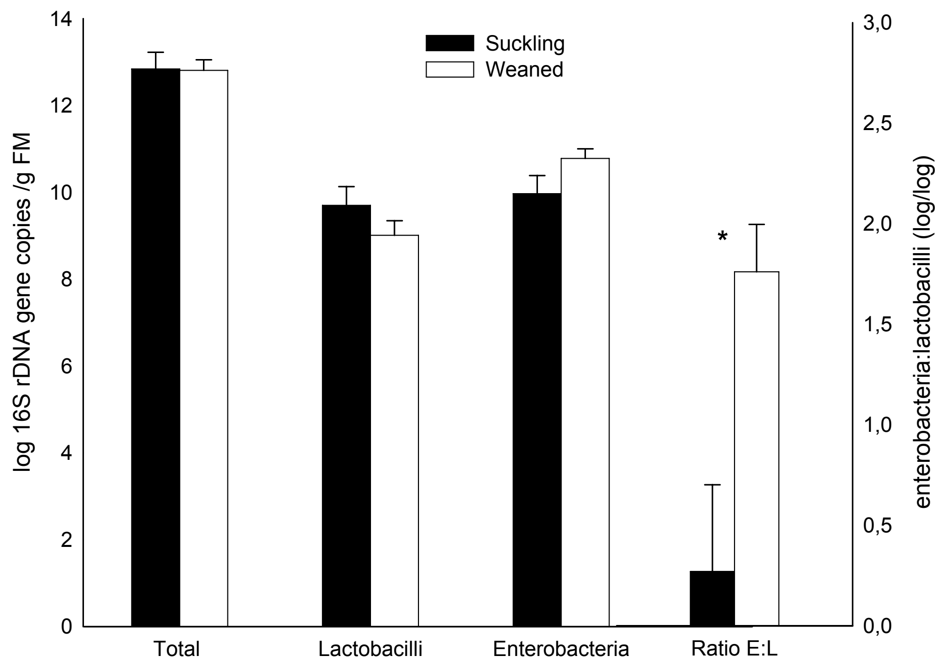
The total bacteria counts, expressed as log 16S rDNA copies/g fresh matter (FM), were similar between groups (12.84 and 12.81 log gene copy number/g FM for S and W respectively). Similar results were found by Franklin et al. (2002) in piglets weaned at 24 days where total faecal anaerobic counts were maintained after weaning.

Lactobacilli and enterobacteria have been traditionally selected as microbial groups with a particular significance for gut health. The ratio between these two bacterial groups, firstly proposed by Muralidhara et al. (1977), has been routinely used as a gut health indicator, being desirable that lactobacilli outnumber enterobacteria to improve robustness against opportunistic pathogens.

As expected, animals fed with dry food showed a numerical decrease in lactobacilli population (9.70 *vs.* 9.01 log gene copy number/g FM for S and F group respectively, $P = 0.24$) that was accompanied by an increase in enterobacteria (9.97 vs. 10.78 log gene copy number/g FM for S and F group respectively, $P = 0.13$). The ratio enterobacteria:lactobacilli, expressed as difference of logarithms, was

significantly higher in weaned pigs reflecting the contrary effect of weaning on lactobacilli and enterobacteria populations (0.27 and 1.76 for S group and W group respectively (P = 0.05)). Comparable results have been found before, with an inverse connection between lactobacilli and enterobacteria during the first week post-weaning (Risley et al., 1992; Jensen, 1998). This response is due to marked decreases in lactobacilli in parallel with increases in enterobacteria population (Mathew et al., 1996; Franklin et al., 2002). In fact, abrupt weaning has been associated with a 100-fold drop in the numbers of lactobacilli in the intestine, and 50-fold increase in the numbers of *Escherichia coli* (Huis in't Veld and Havennar, 1993).

Figure 5.1. Bacterial loads in caecum measured by quantitative PCR (log 16S rDNA gene copies /g FM) in suckling or weaned pigs.



The maintenance of lactobacilli population, that is well adapted to utilise substrate from the milk (Hopwood and Hampson, 2003) may be of a great interest considering its related effects promoting gut health by inhibition of some other bacteria, such as *E. coli* (Hillman et al., 1995; Tannock et al, 1999) and also modulating an adequate immune response (Perdigón et al., 2001), specially important during the first stage of life.

TRIAL II

5.3.2. Ecological bacterial changes, t-RFLP results

The similarity indexes of the t-RFLP profiles illustrated in form of a dendogram are shown in Figure 5.2. It shows microbial profiles of 11 pigs, due to the fact that one pig did not present digesta in the cecum at sampling. The effect of weaning on the ecological composition of microbiota was clearly dominating in comparison with other factors that could have been affected, such as litter or individual effects. This was reflected in two clearly separate clusters, one for each experimental group. There was only an exception for one weaned piglet that grouped in the suckling branch of the dendogram that interesting corresponded to the animal that showed liquid faeces. Separation of this piglet in the dendogram might reflect the beginning of some kind of enteric disbiosis in this piglet, although no differences in productive parameters measured were observed. In fact, watery stools are related with malabsorption syndrome that usually appears 3-10 days after weaning (Kyriakis et al., 1989). A poor adaptation to dry feed in this animal might have caused a higher speed in transit time. Besides dry feed introduction, social stress suffered at weaning has also been related with increases in cortisol release and an increased transit time via the sympathetic nervous system (Pluske et al., 2002). Moreover, is well known that bacterial colonisation is highly dependent on flow of digesta, being impaired by a high speed (Stewart et al., 1999). All these factors might have become into a fail of microbial ecosystem to adapt to dry food in this animal, remaining therefore a bacterial community more similar to that of the suckling period.

Weaning is not only a change of diet, it involves a countless of stressors. Dietary components are drastically changed; lipids are substituted by carbohydrates as the main source of energy that with the immaturity of the piglet digestive system may result into an important fermentable substrate for the intestinal bacteria. Also, there is a marked change into the microbial environment, to which the animal is exposed, that before was mainly determined by the sow. All this, with the withdrawal of milk supply, and therefore diverse functional components such as different glycoproteins and oligosaccharides (Pluske et al., 1996b) can also have a crucial effect shaping the profile of the piglet autochthonous microbiota.

In that sense, suckling pigs showed a higher similarity between them (54-78%) than weaned pigs with more heterogeneous microbial profiles (25-76%). This higher homogeneity found in the microflora of suckling piglets could have been determined by a mother effect. In this sense, Katouli and co-workers (1997), studying metabolic fingerprinting of piglet's microbiota, demonstrated the high effect of the sow being

the determinant factor in microbiota establishment within the piglet's first days of life.

On the other hand, the higher variability in microbial profiles in weaned group would also reflect the stress of the pig at weaning, responding each animal individually.

Biodiversity, measured as the total number of bands was similar between both experimental groups (49.34 for S and 53.40 for F respectively, P = 0.22). Different works have described a marked decrease in biodiversity just after piglet weaning (Katouli et al., 1997; Jensen-Waern et al., 1998; Melin et al., 2000) showing that weaning involves a clear disruption in the normal pig microbiota evolution. After that, there is a process of re-establishment that can take more or less time depending on a plethora of factors. A higher biodiversity is desirable due to the fact that a lower diversity implies lower colonization resistance being the piglet more susceptible to intestinal disorders and proliferation of opportunistic pathogens (MacFarlane and McBain, 1999; Melin et al., 2004). In our case, the pigs fed with dry food were probably in the process of reestablishment of a new microbial equilibrium and probably, a later sacrifice could have shown a higher biodiversity. In that sense, Jensen (1998) found that it takes 2 to 3 weeks after weaning before the fermentative capacity of the microbiota in the hindgut has fully developed, moreover Swords et al (1993) found that until 3-4 months after weaning the microbiota is still in evolution. More recently, Inoue and co-workers (2005) found a recovery in piglet bacterial biodiversity 25 days post-weaning.

In silico restriction using Ribosomal Database Project II was used to infer potential ecological changes in the samples. Before considering these results it is needed to remind that dispersed phylogenetic groups of bacteria may produce T-RFs of identical size (Liu et al., 1997) and that a single t-RF in a profile may represent more than one organism in the sample. Results are therefore presented as potential compatible bacterial species and always have some of speculative as direct attribution of specie to one peak is not completely possible.

In our case, we did an attempt to assign compatible bacteria species among those major pig gut bacterial groups that have been recently described (Leser et al., 2002). Figure 5.3 shows an example of the electropherogram for one pig of each experimental group with inference of some compatible bacteria. Table 5.2 shows the relative abundance (as % of total area) of compatible bacteria that were at least represented in 3 animals.

Figure 5.2. Dendogram illustrating the effect of weaning in t-RFLP banding patterns. The dendogram represents results from 11 piglets sacrificed on day 28 of life. The dendogram distances are in percentage of similarity.

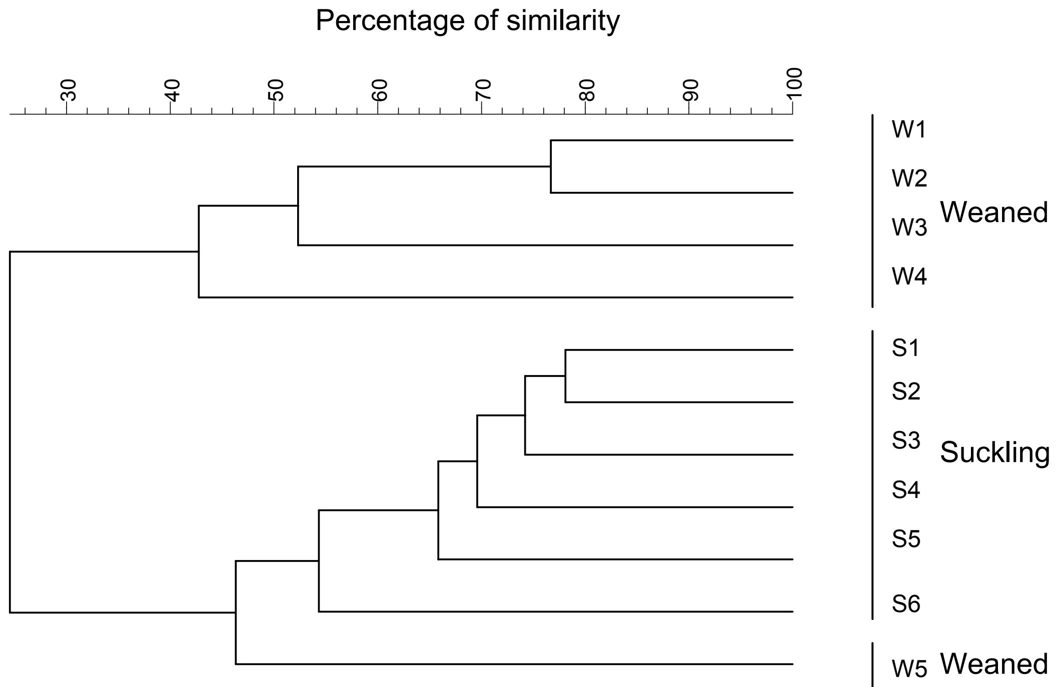
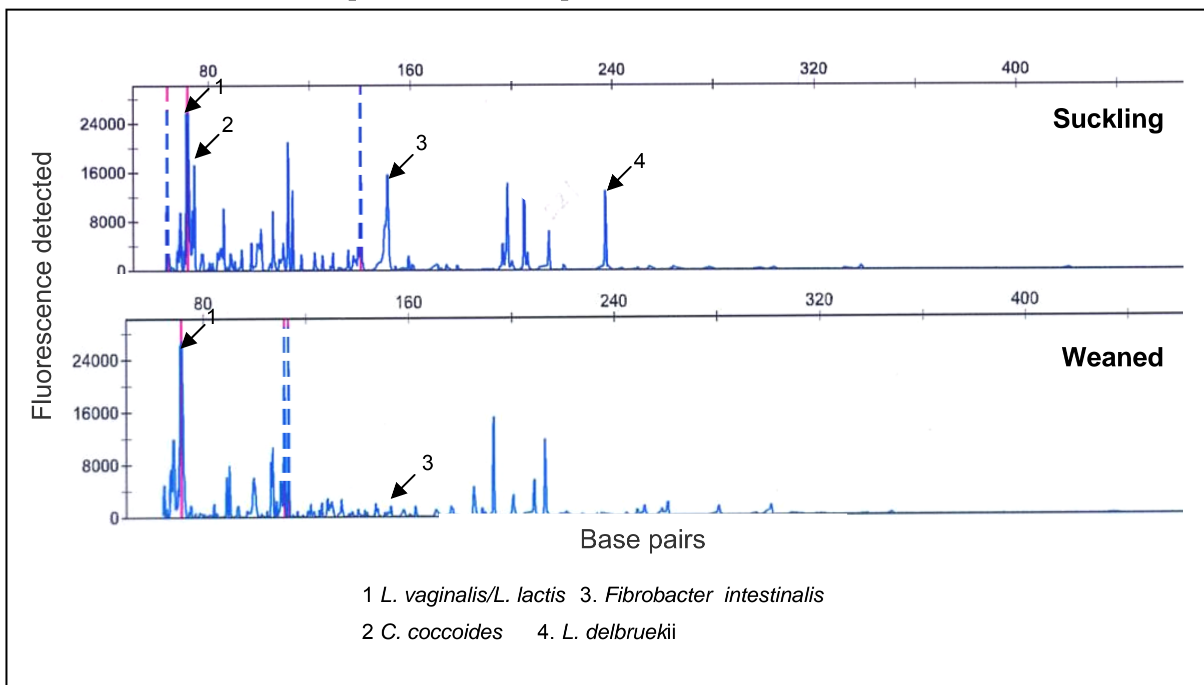


Figure 5.3. Electropherogram produced from Hha I digestion of 16S rDNA PCR products from caecum digesta from one suckling pig and one weaned piglet. The size and intensity of each band were determined by using Genescan software. Arrows show the most abundant peaks in the samples.



Lactic acid bacteria. *Lactobacillus*, *Lactococcus, Streptococcus*, and *Enterococcus,* belong to the Firmicutes with low mol% G + C content in DNA, that are also characterized by the formation of lactic acid (Aguirre and Collins, 1993) and are described as one of the major groups in the pig gastrointestinal tract (Hill et al., 2005).

Analysis of electropherograms revealed compatible TRFs with different lactic acid bacteria including *L. adidophilus, L. bifermentum, L. brevis, L. casei, L. rhamnosum, L. vaginalis, Lactococcus lactis, L. delbruekii sp. lactis, L. delbruekii sp. delbruekii,* and *L. fructivorans*. It is also interesting to remark the fragment of 62 base pairs compatible with both *L. lactis* and *L. vaginalis* that was present in all the animals with a mean contribution around 15 – 20% of total area (see Figure 5.3). Mean area for total lactobacilli was similar between both groups (23.1 % and 21.5 % for suckling and weaned pigs respectively).although suckling pigs showed higher biodiversity in compatible TRFs with different lactobacilli species than weaned pigs. Particularly *L. delbruekii* sp. *lactis* was present in five animals from this group, representing near the 5% of total area, whereas no animal of the W group showed any fragment with compatible size. Similarly, *L. delbruekii* sp. *delbruekii* was present in four suckling pigs, and only appeared in one weaned pig.

The presence of a higher diversity of lactobacilli in suckling pigs has been described before (Krause et al., 1995). It has been related with the different feeding behaviour of suckling piglets, much more frequent than weaned pigs (Moran, 1982) that usually refrain from eating (Le Dividich and Herpin, 1994). This might result in a higher and continous amount of substrate available for fermentation in the upper gastrointestinal tract in suckling pigs. Moreover, the casein component of sow's milk results into the formation of milk clots in the stomach that could act somehow as a carrier niche for lactobacilli from stomach to small intestine. All these factors could result in a more beneficial environment for the growth of lactobacilli in the upper gastrointestinal in suckling pigs tract that could be behind the greater diversity of lactobacilli species observed in posterior sections.

In the case of *Enterococcus* sp., five of the six suckling pigs showed a peak that may be compatible with this group (2.18 %) that only appear in two pigs from the weaned group (1.35%). In the same way, this bacteria group is often described in newborn babies (Favier et al., 2002) and is also in agree with Jensen (1998) who found a decrease in enterococci when piglets were weaned.

Bacteroides and relatives. Different species from the phylogenetic group Cytophaga-Flexibacter-Bacteroides (CFB) phylum (Gherma and Woese, 1992) can be compatible with a series of TRFs of similar size ranging from 89 to 104 bp (see Table 5.2). Summed area of these peaks represent 4.0 % of total peak area for suckling and 5.2 % for weaned pigs being therefore the second group in importance behind lactobacilli.

Savage et al. (1977) demonstrated that *Bacteroides* spp is on of the predominant gram negative anaerobes in the adult pig caecum, and have been described also as one important bacteria in young piglets (Adami and Cavazzoni, 1999) with a marked increase after weaning (Swords et al., 1993).

Clostridium and relatives. Clostridia represents a phenotypically and phylogenetically extremely complex and heterogeneous group of organisms. Sequences of the 16S rRNA have demonstrated deeply branching lineages within the clostridia, which included nonclostridial species (Sharp and Ziemer, 1999; Lawson, 1999). All members of the genus *Ruminococcus* fall within the genus, as well as *Eubacterium* species that are scattered throughout the Clostridium genera too (Collins et al., 1994; Rainey and Janssen, 1995).

In our study, a peak compatible with *Clostridium coccoides* only appear in three suckling pigs representing 1.67 % of total peak area. In the same way, a peak compatible with *C. butyricum* (0.86 %) was only found in the suckling group. Phylogenetically *C. butyricum* is classified into the Cluster I, Clostridium *sensu stricto*, where are found the majority of species of the genera (Collins et al., 1994).

Some other authors agree with our results and have described *Clostridium* as one of the main anaerobic bacteria during suckling period, declining progressively in abundance with the age (Swords et al., 1993). The presence of C. coccoides may be considered as beneficial for the piglets due to its production of SCFA. In fact, it has been used as a probiotic both in animals and humans (Han et al., 1984: Seki et al., 2003).

Other compatible peaks with different species from the *Clostridium* clusters I, IV and XIVa and XVIII were found in both groups of animals representing as a mean of 2.49 and 1.99 % of total area for suckling and weaned pigs respectively. However, it is difficult to conclude potential changes in bacteria belonging to any of those groups related to weaning process.

Fibrobacter. Compatible peaks with *Fibrobacter succinogenes* and *Fibrobacter intestinalis* were found in both groups of animals (5.3 % of total peak area for suckling and 3.4 % for weaned pigs).

Until recently, these bacteria were grouped into the Bacteroides sub-phylum, being recently reclassified into the specifically named genus Fibrobacter (Amann et al., 1990a). Bacteria belonging to this genera are one of the major indigenous fibrolytic bacteria in ruminants (Griffiths and Gupta, 2001) although have been also found in the pig gastrointestinal tract (Varel et al., 1984; Varel and Yen, 1997); in particular, high numbers of these bacteria have been described in adult pigs (Varel et al., 1997). Those bacteria show high cellulolityc and hemicellulolityc enzymatic activities (Gokarn et al., 1997). Therefore, the presence of this both bacteria in both experimental groups may point out the high potential that the pig have to effectively utilize dietary fibre especially important in adult animals.

Proteobacteria. Proteobacteria phylum includes *Enterobacteriaceae* family to which belong different bacteria such as *E. coli, Shigella, Klebsiella, Salmonella* that have been routinely described as members of the indigenous pig gut microbiota (Ewing and Cole, 1994). However, we found potential compatible peaks only in four animals, even that we determined enterobacteria counts by qPCR in all the animals. A bias in the amplification of particular sequences, caused by preferential annealing of particular primer pairs to certain templates (Suzuki and Giovanonni, 1996) and also the complexity of amplifying bacteria in lower proportions in complex samples like digesta content might explain absence of compatible TRFs although being counted by real-time PCR in all the pigs. It is also fair to remark that the presence of different sequences in the databases are undoubtedly biased by the investigation interests. In that sense bacterial groups like lactic acid bacteria have received much attention and huge amount of sequences have been deposited, whereas other groups are less represented. This fact might explain, at least partially, the low abundance found for enterobacteriacea family compared to lactic acid bacteria and inconsistence of T-RFLP with qPCR results..

Three peaks (161, 173 and 238 base pairs) were found compatible with *Mycoplasma arthritidis*, *Mycobacterium* sp. and *Staphylococcus* sp. respectively. These three bacteria were not considered in the study taking into account that did not represent typical pig gut bacteria (Leser et al., 2002).

5.4. Conclusions

The results obtained agree with previous works concluding that commercial weaning produce marked changes in pig caecum microbiota, with an increase in enterobacteria:lactobacilli ratio after weaning and changes in T-RFLP bacterial profiles. Even though only presumptions can be made, suckling pigs showed a higher diversity of compatible TRFs with different lactic acid bacteria than weaned group, and showed peaks compatible with *C. coccoides*, and *C. butyricum* species that were absent in weaned pigs. In the light of these tentative results, an interesting way to maintain post-weaning piglet gastrointestinal health at weaning could be to avoid marked shifts in these characteristic suckling bacteria. In that sense, alternatives to antibiotics may focus in maintaining as far as possible the weaning microbial profile at least during the post-weaning transition, by leading to a more favorable equilibrium. However, more studies are required to increase our knowledge regarding the microbiota changes at weaning.

Chapter 6

MOLECULAR ANALYSIS OF BACTERIAL COMMUNITIES ALONG THE PIG GASTROINTESTINAL TRACT

6.1. Introduction

The gut microbial ecosystem in mammals is highly complex, typically comprising more than 400-500 species and viable counts up to 10^{12} per gram of gut content in the large intestine (Moore and Holdeman, 1974; Eckburg et al., 2005). Several studies have investigated the species diversity of the pig intestine through phenotypic analysis of isolates obtained by anaerobic culturing (Tannock et al., 1970; Salanitro et al., 1977; Robinson et al., 1981; Varel et al., 1987). Culturing however is likely to recover some bacteria more readily than others, and is laborious (Zoetendal et al., 2004). The development of new molecular tools has revolutionized our knowledge of gut microbial diversity (Pryde et al., 1999; Vaughan et al., 2000). Leser and co-workers (2002) sequenced more than 4 200 cloned 16S rDNA sequences from digesta samples of 52 pigs. This work confirmed the complexity of the pig intestinal microbial community and indicated that much of the pig gut microflora remains uncultured. Meanwhile profiling techniques, such as DGGE and T-RFLP that exploit 16S rRNA sequence differences have also contributed to our understanding of population dynamics along the different compartments of the gastrointestinal tract of the pig (Simpson et al., 1999). The availability of 16S rRNA sequences has also facilitated the design of phylogenetically targeted oligonucleotide probes that can be used for fluorescent *in situ* hybridization (FISH). FISH has the advantage that it avoids potential PCR-bias, while also giving information about the spatial distribution of gut microorganisms. FISH has been used to quantify different microbial groups in the human gut (Harmsen et al., 1999; Hold et al., 2003; Takada et al., 2004) but there is relatively little information on pig gut microbiology although this technique has been used to study the abundance of lactobacilli (Konstantinov et al., 2004b).

A major incentive to understanding the composition of the gut microflora comes from the impact of commensal bacteria on host health, in particular pathogen exclusion, immune development and gut metabolism (Stewart et al., 1997). Despite recent advances, our knowledge of a healthy pig gut microbiology is still far from complete. Gaining an understanding of population dynamics and responses to different dietary and environmental changes could help to design strategies to promote health particularly in young animals. Nutrients that escape digestion in the upper gastrointestinal tract represent the main growth substrate for gut bacteria with complex plant carbohydrates as their primary available substrates (Salyers et al., 1979). These include soluble non-

starch polysaccharides (sNSP), insoluble non-starch polysaccharides (iNSP), and resistant starch (RS) (Englyst and Cummings, 1987). In this way, manipulation of the quantity or the type of fibre administered to a pig can be a potential mechanism to change the structure of the microbial population and make it more resistant to the establishment of opportunistic pathogens.

The aim of this project was, firstly, to study the quantitative importance of major bacterial groups along the different sections of the pig gastrointestinal tract and secondly, to evaluate the potential of dietary fibre to modulate the gut microbial ecosystem.

6.2 Material and methods

6.2.1. Animals and diets

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

A total of 32 pigs (Pietrain x (Large White x Landrace) of 15 ± 0.38 kg of body weight, were distributed into 32 pens with forced ventilation. Pens were distributed into four experimental diets that included a control diet (CT) (54% corn, 15% barley, 28% soya-44, 0.7 % vegetable oil, 3% vitamins, minerals and aminoacids), a diet enriched in resistant starch (GC) by substitution of fine-grounded corn of basal diet (2.5 mm) by coarse-grounded corn (4 mm), a diet enriched in soluble fiber (BP) by partial substitution of the corn by 8% of sugar beet pulp or a diet enriched in insoluble fiber (WB) by partial substitution of corn by 10 % of wheat bran. Animals were fed ad libitum for 6 weeks.

6.2.2 Sample collection and processing

At the end of the experimental period, twenty animals (5 per treatment) were euthanised with an intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg kg^{-1} BW). Animals were bled, the abdomen was immediately opened and samples of the intestinal content were taken.

For FISH analysis, samples of the stomach, distal jejunum, distal colon and rectum digesta were taken (500 mg). Immediately after the collection, the samples were

homogenised and diluted ten times with PBS. To remove gross material, the samples were centrifuged (700 x g, 3 min) and 1 ml of supernatant was fixed with freshly prepared 4% paraformaldehyde. To fix the cells, the samples were incubated overnight 4 ºC and finally stored at -80 ºC until use.

Digesta from proximal colon was homogenized and the pH determined. For short chain fatty acids (SCFA) analysis, samples were collected (5 g) and kept frozen (-20 ºC). For purine bases analysis (guanine plus adenine) used as microbial marker, approximately 50 g were taken, frozen and lyophilised until analysis. For DNA analysis, samples of approximately 1 g of digesta were kept in tubes with 3 ml of ethanol as a preservative.

Fluorescent in situ hybridization (FISH). Samples from the stomach, distal jejunum, proximal colon and rectum digesta were assessed with probes for the following groups: Total bacteria (Eub 338), *Bacteroides/Prevotella* group (Bac303), *Ruminococcus flavefaciens* (Rfla729), *R. bromii* (Rbro730), clostridia cluster XIVa (Erec482), clostridia cluster IV species related to *Faecalibacterium prausnitzii* (Fprau645), clostridia cluster IX (Prop853), *Streptococcus/Lactococcus* sp. (Str493) and *Lactobacillus/Enterococcus* sp. (Lab158) (Table 6.1) following the method described by Harmsen and co-workers (2002).

Diluted cell suspensions (10µl) were applied to gelatin coated slides and hybridised with 10 µl of each oligonucleotide probe (50 ng μl^{-1} stock solution) in 110 µl of hybridisation buffer overnight (except for Bac303 probe, which was hybridised for 2 h). To prevent fading of fluorescence Vectashield (Vector Laboratories, Burlingame, California) was added to each sample. Fluorescent cells were counted with a Leica DMRXA epifluorescence microscope. Twenty five fields were counted for each sample (in duplicate).

DNA extraction and purification. Digesta samples (400 mg) preserved in ethanol were precipitated by centrifugation (13 000 g x 5 min) and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90 ºC and a posterior incubation step with lysozyme was added (10 mg ml^{-1}, 37 ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 µl of Qiagen Buffer AE (Qiagen, West Sussex, UK) and was stored at -20º C. The purified DNA was

stabilized with the addition of 4 µl of 40 mg ml^{-1} BSA (Bovine Serum Albumin, Sigma-Aldrich Química S. A., Madrid) plus 2 µl of ribonuclease A (Sigma-Aldrich).

PCR-RFLP analysis . To analyze the total bacteria in the proximal colon digesta, a 580 bp fragment of 16S-rDNA gene was amplified from DNA extracts by PCR using primers specific to conserved sequences flanking variable regions V3, V4 and V5: 5'-CTACGGGAGGCAGCAGT-3' (forward) and 5'- CCGTCWATTCMTTTGAGTTT-3' (reverse). Primers and PCR reaction conditions were those described by Lane and co-workers (1991). The reaction was performed using a GeneAmp PCR System 9700 (PE, Biosystems, Warrington, UK) thermocycler. The DNA amplification conditions were 94 ºC (4 min); 35 cycles of denaturation at 94 ºC (1 min), annealing at 45 ºC (1min) with an increment of 0.1 ºC per cycle, extension at 72 ºC (1 min 15 s); and a final extension at 72 ºC (15 min). Following visual confirmation of PCR products with agarose gel electrophoresis, four independent enzymatic restrictions were carried out (*AluI, RsaI, HpaII, CfoI* (F.Hoffmann-LaRoche Ltd Group, Basel, Switzerland). The digestions were performed as recommended by the manufacturer, with the appropriate restriction buffer at 37 ºC for 3 hours. Different fragments were separated using a 2% high resolution agarose gel.

The size and the intensity of the bands within each lane of a gel was analyzed by the Gene Tools software (Syngene, Cambridge, UK) and the degree of microbial biodiversity was measured as the total number of different bands obtained from the four independent restriction digestions. For pair-wise comparisons of the banding patterns and the construction of dendograms, similarity matrices were generated based on the Manhattan distance (Kaufmann and Rousseaw, 1990) that takes into account the size and the height of the bands generated.

TRIAL III

Fermentation product analysis. Analysis of SCFA was performed by GLC using the method of Richardson and co-workers (1989) modified by Jensen and co-workers (1995). Purine bases (adenine and guanine) in lyophilised digesta samples (40 mg) were determined by HPLC (Makkar and Becker, 1999). For their analysis purine bases were hydrolyzed from the nucleic acid chain by their incubation with 2 ml 2 M-$HClO_4$ at 100ºC for 1h, including 0.5 ml of 1 mM-allopurinol as an internal standard.

Table 6.1. Sequence of oligonucleotide probes used in this study.

Probe	Sequence (5'→ 3')	Targeted bacterial group	Reference
Eub338	GCTGCCTCCCGTAGGAGT	Domain bacteria	Amann et al. 1990b
Bac303	CCAATGTGGGGGACCTT	*Bacteroides-Prevotella* group	Manz et al. 1996
Rfla729	AAAGCCCAGTAAGCCGCC	*Ruminococcus flavefaciens*-like	Harmsen et al. 2002
Rbro730	TAAAGCCCAGYAGGCCGC	*Ruminococcus bromii*-like	
Erec482	GCTTCTTAGTCAGGTACCG	Clostridium cluster XIVa	Franks et al. 1998
Lab158	GGTATTAGCA(C/T)CTGTTTCCA	*Lactobacillus-Enterococcus* group	
Fprau645	CCTCTGCACTACTCAAGAAAAAC	*Faecalibacterium prausnitzii* group	Suau et al. 1999
Str493	GTTAGCCGTCCCTTTCTG	*Streptococcus and Lactococcus* sp.	Franks et al. 1998
Prop853	ATTGCGTTAACTCCGGCAC	Clostridium cluster IX	Walker et al. 2005

6.2.3. Statistical Analysis

The effect of the diet on bacterial, biodiversity, SCFA concentration, pH and purine bases concentration in a given intestinal segment was tested with an ANOVA using the GLM procedures of a SAS statistic package (SAS Institute, INC. 8.1, Cary, NC). Treatment means were assessed with least significant difference test (LSD) when overall treatment effects were $P < 0.05$. Statistical significance was accepted at $P < 0.05$.

6.3. Results

6.3.1. Microflora structure along the gastrointestinal tract as analyzed by FISH

Samples were analyzed from stomach, jejunum, proximal colon and rectum of animals maintained on four different diets. Counts were obtained for each sample with the broad eubacterial probe, eub338, and with seven non-overlapping, group-specific probes. These targeted Firmicute bacteria belonging to clostridial clusters XIVa (Erec482), cluster IX (Prop853) and cluster IV bacteria related to *Faecalibacterium prausnitzii* (Fprau645) or to *Ruminococcus flavefaciens/bromii* (Rbro730/Rfla729), as well as streptococci (Strc493) Lactobacilli (Lab) and *the Bacteroides/Prevotella* group of Gram-negative bacteria (Bac303). As expected, total bacteria per gram of digesta measured with Eub338 probe increased from proximal to distal sections being at least 100-fold higher in proximal colon and rectum (averaging approximately 4.0×10^{10} and 5.8×10^{10} respectively) than in stomach and jejunum (3.6 $\times 10^{8}$ and 2.4×10^{8} respectively).

Eub338 counts for the stomach averaged around 4×10^{8} g^{-1} over the four diets. In this site the little studied clostridial cluster IX group made up a highly significant fraction (14-41 %) when compared with the total eubacterial count. Streptococci (15 - 37 %) and lactobacilli (8-26 %) were also abundant, while bacteria related to clostridial cluster IV ruminococci were also abundant on diets BP and WB (27 and 11 % respectively). The probe set used on average accounted for 56-93 % of the eub338 count in the stomach.

By contrast, in the jejunal samples the probe set coverage was lower than in the stomach and particularly on CT diet only covered 32 % of the total bacteria. Lactic acid bacteria measured (streptococci and lactobacilli) were the main groups and both together exceeded 24 % of the eub338 counts. In contrast, the rest of groups

measured were less important in this gastrointestinal section and were always below the 6% of total counts. As found in the stomach, cluster IV *F. prausnitzii* relatives counts were below the detection limits (counts < 2×10^6 g^{-1}).

Bacterial profiles in the proximal colon and rectum were similar, with cluster XIVa bacteria amounting to 10-19 %*, Bacteroides/*Prevotella relatives 4.5-10 %, cluster IV *F. prausnitzii* relatives 1.4-3.6% and cluster IX bacteria 4.7-7.7% compared to the eub338 count. *Streptococcus* and *Lactobacillus* numbers averaged 1-2%, and less than 0.5%, of the eub338 count respectively. These results confirm the dominance of anaerobic bacteria related to clostridial clusters XIVa and to the clostridial cluster IV *F. prausnitzii* relatives in the dense communities of the large intestine, by comparison with stomach and jejunum. On the other hand, it is also clear that a major fraction of the bacterial variation at these sites is still not accounted for by the probes used.

6.3.2. Effects of fibre on microbial composition as estimated by RFLP and fermentation profiles

The level of biodiversity of the proximal colonic microbial ecosystems expressed as number of bands obtained with the four independent enzymatic restrictions showed differences related to the dietary source of fiber. Animals fed on diet including wheat bran showed the lowest biodiversity level (28 ± 0.66 number of bands) compared to control diet (34 ± 1.12), GC (38 ± 2.48) or BP (37 ± 2.15) diets (P = 0.008). Dendograms (Figure 6.1) evidenced that animals receiving WB clustered separately having the most similar RFLP patterns (95-97 % similarity) followed by CT (91-94 %); GC (86-91 %) and BP (83-95 %) diets.

The pH and purine base concentrations of proximal colon samples were similar between diets (Table 6.2). Total SCFA concentration was higher for diets enriched with additional fibre although differences did not reach statistical significance (CT *vs.* rest of diets, P = 0.06). Diets enriched in non-starch polysaccharides (BP and WB) showed lower molar percentages of branched chain fatty acids (BCFA) (P = 0.10) and valeric acid (P = 0.002).

Figure 6.1. Dendogram illustrating the percentage of similarity of PCR-RFLP banding patterns in samples of proximal colon digesta. The dendogram represents results from 20 pigs euthanised.

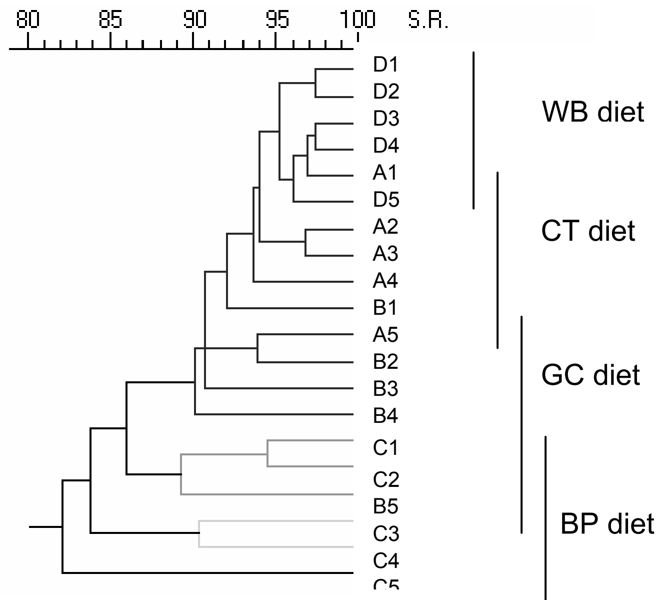


Table 6.2. (A) Fermentation parameters (pH, total SCFA concentration and purine bases), in the proximal colon contents from pigs receiving experimental diets.

	DIETS					
Parameter	CT	GC	BP	WB	SEM	*P*-value
pH	5.85	6.05	5.78	5.92	0.174	0.72
PB (µmol g^{-1})	33.0	31.2	34.3	33.0	6.09	0.98
SCFA (µmol g^{-1})	128	141	155	147	19.5	0.17

(B). Major proportions of the different SCFA

	DIETS					
SCFA (%)	CT	GC	BP	WB	SEM	*P*-value
Acetate	63.8	59.5	63.1	60.2	4.78	0.40
Propionate	20.2	25.1	24.9	26.3	5.73	0.35
Butyrate	13.3	13.3	10.4	11.9	2.30	0.16
Valerate	2.78^{a}	2.10^{ab}	1.56^{b}	1.68^{b}	0.463	0.002
Branched-SCFA	1.72	1.17	0.74	0.75	0.694	0.10

CT: control diet; GC: control diet with coarse-grounded corn; BP: control diet enriched in soluble fiber by addition of 8% of sugar beet bulp and WB: control diet enriched in insoluble fiber by addition of 10% of wheat bran.

Each mean represents five individual pigs. Least-squares means within a row lacking a common superscript letter differ ($P < 0.05$)

TRIAL III

Table 6.3. Proportions of specific bacterial groupings in different regions of the porcine digestive tract estimated by fluorescent *in situ* hybridization.

	Count ($\times 10^9$)/ml	Counts as % Eub338						
	Eub338	**Bac303**	**Erec482**	**Fprau645**	**Rbro/Rfla**	**Prop853**	**Str493**	**Lab158**
Stomach								
CT	0.12 ±0.11	2.4 ±0.9	2.6 ±1.7	bd	3.4c ±1.6	41.1a ±18.1	20.2 ±9.4	8.0 ±17.5
GC	0.29 ±0.27	1.8 ±0.5	2.8 ±1.1	bd	3.3c ±1.7	17.0b ±4.4	36.5 ±41.4	26.2 ±37.1
BP	0.53 ±0.49	2.2 ±1.6	2.1 ±1.0	bd	26.9a ±1.1	25.3ab ±14.6	14.9 ±5.3	21.7 ±16.2
WB	0.49 ±0.63	1.9 ±0.5	2.1 ±1.0	bd	10.5b ±1.5	14.4b ±1.7	15.5 ±8.7	11.4 ±12.8
Jejunum								
CT	0.22 ± 0.13	4.7 ±0.4	1.6 ±0.6	bd	2.0 ±1.1	3.3 ±3.4	4.9 ±5.8	15.3 ±15.3
GC	0.37 ±0.50	3.5 ±1.2	1.5 ±0.4	bd	3.3 ±1.5	3.7 ±2.2	25.7 ±22.6	11.0 ±12.4
BP	0.25 ±0.31	5.5 ±1.9	2.9 ±1.4	bd	2.9 ±1.1	2.1 ±2.0	12.9 ±17.7	11.4 ±23.6
WB	0.12 ±0.11	5.1 ±2.5	2.0 ±0.7	bd	2.8 ±1.3	1.0 ±0.5	31.1 ±36.2	25.0 ±47.1
Proximal colon								
CT	39.6 ±15.8	8.1ab ±2.0	12.2 ±1.4	2.0ab ±0.5	8.9 ±2.4	5.1 ±1.4	1.4 ±0.8	0.2 ±0.1
GC	44.5 ±10.0	6.8b ±0.7	13.6 ±2.9	1.4c ±0.3	8.5 ±1.8	5.1 ±1.3	1.2 ±1.1	0.1 ±0
BP	37.7 ±6.8	8.6a ±1.5	11.0 ±3.9	2.5a ±0.4	9.6 ±1.3	4.7 ±0.7	1.5 ±1.3	0.5 ±0.5
WB	37.7 ±6.4	10.0a ±0.7	12.1 ±2.6	1.6bc ±0.3	9.4 ±1.6	5.8 ±1.7	1.0 ±0.3	0.1 ±0.2
Rectum								
CT	61.3 ±15.6	8.9 ±1.8	10.4 ±4.2	2.4 ±0.7	4.6 ±1.6	5.5 ±1.3	1.2 ±1.1	0.1 ±0.1
GC	49.6 ±9.5	9.3 ±1.6	16.7 ±9.2	2.1 ±0.5	4.7 ±1.0	6.4 ±2.6	1.8 ±1.5	0.1 ±0.1
BP	44.6 ±14.0	8.9 ±2.2	16.9 ±7.9	3.6 ±2.1	3.7 ±1.3	6.1 ±1.4	2.1 ±1.0	Bd
WB	47.7 ±25.5	4.5 ±2.3	18.7 ±8.4	3.6 ±0.5	5.3 ±1.3	7.7 ±4.5	1.3 ±0.8	0.1 ±0.1

TRIAL III

CT: control diet; GC: control diet with coarse-grounded corn; BP: control diet enriched in soluble fiber by addition of 8% of sugar beet bulp and WB: control diet enriched in insoluble fiber by addition of 10% of wheat bran.

Values are average ± SD. Values sharing the same superscripts did not show a significant effect of diet ($P > 0.05$); where no superscripts are shown, there was no significant effect of diet.
Bd=below detection (counts <2 × 10^6 g^{-1}).

6.4. Discussion

Molecular analyses of the diversity of the porcine intestinal microflora have revealed that a high proportion of the resident bacteria do not correspond closely to known cultured species (Pryde et al., 1999; Vaughan et al., 2000). Together with similar 16S rRNA-based work from other mammalian gut communities (Harmsen e al., 2002; Lay et al., 2005) these analyses are helping to provide a valuable array of probes and primers that facilitate studies on the distribution of particular phylogenetic groups within the intestine, and their responses to different dietary regimes.

The present study shows that particular phylogenetic groupings, detected here by specific FISH probes, preferentially colonize different regions of the gut. In some cases the observed distribution agrees with expectations based on the characteristics of cultured representatives, but other findings were unexpected. The group targeted by the Erec482 probe represents Firmicute bacteria that belong Clostridium cluster XIVa, and was significant mainly in the hind gut (proximal colon and rectum) rather than in the stomach and jejunum. Most isolates of this group from the human intestine are highly oxygen sensitive, and are presumed to depend on anaerobic conditions found only in the dense community of the lower gut. A similar distribution was observed for *F. prausnitzii*-related bacteria, which are also strict anaerobes, and belong to the clostridial cluster IV. These two groups include the main butyrate-producing species from the human gut (Barcenilla et al., 2000). The *Bacteroides/Prevotella* group was less abundant in the pig rectum than has been reported in comparable studies using automated microscopic analysis of human feces (Harmsen et al., 2002) although lower estimates for human feces have been reported based on Fluorescence Activated Cell Sorting (FACS) analysis (Lay et al., 2005). Conversely, the *Lactobacillus* and *Streptococcus* groups, that comprise microaerophilic and facultatively anaerobic species, made up significant proportions

of the microflora of the stomach and small intestine, but made a very small contribution to the microflora of the proximal colon and rectum.

Little is known from cultural studies about the two remaining targeted groups of bacteria in the pig gut. The probe used here to detect cluster IX bacteria detected around 5% of total bacteria found in human faeces (Walker et al., 2005) and a similar proportion here in pig rectal contents and proximal colon. The targeted group includes a diverse collection of anaerobes, including *Selenomonas*, *Veillonella*, *Megasphaera* and *Mitsuokella*, most of which produce propionate as an end product. Remarkably, however, this group accounted for 14-41% of total bacteria detected in the stomach. It seems unlikely that the same species would account for the populations of cluster IX bacteria found in the very different environments of the stomach and large intestine, but further investigations will be needed to establish this.

Cluster IV bacteria classified as ruminococci from the rumen or human large intestine include specialist fibre-degrading, often cellulolytic, bacteria such as *R. flavefaciens*, and starch- degrading species such as *R. bromii*. These species are highly oxygen sensitive and their significant populations (8-10%) in the proximal colon of the pig correspond with what is presumed to be the site of most efficient breakdown of structural plant polysaccharides. On the other hand, the very high populations of *Ruminococcus*-related organisms in the stomach on diets BP and WB were quite unexpected. Again, it is a possibility that the stomach representatives belong to distinct strain/ species. It should also be noted however that the overall numbers are far lower in the stomach than in rectal contents, and that FISH detection can detected metabolically inactive or inviable, as well as viable, cells. Thus there is some possibility that re-ingestion of faecal material might produce transient populations in the stomach, and that ruminococci might be relatively more resistant to lysis in the stomach than some of the other groups of anaerobic bacteria found in faeces.

Coprophagy has been described in suckling piglets (Swanson and Gleed, 1981) and may reasonably be expected in animals allocated in small pens. It could be also hypothesized that a high fiber content in form of NSP could stimulate this behaviour, explaining the higher amounts of ruminococci in the stomach of BP and WB diets. Increases in dietary cellulose have been demonstrated to increase the amount of faecal pellets eaten from the cage floor in captive wild water voles (Woodall, 1989).

The relatively low probe coverage achieved in this work may partly reflect the fact that most probes had been designed initially against human faecal bacteria. Clearly much more work is required to design probes that target some of the less-studied groups that colonize the pig gut, particularly for sites such as the jejunum.

We were not able to detect marked dietary changes in gastrointestinal microflora using FISH. It could be due partially to the failure of our set of probes to target all microbial groups but also to changes in species composition of the different groups that had not been detected.

The ability of the diets to promote changes in intestinal ecosystem was confirmed by changes in the fermentation pattern between diets and by modifications in the RFLP profiles. It is well known that as carbohydrate sources decreased as fermentable substrate in the large intestine, fermentation becomes more proteolytic (Piva et al., 1995). This could explain the differences in SCFA profiles observed in animals fed diets rich in NSP (BP and WB). Similar decreases in BCFA with the inclusion of higher amounts of NSP in the diet have been described by other authors in pigs (Bach Knudsen et al., 1993). All these fermentation products, which mostly came from the fermentation of amino acids, reflect somehow the higher availability of carbohydrates as fermentable substrate in those animals receiving diets with additional amounts of fibre. These changes could come from both, a change in species composition and/or in metabolic activity of microflora. Analysis of ecological composition of colonic community revealed some changes in composition that were particularly evident with the WB diet. The inclusion of wheat bran decreased the biodiversity of the ecosystem and also promoted a much more homogeneous community compared to the other dietary regimes. Wheat bran diet could be considered as a diet enriched in insoluble NSP mostly cellulose and hemicellulose. Similarly to beep pulp or coarse maize, wheat bran provides plenty of substrate to the bacteria but in this case this substrate is difficult and time-consuming to ferment (Bach Knudsen et al., 1991) and this could require a specialized bacterial population. This type of specialization could be the reason for the lower biodiversity and the higher homogeneity in the microflora of these animals. Similar results in terms of biodiversity were found by Högberg and co-workers (2004) comparing the microflora of pigs receiving diets differing in the amount and solubility of NSP. Animals receiving diets with high amounts of insoluble NSP showed the lowest biodiversity defined as number of terminal restriction fragments detected by T-RFLP.

The stability of a bacterial ecosystem seems to be directly related to its diversity index (Atlas et al., 1984), and a highly diverse microflora has been considered to play a key role in its stability avoiding intestinal disorders and proliferation of opportunistic pathogens (Kühn et al., 1993; Katouli et al., 1999; Macfarlane et al., 1999). In this regard, beet pulp or coarse maize would appear as better ingredients than wheat bran to promote a robust microflora and prevent the proliferation of pathogens. However although a higher biodiversity could be considered in broad terms as desirable, the resistance against pathogen colonization offered by the intestinal microflora probably will depend not only on the complexity of the ecosystem but also on many other factors as the particular microbial species presents, the type of pathogen challenge or on the different characteristics of the digesta promoted by different diets. Further studies are needed to determine which kind of fibre is suitable in each situation.

6.5. Conclusions

This study is the first to examine the major bacterial communities along the GIT of pigs using FISH. Important differences are shown between foregut and hindgut. Whereas lactic acid-producing bacteria are abundant mainly in the stomach and jejunum, strict anaerobes such as *F. prausnitzii* are only present in proximal colon and rectum. The abundance of the little studied clostridial cluster IX group is revealed along the length of the tract, and this group was shown to make a significant contribution to the microbiota of the stomach. Dietary changes in fibre composition were not reflected in major bacterial groups quantified by FISH, however fermentation pattern and community profile analyzed by RFLP showed changes related to fibre. Globally, increased amounts of fibre promoted decreases in fermentation of protein and particularly wheat bran promoted a less diverse and more homogenous microflora.

Chapter 7

THE RESPONSE OF GASTROINTESTINAL MICROBIOTA TO THE USE OF AVILAMYCIN, BUTYRATE AND PLANT EXTRACTS IN EARLY-WEANED PIGS

7.1. Introduction

Early weaning makes the piglets an easy target for microbial aggressions (Wallgren and Melin, 2001). To prevent gastrointestinal disorders and to improve the post-weaning growth rate, feed-grade antibiotics are used regularly. Despite their general use, their exact mode of action is not entirely clear. Different mechanisms have been proposed. Most of them are based on a reduction in bacterial numbers with a decrease in the production of growth depressing microbial metabolites and in the competition for nutrients with the host (Anderson et al., 1999; Hardy et al., 2000). However other mechanisms related to the selection of a healthier microbial community could also be implicated.

Concerns about bacterial resistance to antibiotics and general food safety issues have encouraged intensive research on new feed additives to maintain the growth promotant effects of AGPs without their potential drawbacks. The addition of different organic acids to the feed is one of the most widely used alternatives with effects that have been related to a reduction in the growth of some bacteria (Partanen, 2001). Herbs have been known since ancient times to have antimicrobial, antioxidative and antifungal properties. Some of these compounds have been reported to improve animal performance due to their stimulating effect on salivation and pancreatic enzyme secretions or by having a direct bactericidal effect on gut microflora (Hardy, 2002). Carvacrol from oregano has demonstrated strong antimicrobial properties (Dorman and Deans, 2000), cinamaldehyde from cinnamon has shown antioxidant and also antimicrobial effects (Mancini-filho et al., 1998), and capsaicin from chili stimulates gastric secretions (Platel and Srinivsan, 2000). The experiment reported here aims to evaluate the effect of an antibiotic, an acidifier, and a plant extract mixture, on the load, metabolic activity and community structure of the early-weaned pigs' gastrointestinal microbiota.

7.2. Material and Methods

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

7.2.1. Animals and Housing

A total of 40 early-weaned pigs (Pietrain x (Large White x Landrace), mixed males and females) from a commercial herd were selected from 10 different litters. No creep feeding was provided during the lactation period. The animals were weaned between 18 to 22 d of age with an average initial BW of 5.9 ± 0.71 kg and were housed in the Universitat Autònoma de Barcelona facilities according to their initial weight in eight pens (five animals per pen). The 40 pigs were allocated in the same room and separated by solid walls of 60 cm in height with bars into the top up to 80 cm. Each pen had its own feeder and nipple drinker. The weaning room was equipped with automatic heating and forced ventilation and the temperature was gradually reduced from 29 to 25 ºC during the experiment.

7.2.2. Dietary treatments and feeding regime

Four dietary treatments were used. A control diet was formulated (CT; Table 7.1) to which three different additives were added: 0.04 % avilamycin, (AB; MAXUS, Elanco Animal Health, Madrid, Spain), 0.3 % sodium butyrate (AC), and 0.03 % plant extract mixture (XT). The plant extract mixture was standardized as 5% (wt/wt) carvacrol (Origanum spp.), 3% cinnamaldehyde (Cinnamonum spp.) and 2% capsicum oleoresin (Capsicum annum) in an inert fatty carrier that represented the remaining 90%. Chromic oxide was included as a digestibility marker in all diets (0.02%). Details of the diet composition are given in Table 7.1. Pigs were fed the experimental diets ad libitum for 3 wk weeks after weaning and they had free access to water throughout the experiment.

Table 7.1. Control diet composition, as fed basis

Ingredient	g/kg
Corn	276
Barley	300
Soybean meal, 44% CP	40
Full fat extruded soybeans	40
Soya protein concentrate	60
Fish meal LT[a]	50
Dried whey	40
Acid whey[b]	150
Wheat gluten	6.8
Sepiolite	10
Dicalcium phosphate	11
L-Lys·HCl	4.4
DL-Met	2.7
L-Thr	1.9
L-Trp	0.4
Choline chloride, 50% choline	2.0
Chromic oxide	1.5
Vitamin and mineral premixc	3.0
Calculated nutrient composition[d]	
GE, Mcal/kg	4.75
CP, g/kg	183.9
Ether extract, g/kg	51.1
Crude fiber, g/kg	27.8
Ca , g/kg	6.44
P total , g/kg	6.95
P available , g/kg	4.01
Lysine, g/kg	13.87

[a] Fish meal low temperature: product obtained by removing most of the water and some or all of the oil from fish by heating at low temperature (< 70 ºC) and pressing.

[b] Acid whey: product obtained by drying fresh whey (derived during the manufacture of cheeses) that has been pasteurized.

[c] Provided the following per kilogram of diet: vitamin A, 13,500 IU; vitamin D3, 2000 IU; vitamin E, 80 mg; vitamin K3, 4 mg; thiamine, 3 mg; riboflavin, 8 mg; vitamin B6, 5 mg; vitamin B12, 40 μg; nicotinic acid, 40 mg; calcium pantothenate, 15 mg; folic acid, 1.3 mg;

TRIAL IV

biotin, 150 µg; Fe, 120 mg as iron carbonate; Cu, 175 mg as copper sulfate 5H2O; Zn, 110 mg as zinc oxide; Mn, 65 mg as manganese sulfate; I, 1 mg as potassium iodate; selenium, 0.10 mg as sodium selenite.

[d] Based on composition values from NRC (1998)

7.2.3. Collection Procedures and Measurements

On d 19 and 21, a total of 32 animals (eight per treatment) were euthanized with an intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain; 200 mg/kg BW). Each slaughter day an equal number of pigs per treatment were included. The pigs selected from each pen were those with individual BW closest to the mean pen weight. Animals were bled, the abdomen was immediately opened, and samples of the intestinal content were taken.

For DNA analysis, samples of about 1 g of digesta from the stomach, distal jejunum, cecum, and the distal colon were kept in weighed tubes with 3 mL of ethanol as a preservative. Samples were also taken from the jejunum mucous layer: a segment of 4 cm was longitudinally cut and gently washed with sterile saline solution. The mucous layer was scraped with a spatula (250 to 500 mg), placed in weighed capped tubes, and immediately snap-frozen in liquid N. Samples were kept at -80 ºC until analysis. Digesta samples (approximately 50 g) from the ileum, cecum, proximal colon, distal colon, and rectum were taken for purine base analysis. Samples were frozen and lyophilized until analysis. For the study of microbial enzymatic activities, samples of 5 g of cecum and distal colon digesta were snap-frozen in liquid N and kept at -80 ºC until analysis.

DNA Extraction. Digesta samples (400 mg) preserved in ethanol were precipitated by centrifugation (13,000 x g for 5 min), and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lysis temperature was increased to 90ºC and a posterior incubation step with lysozyme was added (10 mg/mL, 37 ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 mL of Qiagen Buffer AE (Qiagen, West Sussex, UK) and was stored at -80º C. The DNA from the mucous layer scrapings was harvested using the same commercial kit. The DNA from pure cultures of Lactobacillus acidophilus (CECT 903NT) and Escherichia coli (CECT 515NT) was harvested using the same Qiagen Kit. Pig genomic DNA was obtained from blood samples using the Mammalian Genomic

DNA extraction kit (CAMGEN, Cambridge Molecular Technologies Ltd., Cambridge, UK).

Quantitative Polymerase Chain Reaction (qPCR). The primers used to quantify the different bacterial groups are listed in Table 7.2. The oligonucleotides were based on regions of identity within 16S rDNA and were adapted from published specific primers or probes using the Primer Express Software (Applied Byosistems, CA, USA). This software was used to check for primer-dimer, internal hairpin configurations, the melting temperature, and percentage guanine and cytosine values within possible primer/probe sets. The different primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST (Altschul et al., 1990) and the absence of amplification of porcine DNA was tested empirically by PCR using DNA extracted from pig blood.

Amplification and detection of DNA by quantitative real-time PCR were performed with the ABI 7900 HT Sequence Detection System using optical grade 96-well plates and SYBR Green dye (PE Biosystems, Warrington, UK). Duplicate samples were routinely used. The PCR reaction was performed in a total volume of 25 µl using the SYBR Green PCR Core Reagents kit (PE Biosystems, Warrington, UK). Each reaction included 2.5 µl 10x SYBR Green buffer, 3 µL $MgCl_2$ (25 mM), 2 µL dNTPs (2.5 mM), 0.25 µL AmpErase UNG (1 U/µL), 0.125 µl AmpliTaq Gold (5 U/µL) (PE Biosystems, Warrington, UK), 1 µL of each primer (12.5 µM), and 2 µL of DNA samples (diluted 1/10). The reaction conditions for amplification of the DNA were 50ºC for 2 min, 95ºC for 10 min, and 40 cycles at 95ºC for 15 s and 60ºC for 1 min. To determine the specificity of amplification, the product melting curve was analyzed.

For absolute quantification, PCR products obtained from the amplification of the whole 16S rDNA of Escherichia coli (CECT 515NT) and Lactobacillus acidophilus (CECT 903NT) were used to construct the standard curves, the PCR conditions corresponded to those published by Leser et al. (2002). An amplified gene from E. coli was used for absolute quantification of the total bacteria and enterobacteria and an amplified gene from L. acidophilus for quantification of the lactobacilli. The functions describing the relationship between Ct (threshold cycle) and x (log copy number) for the different assays were: $Ct = -3.19\ x + 53.66$; $R^2 = 0.99$ for total

bacteria; Ct = - 2.60 x + 46.82; $R2$ = 0.99 for lactobacilli; and Ct = - 2.32 x + 43.88; $R2$ = 0.99 for enterobacteria.

PCR-RFLP analysis. To analyze the total bacteria, a fragment of 16S-rDNA gene was amplified from DNA extracts by PCR using primers specific to conserved sequences flanking variable regions V3, V4 and V5: 5'-CTACGGGAGGCAGCAGT-3' (forward) and 5'-CCGTCWATTCMTTTGAGTTT-3' (reverse). Primer and PCR reaction conditions were those described by Lane et al. (1991). The reaction was performed using a GeneAmp PCR System 9700 (PE, Biosystems, Warrington, UK) thermocycler. The DNA amplification conditions were 94ºC (4 min); 35 cycles of denaturation at 94ºC (1 min), annealing at 45ºC (1 min) with an increment of 1ºC per cycle, extension at 72ºC (1 min 15 s); and a final extension at 72ºC (15 min). After visual confirmation of the PCR products with agarose gel electrophoresis, four independent enzymatic restrictions were carried out (AluI, RsaI, HpaII, CfoI (F.Hoffmann-LaRoche Ltd Group, Basel, Switzerland). The digestions were carried out as recommended by the manufacturer, with appropriate restriction buffers at the recommended temperature for 3 h. Different fragments were separated using a 2% high resolution agarose gel.

The size and the intensity of the bands within each lane of a gel were analyzed by the Gene Tools software (Syngene, Cambridge, UK), and the degree of microbial biodiversity was measured as the total number of different bands obtained from the four independent restriction digestions.

For pairwise comparisons of the banding patterns and the construction of dendograms, similarity matrices were generated based on the Manhattan distance (Kaufmann et al., 1990) that takes into account the size and the intensity of the bands generated.

Purine Bases Analysis. Purine bases (adenine and guanine) in lyophilized digesta samples (40 mg) were determined by HPLC (Makkar & Becker, 1999). For their analysis, purine bases were hydrolyzed from the nucleic acid chain by their incubation with 2 mL 2 M-HClO4 at 100ºC for 1h, including 0.5 mL of 1 mM-allopurinol as an internal standard.

Microbial enzymatic activities. The microbial enzymes were extracted from the digesta contents by hydrolysis of bacterial cells with lysozyme (5 mg/mL, 37ºC, 3h) following the method described by Silva et al. (1987). After the incubation period,

samples were centrifuged (23,000 g for 15 min) and the enzymes, from the supernatant were kept frozen (-80ºC) until analysis. Polysaccharidase activities of the enzymatic extract were determined by assay of reducing sugars released from purified substrates according to the Nelson-Somogyi method (Ashwell, 1957). The substrates were suspended in 0.1N sodium phosphate buffer (pH 6.7). The samples (0.05 mL) were incubated (30 min, 40ºC) with 0.45 mL of each substrate solution containing carboxymethylcellulose (Sigma-Aldrich Química S. A., Madrid), xylan from oat spelts (Sigma-Aldrich Química), soluble starch from potato (Panreac, Barcelona, Spain), and waxy starch from corn (Sigma-Aldrich Química). Activities against these four substrates were referred to as CMCase, xylanase, amylase, and amylopectinase respectively. After the incubation period, the reaction was stopped by denaturing enzyme proteins (100 ºC for 10 min) and the amount of reduced sugars was quantified spectrophotometrically at 600 nm. Dilutions of glucose (0, 25, 50, and 100 μg/mL) were used as a standard curve. The activity of the enzymatic extract was expressed as μmoles of neutral sugars released per mL of extract per minute and referred to the purine bases concentration (bacterial enzymatic activity).

7.2.4. Statistical Analysis

The effect of diet on microbial counts, biodiversity, purine base concentration, and enzymatic activities in a given intestinal segment was tested with an ANOVA using the GLM procedures of a SAS statistic package (SAS Inst., Inc. 8.1, Cary, NC). The individual pig was used as the experimental unit. When treatment effects were established ($P < 0.05$), treatment least square means were separated using the probability of differences (PDIFF) function adjusted by Tukey-Kramer (SAS Inst. Inc.). Purine bases concentrations along different intestinal segments in each animal were analyzed as repeated measures using the PROC MIXED procedure of SAS. Statistical significance was accepted at $P < 0.05$.

Table 7.2. Material and conditions for the quantification of total bacteria, enterobacteria, and lactobacilli

Group	Name[a]	Sequence (5'→ 3')[b]	Melting T[c] (C°)	Position in E. coli gene	Amplicon length (bp)[d]	Reference
Total	F-Tot	GCAGGCCTAACACATGCAAGTC	61	23	315	Marchesi et al. (1998)
	R-Tot	CTGCTGCCTCCCGTAGGAGT	60	337		Amann et al. (1995)
Enterobacteria	F-Ent	(G)ATGGCTGTCGTCAGCTCGT	58	1035	385	Leser et al. (2002)
	R-Ent	CCTACTTCTTTTGCAACCCACTC	58	1419		Sghir et al. (2000)
Lactobacilli	F-Lac	GCAGCAGTAGGGAATCTTCCA	58	373	349	Walter et al. (2001).
	R-Lac	GCATTYCACCGCTACACATG	59	721		

[a] Oligonucleotides used as primers (F, forward; R, reverse) for the quantification of 16S rDNA genes from the total bacteria (F-Tot, R-Tot), lactobacilli (F-Lac, R-Lac), and enterobacteria (F-Ent, R-Ent).

[b] Italicized bases denote added nucleotides and in brackets deleted nucleotides from previous published primers.

[c] Melting temperature estimated by Primer Express Software (Applied Byosistems, CA, USA).

[d] Length of PCR product expressed in base pairs.

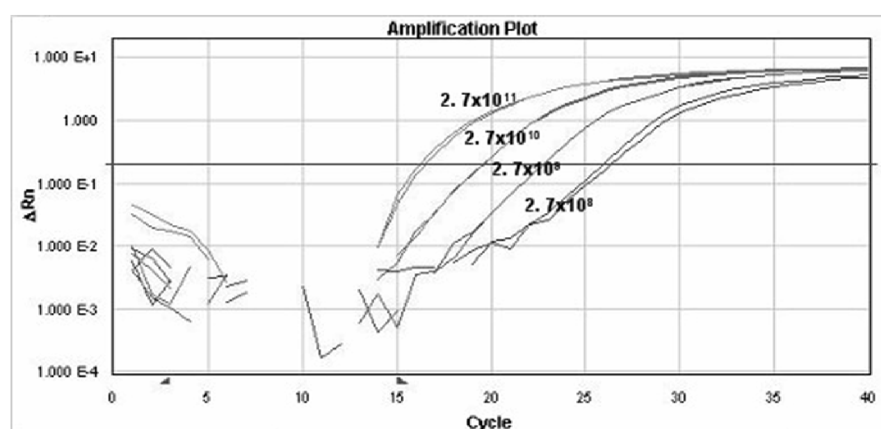
7.3. Results

The animals remained healthy throughout the experiment and diarrhea was not detected in any of the pigs. Animals receiving the different additives tend to had greater ADG (P = 0.069) at the end of the experimental period (124.7, 177.4, 177.6, and 165.9 g for CT, AB, AC and XT respectively; E. G. Manzanilla, personal communication).

7.3.1. Changes in the total microbial counts

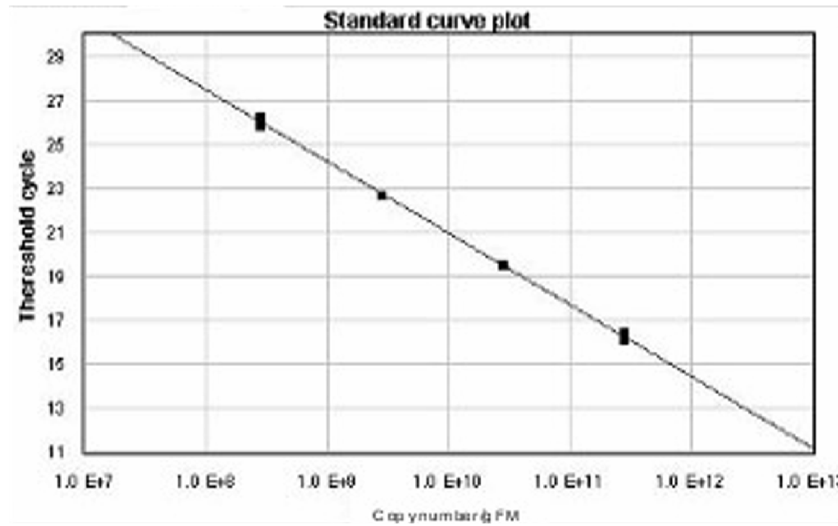
The total microbial population was quantified along the whole gastrointestinal tract using qPCR (Figure 7.1). In the foregut, the counts, expressed as log 16S rDNA copies/g fresh matter (FM), increased from 8.0 ± 1.16 in the stomach to 11.1 ± 0.88 in the jejunum, showing a considerable increase of more than three log units. The microbial population intimately attached to the jejunum mucous membrane was also quantified and although mean values were lower than counts in the lumen (10.2 ± 0.94 log 16S rDNA copy number/g FM) differences did not reach statistical significance. The cecum and colon digesta showed mean values of 12.4 ± 0.13 and 12.3 ± 0.93 log 16S rDNA copy number/g FM respectively. These values represent an increase of more than one log unit compared to the total counts in the jejunum. No significant differences in total bacterial loads related to experimental diets were found in any part of the analyzed gastrointestinal tract.

Figure 7.1. Quantitative PCR for total bacteria. **(A)** The amplification plot of the standards used to quantify total bacteria: 2.7 x 10^{11}, 2.7 x 10^{10}, 2.7 x 10^{9}, and 2.7 x 10^{8} 16S rDNA gene copies/g fresh matter (FM). Threshold cycle was plotted versus ΔRn (magnitude of the signal generated by the PCR conditions).

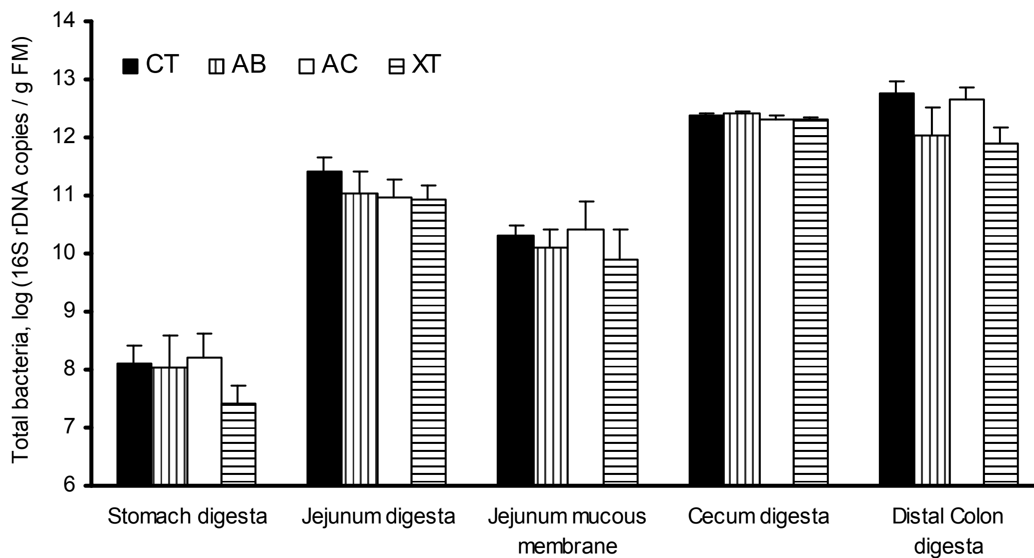


TRIAL IV

(B) DNA concentrations (16S rDNA gene copies / g FM) were plotted vs. threshold cycle value to construct the standard calibration curve.



(C) Bacterial loads in the stomach, jejunum, cecum, distal colon digesta, and in the jejunum mucous layer, measured by quantitative PCR (log 16S rDNA gene copies /g FM) in early-weaned pigs receiving a control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC) or 0.03 % plant extract mixture (XT). Bars represent means and standard error of the means.



TRIAL IV

7.3.2. Changes in the microbial ecosystem

In order to find possible effects of the additives on particular microbial groups, we also quantified enterobacteria and lactobacilli in the jejunum and cecum using qPCR (Table 7.3). Both groups increase in number from the jejunum to the cecum; however, while the enterobacteria showed an increase of around four log units, lactobacilli increased only by around two logs. Expressing the difference between both bacteria groups as a ratio of logarithms (lactobacilli:enterobacteria ratio) the cecum showed lower ratio values than the jejunum. Between dietary treatments, XT promoted an increase in the lactobacilli:enterobacteria ratio in the cecum when compared to the CT (*P* = 0.02), which can be explained by an increase in lactobacilli numbers (*P* = 0.02). The AC diet also showed higher lactobacilli:enterobacteria ratio mean values; however, differences with control diet were not significant (*P* = 0.49).

Table 7.3. Bacterial populations, size of lactobacilli, and enterobacteria in the distal jejunum and cecum measured by qPCR (log (16S rDNA gene copies / g FM) in early-weaned pigs[a]

		Diets[b]				
Segment	**Bacteria**	**CT**	**AB**	**AC**	**XT**	**SEM**
Jejunum	Enterobacteria	8.2	8.6	8.6	8.0	0.20
	Lactobacilli	11.5	10.6	10.9	10.2	0.59
	lactobacilli:enterobacteria[c]	3.30	2.02	2.32	2.23	0.583
Cecum	Enterobacteria	12.4	12.5	12.4	12.4	0.05
	Lactobacilli	12.9^{z}	12.9^{z}	13.1^{yz}	13.5^{y}	0.11
	lactobacilli:enterobacteria	0.48^{z}	0.43^{z}	0.75^{yz}	1.10^{y}	0.129

[a] Each mean represents eight individual pigs.

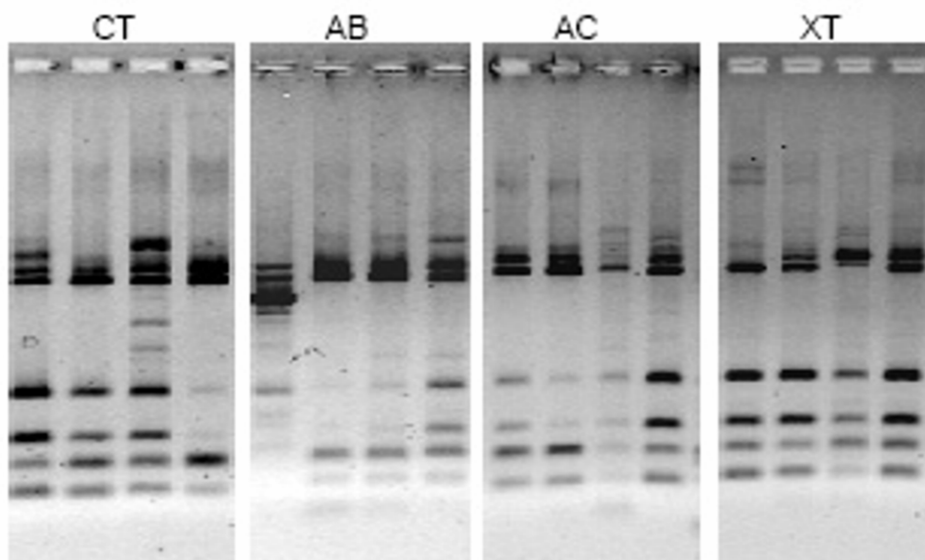
[b] Experimental diets: CT, control diet; AB, control diet with 0.04 % avilamycin; AC, control diet with 0.3 % butyrate; and XT, control diet with 0.03 % plant extract mixture.

[c] Relation between lactobacilli and enterobacteria populations expressed as ratio of logarithms.

[y,z] Least-squares means within a row lacking a common superscript letter differ (*P* < 0.05).

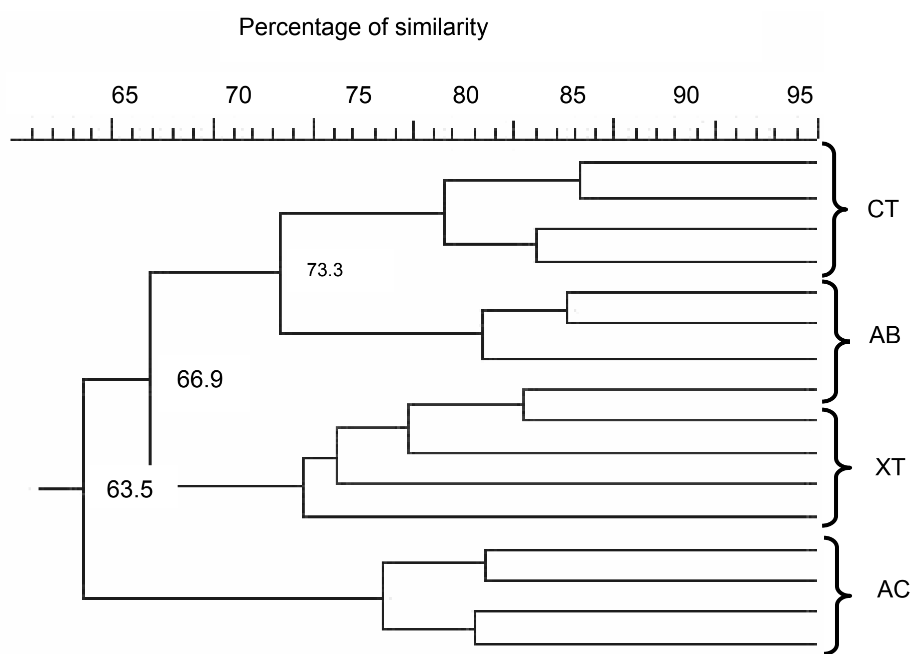
In samples of the jejunum digesta, a PCR-amplified region from the microbial 16S rDNA was analyzed using the RFLP method (Figure 7.2). The effect of different additives on biodiversity, measured as number of bands, was evident when considering the decrease in number of bands for the control diet (28.9, 38.5, 38.8, and 32.0 for CT, AB, AC, and XT respectively; *P* = 0.03). The effects of the experimental diets on microbial composition were more clearly distinguished by the cluster analysis. A dendogram comparing different banding patterns is shown in Figure 7.2. Distinct clusters according to the different diets were observed. The acidifier diet promoted the biggest structural changes (63.5% similarity) followed by the XT diet (66.9 %), and then the AB diet (73.3 %).

Figure 7.2. Ecological changes in microbial population of jejunum digesta measured by RFLP. **(A)** Example of gel electrophoresis of the PCR-amplified V3, V4, and V5 regions of the 16S rDNA restricted with the enzyme Hpa II (for more details see materials and methods). Each line represents different animals receiving the control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC); or 0.03 % plant extract mixture (XT).



TRIAL IV

(B) Dendogram illustrating the correlation between experimental diets in PCR-RFLP banding patterns. The dendogram represents results from 16 piglets killed during the second sampling day (d 21). The dendogram distances are in percentage of similarity



7.3.3. Changes in metabolic bacterial activity

Total microbial activity along the hindgut was also studied according to the concentration of all purine bases in the digesta. Evolution of the purine bases concentration along the ileum, cecum, proximal colon, distal colon, and rectum, according to the different diets is shown in Figure 7.3. All the treatments show an increase in PB concentration from the ileum to the cecum that is quantitatively larger for the CT and AB diet than for the AC and XT diet. In the hindgut, the evolution of the PB concentration also shows differences between diets (diet x intestinal section, $P = 0.01$). While the CT diet reached maximum values at the end of the colon, with an abrupt decline from the colon to rectum, the rest of the treatments reached maximum values in previous intestinal sections. Finally, PB concentration in the rectum was similar for all the treatments.

In order to detect changes in the microbial metabolic activities between diets, different carbohydrase microbial activities in the cecum and distal colon digesta were also measured. We could not detect enough carboxymethylcellulase or xylanase activity in most of the samples and therefore data are not shown. However, we did find detectable amylase and amylopectinase activity, which is shown in Table 7.4. The data showed high variability, precluding the ability to find any differences between treatments.

Figure 7.3. Purine bases (PB; adenine and guanine) concentration (µmol/g DM) in digesta samples from the ileum, cecum, proximal colon, distal colon and rectum in early-weaned pigs receiving a control diet (CT) or the same diet with 0.04 % avilamycin (AB); 0.3 % butyric acid (AC); or 0.03 % plant extract mixture (XT). The asterisks show that diets within an intestinal section differ (*P* < 0.05). Differences also occurred between intestinal sections (*P* < 0.001) and in relation to diet x intestinal section interaction (*P* = 0.01).

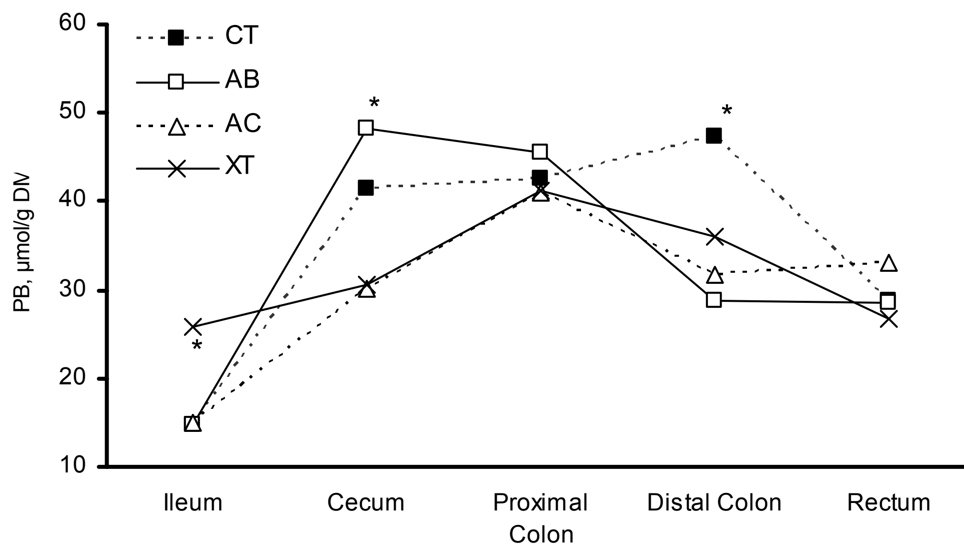


Table 7.4. Bacterial enzymatic activity in samples of the cecum and distal colon contents from early-weaned pigs[a]

		Diets[b]				
Enzyme	**Segment**	**CT**	**AB**	**AC**	**XT**	**SEM**
Amylase	Cecum	1.11	0.27	0.33	1.49	1.112
	Distal Colon	0.67	0.88	0.99	0.85	0.344
Amylopectinase	Cecum	1.80	1.50	2.08	4.34	1.810
	Distal Colon	0.99	1.72	1.64	0.58	0.336

[a] Each mean represents eight individual pigs.

[b] Experimental diets: CT, control diet, AB; control diet with 0.04 % avilamycin, AC; control diet with 0.3 % butyrate; and XT, control diet with 0.03 % plant extract mixture.

7.4. Discussion

We found that the antibiotic did not reduce the total microbial counts along the GIT, which was also true for butyrate and the plant extracts. Although it is generally accepted that antibiotics reduce the number of bacteria in the gut at growth promoting doses, results obtained in the literature are not always consistent and probably depend on the type and doses of antibiotic administered. Collier et al. (2003) using a similar PCR methodology described a decrease in the total bacteria in the ileum of growing pigs after 2 wk of treatment with 40 ppm of tylosin. This decrease was observed until d 21, however, the effect disappeared after 28 d, which reflects the microbial community's adaptive response, replacing susceptible strains with resistant organisms. In our case, this new equilibrium could have been reached sooner (2 wk), which would explain the absence of an antibiotic effect on the total bacteria counts.

The values for the total bacteria in the stomach, jejunum, cecum, and colon estimated by qPCR were similar to those described by other authors for culturable bacteria in pigs of a similar age (Jensen and Jorgensen, 1994; Krause et al., 1995; McFarland, 1998). It is fair to remark that values were always close to the highest levels, probably due to the trend of qPCR to overestimate microbial populations.

Some authors, when comparing qPCR with culture methods, have also described discrepancies of one or even two log units (Nadkarni et al., 2002; Huisjdens et al., 2002). These discrepancies could be explained mainly by the presence of a high number of viable but not culturable bacterial cells in the digesta samples (Rigottier-Gois et al., 2003), the amplification and later quantification of free DNA from dead bacteria, and the multiplicity of 16S rDNA genes per genome in prokariotic organisms (Fogel et al., 1999).

In the light of the absence of significant effects on the total microbial counts, it seems feasible that antibiotics and other alternatives such as organic acids or plant extracts, could act not by reducing the total size of the microbial population but by promoting the selection of particular bacteria. In this respect, the different spectra that antibiotics have and also the specific susceptibility of bacteria to different organic acids is well known (Cherrington et al., 1991). Possemiers et al. (2004) using qPCR could not detect changes in the total microbial population after adding an antibiotic to an in vitro simulator of the human microbial ecosystem; however, using group specific primers they could detect a decrease in the number of bifidobacteria.

Looking for ecological changes, the lactobacilli and enterobacteria populations were quantified in the jejunum using qPCR. The relationship between these bacterial groups has traditionally been considered as an index of desirable or undesirable bacteria in pigs, relating a high index with a higher resistance to intestinal disorders (Ewing and Cole, 1994). From the additives tested, XT showed the clearest effect, increasing the lactobacilli:enterobacteria ratio compared to the control. Increases were observed in the cecum (P = 0.006) mainly due to an increase in the number of lactobacilli. Previous results with the same plant extract mixture also showed increases in the lactobacilli:enterobacteria ratio in the jejunum of weaned pigs due to an increase in lactobacilli numbers (Manzanilla et al., 2004). It is difficult to find an explanation for this promoting effect taking into account that most of the in vitro studies with plant extracts have shown an unspecific antimicrobial effect (Hammer et al., 1999). However these consistent results seem to point to some kind of prebiotic effect on the lactobacilli population, either by a direct or indirect effect through an ecological change in the intestinal microbiota. Adding butyrate to diets also promoted higher mean values in the lactobacilli:enterobacteria ratio although in this case differences compared to the control were not significant (P = 0.17). There are few publications studying the inclusion of n-butyrate in diets for weaned piglets and its

effects on microbial populations. Galfi and Bokori (1990) using 0.17 % sodium n-butyrate in the diets of weaned piglets observed changes in the ileal microbiota with a decrease in the proportion of coliform bacteria with a simultaneous increase in lactobacilli, however these authors also found an increase in the ileal concentration of butyrate that we did not observe (data not shown). Van Immersel et al. (2004) using microencapsulated butyric acid in young chickens could also demonstrate a decrease in Salmonella in the cecum colonization after an experimental infection. It is interesting to note that the same authors using other organic acids like formic and acetic acid observed the opposite effect with an increase in Salmonella in the cecum colonization. Probably many other factors, such as the activation or inhibition of different metabolic routes with different organic acids, are involved in the changes observed with the acidifiers and not only a simple effect caused by a lower pH. This complexity could explain the diverse and sometimes contradictory effects of different acidifiers on microbial populations described in the literature (Hebeler et al., 2000; Canibe et al., 2001; Février et al., 2001).

Avilamycin is an antibiotic mainly active against gram positive bacteria; therefore, we could expect a decrease in lactobacilli numbers. However, we did not find this effect. Other authors using avilamycin (50 ppm) also did not find differences in bacterial numbers (Decuypere et al., 2002). Similarly, Collier et al. (2003) using tylosin (another macrolide active against gram positives) could not detect any decrease in lactobacilli but rather an increase, which is particularly intriguing and could reflect complex interactions between different species in the bacterial ecosystem. When using RFLP to analyze variations in the bacterial community, we evidenced changes in band patterns related to dietary treatments. It is interesting to point out how profiles for each treatment were clustered separately, the cluster for the animals that received the AC diet showed the most difference. Differences in the RFLP patterns were due to an increase in the biodiversity in the microbial ecosystem with the use of additives (number of bands) and also to a change in the species composition of the community (type of bands). From our results, it could be suggested that a more complex microbial community would have a higher robustness in response to changes in the intestinal environment promoted by different dietary ingredients or stress and that the beneficial effects of antimicrobial additives could be related, to an improvement of the adaptive capacity of commensal microbiota as a natural barrier defense against the overgrowth of pathogens, more than to a reduction

in bacteria numbers. Other authors using similar fingerprinting techniques (Denaturant Gradient Gel Electrophoresis (DGGE); McCracken et al., 2001) and comparing fecal microbial populations from rats receiving diets supplemented or not with antibiotics, did not detect changes in the biodiversity but they did detect how bacterial species that form each microbial community were significantly altered by the antibiotic. Similarly, Collier et al. (2003) working with pigs receiving 40 ppm of tylosin for more than 21 d, found that the number of DGGE bands in ileal samples was similar to the number in the control diet but that the banding patterns were treatment dependent.

The evolution of purine bases concentration along the hindgut showed that the main differences were between diets CT and AB. While in the CT diet, the purine bases concentration reached its maximum value at the distal colon, decreasing afterwards, the AB diet purine concentration reached its maximum values at the cecum. Previous results from our group described similar patterns when comparing animals receiving diets differing in the amount of resistant starch (Martinez-Puig et al., 2003). In that case, the purine bases concentration decreased earlier in animals receiving the diet with a lower amount of fermentable starch. In the present work, experimental diets have the same ingredient composition except for the added additives therefore changes in fermentation should not be attributed to differences in dietary carbohydrates. However, changes in the extent of digestion and absorption of nutrients at the foregut level could potentially have promoted the arrival of different amounts of fermentable material to the hindgut and therefore changes in microbial carbohydrases activity, but we were unable to observe that occurrence. If additives did promote differences, they were not big enough to be detected by this methodology. It is interesting to point out that while the amylase and amylopectinase activities were comparable to those described by other authors in growing pigs (Morales et al., 2002), cellulase or xylanase activities were not detected. This lack of enzymatic bacteria activity could be related to an insufficient adaptation of microbiota to digesting complex carbohydrates like cellulose or hemicellulose in young animals.

7.5. Implications

Results suggest that the effects of some growth promoters could be related more with changes in the species and complexity of the microbiota than to a simple decrease in bacterial colonization of previous sections of the gastrointestinal tract. More specific studies are required to clarify how these products modify pig gastrointestinal bacteria, which would facilitate their most judicious use in field conditions.

Chapter 8

USE OF MANNAN-OLIGOSACCHARIDES AND ZINC CHELATE AS GROWTH PROMOTERS AND DIARRHEA PREVENTATIVE IN WEANING PIGS: EFFECTS ON MICROBIOTA AND GUT FUNCTION

8.1. Introduction

Reducing post-weaning diarrhea is one of the main challenges for the pig industry. In commercial practice, the use of different in-feed additives has been traditionally recommended as a way to help the piglet during this stage. Among these agents, mannan-oligosaccharides (MOS) and supplements of zinc have been some of the proposals with probed positive results.

Mannan-oligosaccharides, Bio-Mos (Alltech Inc, USA; BM) derived from the outer cell wall of a selected strain of yeast have been extensively used to enhance gut health (Pettigrew, 2000; Miguel et al. 2004). Research suggests that BM interferes with bacterial attachment to the epithelial cell (Spring et al., 2000) and can also enhance immunity (Newman and Newman, 2001; O'Quinn et al., 2001). Zinc oxide (ZnO) at pharmacological levels (2,000 to 3,000 ppm) has been used to prevent diarrhea during weaning (Poulsen, 1995) and actually, it has become routinely used in nursery diets as a growth promotant, although its mode of action is not entirely clear. Various studies suggest that it could be mediated by a luminal (Katouli et al., 1999), an intestinal (Carlson et al., 1999) or a systemic effect (Case and Carlson, 2002). Recently, some experimental trials have studied the possible synergistic effect when high levels of ZnO are associated with BM (Davis et al., 2002). However, the use of high doses of inorganic Zn has raised some environmental concerns due to the elevated excretion levels in faeces. If the mode of action of Zn is based on a systemic effect, then the use of alternative sources of organic forms of Zn, with a higher bioavailability, would allow a reduction in its feed concentration, and also in its release to the environment whilst maintaining the benefits to the animal. Case and Carlson (2002) demonstrated equivalent efficacy of low doses of organic sources of Zn compared to pharmacological levels of ZnO under certain conditions.

The main objective of this work was to study the growth promoting effect on weaning pigs of BM derived form the yeast cell wall of a selected strain of Saccharomyces cerevisiae , and a Zn chelate and the combined use of both additives.

8.2. Material and methods

The experiment was carried out in the Experimental Farms of the Universitat Autònoma de Barcelona in Spain and received prior approval from the Animal Protocol Review Committee of this Institution.

8.2.1. Animals and diets

A total of 128 early-weaned pigs (Pietrain x (Large White x Landrace, 66 males and 62 females) were selected from a commercial herd. The animals were weaned between 18 to 22 days of age with an average initial BW of 6.7 ± 1.17 kg.

The animals were housed into 32 pens (four pigs per pen) taking litter and initial body weight into account. Animals received four dietary treatments. A control diet was formulated (CT) to which 0.2 % of a commercial source of mannan-oligosaccharide (Bio-Mos® Alltech Inc, USA; BM), 0.08% organic Zn equivalent to 80 ppm of Zn: (Bioplex Zn™ Alltech Inc, USA; BP') or both additives (BMP) were added. No medication or other additives were included in any of the diets.

The experiment lasted for 5 weeks including a pre-starter period of two weeks and a starter period of three weeks. During the starter period, diets were slightly modified according to the requirements of the animals but maintaining constant levels of BM and organic Zn. The composition of the control diets are showed in Table 8.1. Diets were formulated following the recommendations of NRC (1998). Animals were fed ad libitum and had free access to feed and water. At the end of the second week, and just before changing to the starter diet, animals were challenged by a controlled stress. Stress consisted of lowering the room temperature from 27 to 17 ºC and feed deprival over a period of 10 hours.

8.2.2. Performance and collection procedures

Body weight was recorded on a weekly basis and feed intake (by pen) was recorded daily during the first week and weekly thereafter. Average daily gain (ADG) and gain:feed data were calculated individually and by group respectively.

Two persons who were blind to treatment modality monitored faecal consistency daily during the first three weeks. Faecal morphology was classified using a scale ranking form 0 to 3 with 0 = normally shaped faeces, 1= shapeless faeces, 2 = soft faeces and 3 = thin, liquid faeces.

On day 14, 32 animals, one from each pen, were sacrificed with an intravenous injection of sodium pentobarbitone (Dolethal, Vetoquinol, S.A., Madrid, Spain) (200mg/kg BW). . Animals were bled, the abdomen was immediately opened and the whole gastrointestinal tract (GIT) tied and excised. Weight of the whole GIT, full and empty stomach, and small intestine, empty ileum, and full hindgut were recorded. Lengths of the whole small intestine and ileum were also registered. Samples for histology were taken from the distal jejunum and transferred to 10 % neutral buffered formaldehyde.

Digesta from the stomach, ileum and caecum was homogenised and pH determined. Samples (approximately 5 g) were kept frozen (-20 ºC) until analysis for short-chain fatty acids (SCFA). Digesta samples from the ileum, caecum and rectum were also frozen (-20 ºC) and lyophilised until analysis of purine bases (PB). Lyophilised ileal samples were also analyzed for protein contents and IgA concentration. Samples of jejunum digesta were taken and preserved in ethanol for DNA extraction and posterior microbiological studies.

8.2.3. Analytical methods

Short-chain fatty acid analysis. Analysis of SCFA was performed by GLC using the method of Richardson et al. (1989) modified by Jensen et al. (1995).

Purine Bases analysis. Purine bases (adenine and guanine) in lyophilised digesta samples (40 mg) were determined by HPLC (Makkar & Becker, 1999). For the analysis, purine bases were hydrolysed from the nucleic acid chain by their incubation with 2 mL 2 M-HClO4 at 100ºC for 1h, including 0.5 mL of 1 mM-allopurinol as an internal standard.

Immunoglobulins. IgA, IgG and IgM concentration in serum was quantified using Pig IgA, Ig G and IgM ELISA Quantitation Kits. (Bethyl Laboratories, Inc., Montgomery, TX). For the determination of IgA in ileum digesta samples, the method of Swanson et al., (2002) was used. Samples (2g) were lyophilised and

crushed with a mortar before being placed in an Erlenmeyer flask along with 20 mL PBS solution, pH 7.2. Samples were mixed for 30 min at room temperature and then centrifuged at 20.000 x g for 30 min. The supernatant was collected and ileal Ig concentrations were determined using the same kits used for serum samples. Calculation of Ig concentration per crude protein, was determined in ileum digesta as total N following the Kjeldahl method (AOAC, 1990).

DNA extraction. Digesta samples (400 mg) preserved in ethanol were precipitated by centrifugation (13000g x 5 min) and DNA from the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK).). The recommended lysis temperature was increased to 90 ºC and a posterior incubation step with lysozyme was added (10 mg/mL, 37ºC, 30 min) in order to improve the bacterial cell rupture. The DNA was eluted in 200 mL of Qiagen Buffer AE (Qiagen, West Sussex, UK) and was stored at -80º C.

Quantitative PCR. Microbial populations of enterobacteria and lactobacilli in ileum digesta samples were quantified by real time PCR using SyBR Green following Castillo et al. (2006) specifications.

Histological analysis. Formalin-fixed samples were included in paraffin and slides processed for periodic acid-Schiff (PAS) reaction. For each sample, villus height, crypt depth and intraepithelial lymphocytes (IEL) were measured. The Goblet cells in the villi and crypts were also counted. All measurements were made in 10 well-oriented villi and crypts.

8.2.4. Statistical Analysis

The effect of diet on different parameters was tested with an ANOVA using the GLM procedures of SAS statistics package (SAS Institute, INC. 8.1, Cary, NC). For performance analyses, pig was used as the experimental unit for ADG, and pen for ADFI and feed efficiency. Initial live weight was used as a covariate for productive performance results. In slaughter measurements, the pig was the experimental unit. In the event that significant diet effects were established ($P < 0.05$), multiple comparisons of the means were performed using the PDIFF function of SAS adjusted by Tukey Kramer. Faecal consistency data were analyzed by a $\chi 2$ test using the same statistical software. Statistical significance was accepted at $P < 0.05$.

Table 8.1. Composition as fed basis of pre-starter and starter control diets of Phase 1 and Phase 2.

	Experimental period	
Ingredient, g/kg	**Pre-starter**	**Starter**
Corn flakes	360.6	180.0
Barley	-	215.7
Wheat	-	100.0
Wheat flakes	240.2	120.0
Soybean meal, 44% CP	-	93.0
Full fat extruded soybeans	40.0	93.0
Soya protein concentratea	60.0	60.0
Wheat gluten	60.0	-
Potato protein	30.0	-
Fat-filled sweet whey	30.0	30.0
Sweet whey	150.0	80.0
Calcium carbonate	-	7.0
Calcium phosphate (dicalcium) (18%)	16.0	9.0
Salt	-	2.0
L-Lysine HCl 99%	5.0	3.0
DL-Methionine 99%	0.3	0.9
L-Threonine 98%	0.7	0.7
L-Tryptophan 10%	0.2	-
Choline HCl 50%	2.0	0.7
Vitamin and mineral pre-mixb and additives.	5.0	5.0

[a] Soya HP-300. Hamlet protein A/S (Spain).

[b] Provided the following per kilogram of diet: vitamin A, 10000 IU; vitamin D3, 2000 IU; vitamin E, 15 mg; vitamin B1, 1.3 mg; vitamin B2, 3.5 mg; vitamin B12, 0.025; vitamin B6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K3, 2 mg; Fe, 80 mg; Cu, 6 mg; Co, 0.75 mg; Zn, 185 mg; Mn, 60 mg; I, 0.75 mg; Se, 0.10; etoxiquin, 0.15 mg.

8.3.Results

8.3.1. Growth performance

Results for body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F) are shown in Table 8.2. Body weight or ADG of the animals did not show significant differences between treatments. Intakes were similar between treatments showing a progressive increase from 155 g/d during the first week to 825 g/d during the fifth week. For the first day post-weaning, intake was very low and animals did not eat more than 100 g/d. During the second day there were a compensatory response with a sudden increase in intake up to 170 g/d that normalised thereafter (Figure 8.1). Feed efficiency was increased with the use of the different additives compared to the control diet during the starter period ($P < 0.05$). Regarding mean values for the whole experimental period, both additives and their combination improved efficiency ratio compared to controls ($P < 0.05$), with BP' and BMP treatment animals demonstrating higher values than BM ($P < 0.05$).

Figure 8.1. Voluntary feed intake (kg/day) of pigs receiving a control diet (CT), or the same diet supplemented with Bio-Mos (BM), Bioplex-Zn (BP') or both additives (BMP) during the first week post-weaning.

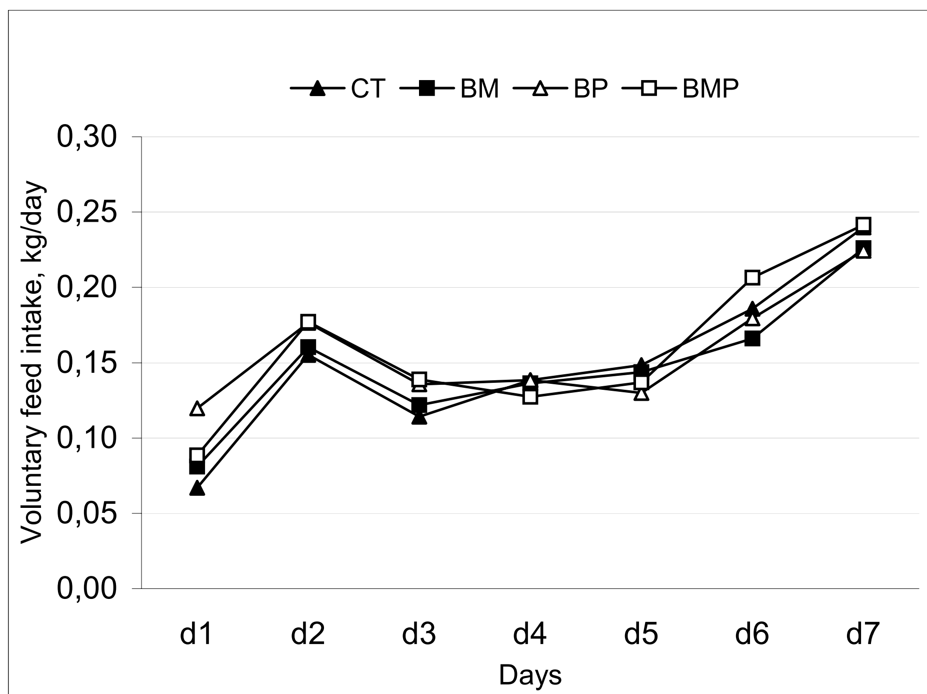


Table 8.2. Initial and final pig body weight (kg), voluntary feed intake (kg/day), average daily gain (kg /day) and feed efficiency in early-weaned pigs

	Diets[a]					
Item	CT	BM	BP'	BMP	SEM	Diet p-value
Pig body weight, kg						
Initial body weight	6.65	6.64	6.63	6.68	0.207	0.99
Finalbody weight	17.81	17.77	18.09	18.73	0.782	0.82
Voluntary feed intake, kg/day						
Pre-starter[b]	0.24	0.22	0.24	0.24	0.011	0.58
Starterc	0.68	0.64	0.66	0.69	0.042	0.82
Whole period	0.50	0.47	0.49	0.51	0.023	0.54
Average Daily Gain, kg/day						
Pre-starter	0.15	0.14	0.15	0.16	0.032	0.77
Starter	0.43	0.44	0.44	0.47	0.039	0.72
Whole period	0.35	0.34	0.35	0.37	0.019	0.72
Feed efficiency						
Pre-starter	0.57	0.58	0.66	0.66	0.033	0.15
Starter	0.63[z]	0.69[x]	0.67[x]	0.68[x]	0.014	0.04
Whole period	0.63[z]	0.64[y]	0.66[x]	0.67[x]	0.009	0.002

[a] Diets: CT (control diet), BM (control diet with 0.2 % Bio-Mos), BP' (control diet with 0.08 % Bioplex Zn) and BMP (control diet with 0.2 % Bio-Mos and 0.08 % Bioplex Zn).

[b] Period between the 1 to 2nd week post weaning.

[c] Period between the 3 to 5th week post weaning.

[d] Period between the 1 to 5th week post weaning.

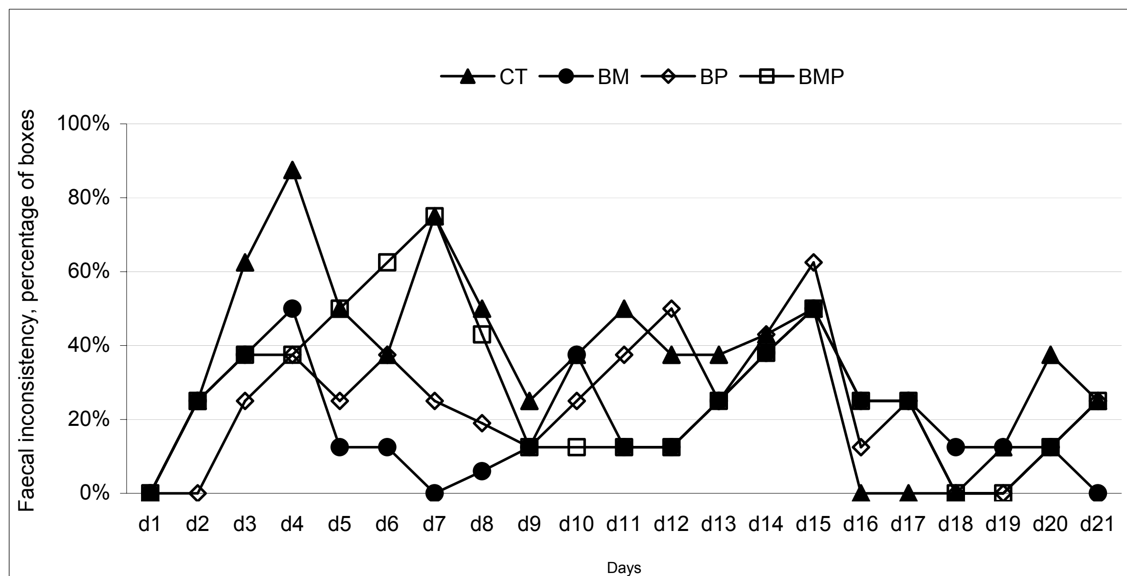
[x, y ,z] Least-squares means within a row lacking a common superscript letter differ (P < 0.05).

8.3.2. Faecal consistency

Faecal consistency was scored during the first three weeks post-weaning. Percentage of pens with score two and three is shown in Figure 8.2. There was an increase in faecal inconsistency at day four for all of the diets that tended to be higher for CT diet (diet overall effect, P = 0.11) with more than 80 % of the pens with faecal inconsistency. During the following days, BM showed the fastest recovery with no

animal with faecal inconsistence at the end of the first week while the rest of diets showed 25, 75, and 75 % for BP', BMP and CT respectively (diet overall effect $P < 0.001$). The controlled stress performed on the 14th day of the experimental period, was reflected by an increase in faecal inconsistency on day 15 that was similar across all dietary treatments.

Figure 8.2. Faecal consistency (% of boxes with a score value 2 and 3) in pigs receiving a control diet (CT), or the same diet supplemented with Bio-Mos (BM), Bioplex-Zn (BP') or both additives (BMP) during the initial three weeks post-weaning



8.3.3. Organ weights and small intestine length

Weights and lengths from different compartments of the gastrointestinal tract at the end of starter period are shown in Table 8.3. In general, the experimental diets did not promote changes in weights or lengths of the different sections studied or their contents. Only the empty ileal weight showed changes related to the diets with BP' treatment having the highest values (8.9, 9.6, 11.9 and 10.3 for CT, BM, BP' and BMP respectively; $P = 0.08$).

8.3.4. Short Chain Fatty Acids (SCFA)

Total SCFA, pH and lactic acid concentrations in the stomach, ileum and caecum are shown in Table 8.4. The pH was not modified by the experimental diets in any of

the sections. As expected, fermentation products increased slightly from the stomach to the ileum and abruptly to the caecum. No differences were found between diets either in total SCFA concentration or in their components (acetate, propionate, butyrate, valerate and branched chain fatty acids, data not shown) in any of the sections. Lactate concentration was also similar between diets.

Table 8.3. Weight (g/kg BW) and length (m) of different parts of the gastrointestinal tract from early-weaned pigs sacrificed two weeks post-weaning

Item		Diets[a] CT	BM	BP'	BMP	SEM	Diet p-value
Weight, g							
Whole intestine	Full	150.5	146.9	150.7	162.2	6.64	0.41
Stomach	Full	30.70	26.85	27.81	31.19	2.66	0.59
	Empty	7.76	7.31	7.43	7.95	0.27	0.35
	Content	22.95	19.55	20.37	23.25	2.56	0.76
Small intestine	Full	84.32	84.04	85.76	84.97	3.88	0.90
	Empty	53.38	55.27	58.47	58.05	2.71	0.51
	Content	30.95	26.77	27.30	26.27	1.99	0.31
	Empty ileum	8.91	9.56	11.92	10.34	0.82	0.08
Large intestine	Full	35.43	38.03	37.11	48.81	4.15	0.12
Length, m							
Small intestine	Ileum	1.55	1.55	2.00	1.63	0.178	0.92
	Whole	10.60	10.23	11.07	9.74	0.324	0.66
	Ratio	0.15	0.15	0.19	0.17	0.016	0.82

[a] Diets: CT (control diet), BM (control diet with 0.2 % Bio-Mos), BP' (control diet with 0.08 % Bioplex Zn) and BMP (control diet with 0.2 % Bio-Mos and 0.08 % Bioplex Zn).

[x, y ,z] Least-squares means within a row lacking a common superscript letter differ ($P < 0.05$)

Table 8.4. pH, SCFA and lactic acid concentration (µmol/g dry matter (DM)) in the stomach, ileum and caecum in pigs sacrificed two weeks post-weaning

		Diets[a]					
Item		CT	BM	BP'	BMP	SEM	Diet p-value
pH	Stomach	3.2	3.09	3.25	3.25	0.293	0.98
	Ileum	6.82	6.7	6.74	6.69	0.107	0.81
	Caecum	5.73	5.59	5.66	5.68	0.135	0.91
Total SCFA	Stomach	8.31	7.48	6.24	9.6	1.048	0.17
	Ileum	14.56	12.93	9.84	12.48	2.009	0.43
	Caecum	130.1	136.3	132.8	137.8	7.478	0.89
Lactic Acid	Stomach	4.35	3.68	3.75	4.80	0.594	0.51
	Ileum	15.84	9.85	14.34	14.20	3.845	0.72
	Caecum	3.29	2.25	1.40	2.10	1.648	0.87

[a] Diets: CT (control diet), BM (control diet with 0.2 % Bio-Mos), BP (control diet with 0.08 % Bioplex Zn) and BMP (control diet with 0.2 % Bio-Mos and 0.08 % Bioplex Zn).

8.3.5. Quantitative changes in microbial population

Table 8.5 shows the concentration of purine bases in the ileum, caecum and rectum digesta as an estimate of the total microbial population size and activity. Population size of lactobacilli and enterobacteria in the distal jejunum are also shown. Purine base concentration increased drastically from the ileum to the caecum, without differences between diets (6.46, 47.65 and 35.04 µmol/g DM for ileum, caecum and rectum respectively). However it is interesting to remark that in both the caecum and rectum, BM promoted the lowest mean values.

Lactobacilli did not show differences between diets, however, enterobacteria showed a significant decrease in their numbers with BM and BMP treatment. Compared to control (CT), enterobacteria decreased from 9.13 to 8.05 gene copies /g FM with BM ($P = 0.05$) and from 9.13 to 7.89 with BMP ($P < 0.05$). Expressed as

lactobacilli:enterobacteria, the BMP diet promoted the highest ratio compared to CT (P = 0.03).

8.3.6. Immune proteins and intestinal morphology

Table 8.6 shows serum concentrations of IgG, IgM and IgA, ileal concentration of IgA and histological measurements performed in the distal jejunum wall samples. No significant differences among diets were found in the serum concentration of immunoglobulins, ileal IgA or in the number of intraepithelial lymphocytes or goblet cells in the jejunum. However, crypt depth showed lower mean values with experimental diets compared to CT, although differences only reached statistical difference for the BMP diet (P = 0.04). The villus height was not affected and this resulted in a response in villus:crypt ratio similar to crypt depth, with significant increases when BM and organic Zn were both added into the diets.

8.4. Discussion

Numerous studies have reported that BM supplementation during the post-weaning phase improves growth performance of pigs (Dvorak and Jacques, 1998; Davis et al., 2002, 2004a). Recently, a meta-analysis of 54 different experiments in nursery pigs fed with Bio-Mos demonstrated a 4.12 % improvement in weight gain, 2.11 % improvement in feed intake and an increase in feed efficiency of 2.29 % (Miguel et al. 2004). In the case of organic sources of Zn, results are scarce and more controversial. Authors such as Carlson et al., (2004) did not demonstrate improvements in growth performance when nursery pigs were fed different sources of organic zinc (Zn-polysaccharide or Zn-chelate) however, other researchers suggest that Zn chelate (Bioplex Zn) may improve growth performance in young pigs (Mullan et al., 2002; 2004, Case and Carlson, 2002). In our case, the inclusion of either BM or BP promoted an increase in feed efficiency compared to controls, although differences promoted in ADG or DFI were not large enough to reach statistical significance. Growth promoting effects of feed additives are normally maximised when animals are reared under field conditions where disease challenges are greater than in an experimental farm situations where hygiene and environment are carefully controlled (Spring, 2004). Considering this, our improvements registered in feed conversion can be considered as a promising result of the potential of both additives

under practical conditions. In relation to the possible benefits of using BM and BP together, from our study we could not detect any significant improvement in growth performance compared with the additives alone. Le Mieux et al. (2003) in a series of four experiments evaluated BM and ZnO supplementation in nursery pigs with variable responses. In general, the response of growth performance to BM was more consistent with low levels of Zn, although in some of the trials BM addition was effective in the presence of an excess of Zn which was manifest as an improvement in gain:feed ratio. Similar variable results were described by Davis et al. (2004b) with a major response of BM when diets did not include an excess of Zn.

8.4.1. Changes on microbial ecosystem

BM has been proposed to promote growth by modifying the gastrointestinal ecosystem, and reducing intestinal pathogen colonisation. This ability seems to be due to the capacity of BM to attach to mucosa binding proteins on the cell surface of some bacteria, preventing colonisation of intestinal epithelium (Spring et al., 2000). In that sense, we found a selective diminution in the enterobacteria population in pigs fed diets supplemented with BM (BM and BMP diets), but no change was registered with Zn addition. Likewise, White et al. (2002) found a lower concentration of coliforms in the faeces of pigs fed diets with MOS. The decline in enterobacteria numbers is noteworthy because of the relationship that this group of bacteria have with post-weaning diarrhea syndrome. The ratio of lactobacilli:enterobacteria was first proposed by Muralidhara et al. (1977) as an index of robustness of commensal microbiota. An inhibition of these bacteria could prevent or decrease the severity of diarrhea that appears during the initial days after weaning (Gianella, 1983). This could be the reason why animals receiving the BM diet showed a more rapid recovery after the outbreak of faecal inconsistency observed at four days post-weaning.

Table 8.5. Purine Bases concentration (µmol/g DM) in the ileum, caecum and rectum, and bacterial populations (Lactobacilli and enterobacteria) from the distal jejunum measured by Real-Time PCR (log 16S rDNA gene copies /g FM) in ileum digesta in early-weaned pigs

Item			Diets[a]					
			CT	BM	BP	BMP	SEM	Diet p-value
Purine Bases	Ileum		6.17	5.62	6.58	7.46	1.38	0.81
	Caecum		50.23	40.38	52.86	47.16	5.64	0.45
	Rectum		41.28	25.28	34.79	40.00	6.25	0.19
Bacteria	Jejunum	Lactobacilli	10.04	9.62	9.76	9.79	0.27	0.75
		Enterobacteria	9.13^{x}	8.05^{y}	8.87^{xy}	7.89^{y}	0.28	0.01
		Lactobacilli : enterobacteria	0.91^{y}	1.57^{xy}	0.89^{y}	1.89^{x}	0.81	0.04

[a] Diets: CT (control diet), BM (control diet with 0.2 % Bio-Mos), BP (control diet with 0.08 % Bioplex Zn) and BMP (control diet with 0.2 % Bio-Mos and 0.08 % Bioplex Zn).

$^{x, y, z}$ Least-squares means within a row lacking a common superscript letter differ ($P < 0.05$).

Table 8.6. Plasma and ileal immunoglobulin (IgA, Ig M and IgG) concentration and jejunum histological parameters in early-weaned pigs sacrificed two weeks post-weaning

Item		Diets[a]				SEM	Diet *P*-value
		CT	BM	BP	BMP		
Inmunoglobulin							
Plasma	Ig G, mg/ml	25.50	20.97	25.10	13.88	4.142	0.26
	Ig M, mg/ml	4.40	5.44	6.81	5.05	0.853	0.32
	Ig A, mg/ml	0.82	1.43	1.48	1.05	0.255	0.34
Ileum digesta	IgA/gDM	1.47	2.01	1.72	1.94	0.470	0.85
	IgA/Gcp	6.19	8.29	7.04	7.42	1.670	0.84
Morphology							
Jejunum wall	Crypt depth, µm	281.3^{x}	241.60^{xy}	240.10^{xy}	234.50^{y}	11.980	0.04
	Villus height, µm	338.5	340.80	328.50	335.10	19.420	0.97
	Villus: crypt ratio	1.23^{y}	1.41^{xy}	1.40^{xy}	1.46^{x}	0.060	0.05
	Lymphocytes/100 enterocytes	4.64	3.60	4.66	5.16	0.940	0.69
	Crypt Goblet cells/100 enterocytes	17.56	15.33	15.70	15.51	1.071	0.44
	Villus Goblet cells/100 enterocytes	3.36	3.35	3.83	2.91	0.604	0.76

[a] Diets: CT (control diet), BM (control diet with 0.2 % Bio-Mos), BP (control diet with 0.08 % Bioplex Zn) and BMP (control diet with 0.2 % Bio-Mos and 0.08 % Bioplex Zn).

Changes in bacterial populations were not reflected in fermentation patterns that were unaffected by the experimental diets. Similarly other authors have related the inability of feed added mannan oligosaccharides to modify faecal pH or VFA concentration in weaning pigs (White et al., 2002) and neither in dogs (Swanson et al., 2002). Although non-digestible fermentable oligosaccharides such as fructo oligosaccharides (FOS) are considered as VFA-promoting compounds due to their high susceptibility to be fermented preferentially by some lactobacilli and bifidobacteria species (Kaplan and Hutkins, 2000), their low inclusion rate in the diet compared to other non-digested carbohydrates probably preclude their potential to promote different fermentation patterns. From our results it seems more plausible that the specific effect of BM on enterobacteria populations is related to an impairment of gut colonisation by these bacteria, presumably by a specific blockage of their binding sites as has previously proposed (Spring et al., 2000).

One of the modes of action proposed for the use of pharmacological levels of ZnO as a growth promotant has been their potential antimicrobial properties (Sordeberg et al., 1990). Katouli et al. (1999) showed that high doses of Zn oxide increased the stability of the intestinal microflora through a reduction in the diversity of coliform species that the authors regarded as an index of a more robust microbiota. However, no significant effect was found in the total number of coliforms. The absence of quantitative effects on coliform populations was also described by Jensen-Waern et al. (1998) using 2500 ppm of ZnO in weaners with no effects on coli and enterococci in faeces. This lack of a direct effect on specific bacterial populations with pharmacological levels of ZnO make it difficult to consider that organic sources of Zn play their role through a direct effect on intestinal microbiota when taking into account that they are added to a much lower doses.

8.4.2. Effect on gut function

The early-weaned piglet has to cope with a massive challenge with antigens associated with the new food and with the establishment of a novel commensal intestinal flora. Facultative pathogens, previously controlled by maternal IgA can expand and colonise the gut. The maintenance of the intestinal integrity and the digestive and absorptive function during this weak period depends on the ability of the immune system to adapt to this new situation and to discriminate between

"harmful" and "harmless" antigens with an appropriate response (Bailey et al., 2001). External aggressions to the enterocyte, as those caused by microorganisms or new feed proteins are normally associated with an atrophy of intestinal villi compensated at least partially by an accelerated turnover of crypt cells which results in a reduced villi:crypt ratio (Miller et al., 1986). In this experiment, the addition of BM or BP to the diets promoted a decrease in the mean values for crypt depth, although differences with control only reached statistical significance when both additives were combined into the BMP diet. A similar response was registered in the villi:crypt ratio as villus height remains unaffected by the experimental treatments. Other authors have previously described the effect of BM addition on gut structure. Similar to the results reported here, Ferket (2002) found that adding 0.1 % Bio-Mos to broilers did not affect villus height, but promoted a decrease in crypt depth with a lower villi:crypt ratio. Other authors such as Iji et al. (2001) also observed an increase in such a ratio in poultry, but due to a significant increase in villi height rather than crypt depth. This beneficial effect of BM on intestinal morphology may respond to the observed reduction in the enterobacteria population, but may also be due to other mechanisms. Ferket et al. (2002) have also proposed an increase in the production of the mucous gel layer promoted by BM as another mode of action of mannan-oligosaccharides. The mucous layer acts as barrier against bacterial aggression thus protecting the host animal from enteric infection. The current experiment did not detect any increase in the number of Goblet cells responsible for the production of mucous on villi or crypts. Stimulating effects on mucin dynamics in the presents of BM could be attributed to an increase in mucin gene expression rather than the increase in actual goblet cells (Smirnov et al 2005).

The use of BM as a tool to modulate immune response has been demonstrated by (O'Quinn et al. 2001) who found increased IgA titers in sow's milk or Davis et al. (2004a) who reported an alteration in the leukocytes populations in piglets fed BM. Preventing the onset of an acute phase immune response by modulating the immune response has a profound impact on growth performance. None of the inmunological parameters included in this study, like plasmatic and intestinal Igs or intraepithelial lymphocytes, responded significantly to the inclusion of BM, however it is possible that these broad indexes are not sensitive enough to detect more subtle effects on immune response.

The mode of action of supplemental Zn as a growth promotant in young pigs remains unclear, and there are doubts if it is based on a luminal, an intestinal or a systemic effect. High feed concentrations of Zn (2000-3000 ppm of ZnO) has become a common practice to control post-weaning diarrhea with probed positive results, but it has raised some environmental concerns because of the high release of Zn into the environment. If Zn promoted growth throughout a systemic effect, the use of organic sources of Zn with higher bioavailability could represent a good alternative. Zinc is know to play a central role in the immune system (Shankar and Prasad, 1998), and also functions as an antioxidant protecting cells from the damaging effects of oxygen radicals generated during immune activation (Bray and Bettger, 1990). Protection against deleterious effects of inappropriate immune responses against bacterial or new antigens after weaning could explain the increase that we observed in villus:crypt ratio. In addition to an increased protection, an improvement of the development of the immune response can also be suggested. We could not find significant differences in the plasmatic immunoglobulins related to diets, however BP diet showed the highest Ig M and Ig A plasmatic mean values, whereas the control diet showed the lowest. In addition, it is interesting to note that animals fed organic zinc showed a heavier empty ileum, which was numerically longer compared with the rest of the treatments. In this work, the ileum was considered as the section of the small intestine showing a continuous Peyer's Patch. Therefore, a higher ileal weight may indicate a higher development of the Peyer's Patches in these animals. In the newborn piglet the mucosal immune system is almost completely absent, and during the first weeks after birth rudimentary Peyer's Patch follicles expand rapidly and a spatially organised architecture of the mucosal immune system takes place (Bailey et al., 2001). Supplementing an additional organic source of Zn could aid the suitable development of the immune response in the young pig. This could explain the positive result observed both in intestinal architecture and in the development of the Peyer's Patches.

8.5. Implications

The use of Bio-Mos® derived from the outer yeast cell wall of a selected strain of Saccharomyces cerevisiae and Bioplex Zn™ supplement improved growth performance in terms of feed efficiency in the weaning pig. The mode of action of BM seems to be related to the inhibition of certain opportunistic gut bacteria from the Entobacteriaceae family, whereas organic Zn could act through an improvement

in the host immunological response, suggested by an increased ileal weight. Complementary actions could explain the highest values in villus:crypt ratio when both additives were used together.

Chapter 9

GENERAL DISCUSSION

The following chapter discusses the overall results obtained in the different experiments included in this thesis (Chapter 4-8):

TRIAL I. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real time PCR.
TRIAL II. Influence of weaning on caecal microbiota of pigs: use of real-time PCR and t-RFLP.
TRIAL III. Molecular analysis of bacterial communities along the pig gastrointestinal tract.
TRIAL IV. The response of gastrointestinal microbiota to the use of avilamycin, butyrate and plant extracts in early weaned pigs.
TRIAL V. Use of mannan-oligosaccharides and zinc chelate as growth promoters and diarrhea preventative in weaning pigs: effects on microbiota and gut function.

9.1. Usefulness of quantitative PCR, FISH and t-RFLP to study the intestinal microbiota

In the last fifteen years, molecular methodology has been increasingly used in gastrointestinal microbiology, greatly improving the knowledge regarding composition, phylogeny and function of this complex ecosystem.

Although traditional methods have greatly contributed to set up the basis of gastrointestinal microbiology, it is well known that these methods are inherently limited by their low sensitivity, reproducibility, labourity and inadequacy in detecting all gut bacteria (Furham et al., 1992; Dutta et al., 2001). Recent works estimate that only a range of around 10-40% of the gastrointestinal microbiota can be accounted for by traditional methods (Zoetendal et al., 1998). In this regard, molecular methods have several advantages compared with traditional ones: the viability of the cells is not required, thus avoiding the need to work in fresh and also the ability of bacterial cells to growth in a medium semisynthetic that appear as a critical point for most of the traditional ones. Moreover, once implemented they are less cumbersome than traditional methods, with high levels of sensitivity and reproducibility (Wang et al., 1996, Raskin et al., 1999; McCartney, 2002). However, when required, isolation of DNA from the digesta can turn on a limitation step that may involve differences in DNA isolation efficiency of different bacteria (McOrist et al., 2002; Anderson and Lebepe-Mazur, 2003), DNA extraction being a source of bias in the representativity of the whole ecosystem. In this regard, a consensus between different working groups in the method used to extract DNA might be extremely useful to obtain results more comparable than at present, avoiding this bias. Also, it is necessary to remark that many of these molecular methods used for characterization of species composition of the microbiota (known as fingerprinting methods) could also be biased on the first PCR amplification of the 16S rRNA gene using universal primers. The proper election of primers (Liu et al., 1997; Osborn et al., 2000), number of PCR cycles (Suzuki and Giovanonni, 1996) and PCR conditions (Kitts, 2001) is essential to achieve a direct proportion between the abundance of amplicons to the abundance of that template in the sample (Clement et al., 2000; Dunbar et al., 2000).

In the different trials included in this thesis, three different molecular methods were used: real-time PCR (qPCR), terminal-Restriction Fragment Length Polymorfism (t-RFLP) and Fluorescent *in situ* hybridization (FISH).

To quantify total and particular bacterial groups, two different methods were used: qPCR (**trial I, II, IV and V**) and FISH (**trial III**), the first one as a rapid method to assess robustness of microbiota measured as lactobacilli:enterobacteria ratio and the second one to obtain an overall picture of the main bacterial groups described in the pig.

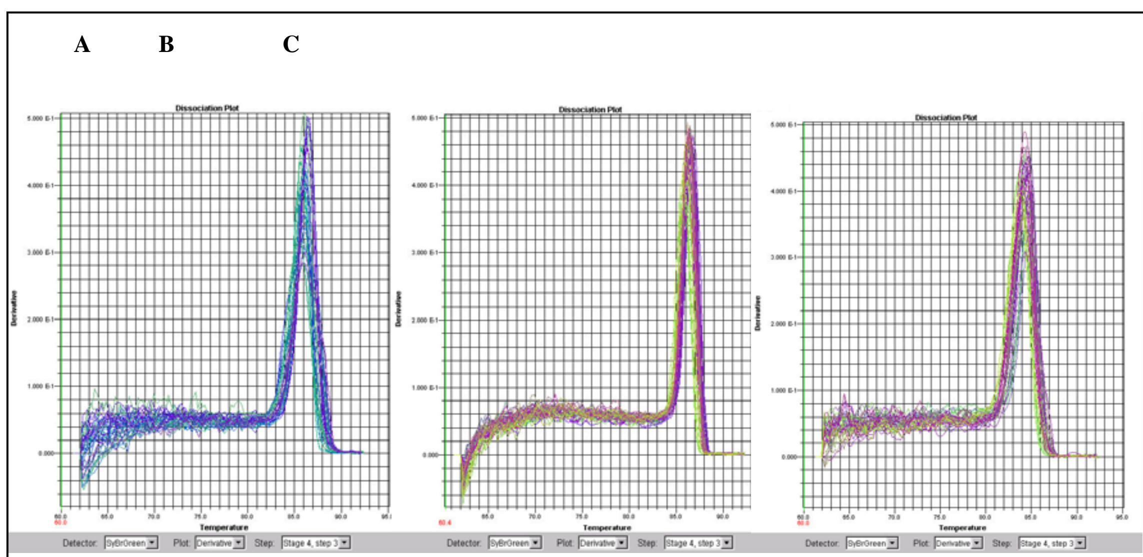
Real-time PCR with Sybr Green® dye was employed to quantify total bacteria, lactobacilli and enterobacteria, as objective bacterial groups in pig microbiology. Recently, other research groups have also described quantification of total bacteria and lactobacilli in pig gut digesta by real-time PCR (Collier et al., 2003; Hill et al., 2005). First results obtained (**trial I**) were compared with those obtained by traditional methods (culture and direct microscopy) to assess its usefulness.

In our case, the results obtained by qPCR were higher than those with traditional methods, although similar discrepancies have often been described (Nadkarni et al., 2002; Bach et al., 2002; Huijsdens et al.,2002). Different facts may be behind differences observed. Firstly, presence of non- viable, viable but not culturable cells and free DNA present in the samples and thus amplified by real-time PCR but not quantified by traditional methods (Rigottier-Gois et al., 2003). Secondly, the multiplicity of 16S rRNA gene copies (7 for *E. coli* and 4 for *Lactobacillus* spp.; Fogel et al., 1999). And thirdly, differences intrinsic to methods, in particular the pre-treatment of digesta in direct microscopy and culture that may involve a loss of an important fraction of the bacteria firmly attached to particulated material when previous dilution is done. In this regard, we were able to detect losses of microbial material up to 90%. Finally, quantification of non-specific amplicons with Sybr Green dye have also been described (Hein et al., 2001), although in our case, melting curve analysis was performed and discarded this possibility (Figure 9.1).

Despite differences in absolute numbers, correlation in total bacteria and in the lactobacilli: enterobacteria ratio between traditional and molecular method used, confirmed validation of the results obtained. For this reason, the method was considered as a useful technique to assess changes in microbial ecosystem rapidly by

the use of the ratio lactobacilli:enterobacteria, avoiding comparison between absolute values.

Figure 9.1. Melting curve obtained after the PCR reaction for total (A), enterobacteria (B), and lactobacilli (C). Dissociation temperature (ºC) for PCR product plotted vs. the signal fluorescence derivative.



This ratio, as previously mentioned, has been routinely used as an indicator of gut health (Muralidhara et al., 1977; Ewing and Cole, 1994) with an increase in the ratio being considered beneficial for the animal gut health. Interest in both these bacteria becomes from the fact that whereas lactobacilli have been associated with favorable effects on animal health, bacteria belonging to the *Enterobacteriaceae* family are related with diarrhea outbreaks (Melin, 2001). Lactobacilli is thought to promote health through inhibition of some opportunistic pathogens, such as *E. coli* (Blomberg et al., 1993; Tannock et al, 1999), by preventing or decreasing the severity of diarrhea that appears during the initial days after weaning (Gianella, 1983) and also by modulating an adequate immune response (Perdigón et al., 2001). Due to this, the objective is to maintain a ratio favorable to lactobacilli, especially in young pigs.

However, it is fair to remark that literature on the ratio is scarce. Results come from previous works that described changes by traditional methods in intestinal bacteria in pigs of different ages and experimental conditions (Chopra et al., 1963, Muralidhara et al., 1977; Reid and Hillman, 1999; Manzanilla et al., 2004). In this

regard, further investigations are needed to verify the usefulness of this ratio to check pig gut health and also to see if it could have some kind of relationship with performance improvement.

Table 9.1. Results of lactobacilli:enterobacteria ratio and its relation with growth performance from pigs included in trial II, IV and V.

Trial	Item	Section /Performance measure	Diet			
			S	**W**		
II	Ratio lactobacilli:enterobacteria	Caecum	-0.27[y]	-1.76[x]		
	Improvement in growth performance[a]	Average daily gain	+			
			CT	**AB**	**AC**	**XT**
IV	Ratio lactobacilli:enterobacteria	Jejunum	3.30	2.02	2.32	2.23
		Caecum	0.48[z]	0.43[z]	0.75[yz]	1.10[y]
	Improvement in growth performance	Average daily gain		t	t	t
		Feed efficiency		+	+	=
			CT	**BM**	**BP'**	**BMP**
V	Ratio lactobacilli:enterobacteria	Jejunum	0.91[y]	1.57[xy]	0.89[y]	1.89[x]
	Improvement in growth performance	Feed efficiency		+	+	+

[a] Overall results observed in growth performance (-, indicates significant impairment, +, = not significant changes and t, a tendency to improve).

In our case, the ratio was determined in **trials II, IV and V** (Table 9.1), where the piglets were always healthy. In general, when the index increased the animals always showed an improvement in performance, compared to control treatment. In particular, in **trial II**, comparing suckling with weaned pigs, those that remain with the mother show the high ratio parallel to higher lower body weight. Similarly, in **trial IV,** animals that received the plant extract showed the highest lactobacilli:enterobacteria ratio in cecum with an improvement in average daily gain. In **trial V**, the ratio was significantly increased in the jejunum digesta of animals that received both additives together (mannan-oligosaccharides plus organic zinc) with an increase also in the

feed:gain ratio. However, when the performance improved we could not always see an increase in the ratio. Undoubtly, several factors are implied in improving pig performance in addition to those related directly with microbiota, that may behind the results obtained.

Besides qPCR, FISH was also used to quantify total bacteria, but in this case the objective was to cover most of the main microbial groups of the pig gastrointestinal tract with the different probes used to give a overall picture of the ecosystem. Bacterial groups determined were: Bacteroides/Prevotella group, *Ruminococcus flavefaciens*, *R. bromii*, clostridia cluster XIVa, clostridia cluster IV species related to *Faecalibacterium prausnitzii*, clostridia cluster IX, *Streptococcus/Lactococcus* sp. and *Lactobacillus/Enterococcus* sp. (**trial III**). The aim of this trial was to study composition of adult pig gut microbiota along the gastrointestinal tract and also the potential of dietary fiber to manipulate its equilibrium. Although this method has been extensively used to study other ecosystems (Amann et al., 1996; Hold et al., 2003; Takada et al., 2004), its application in pig gut microbiology has been scarce; to our knowledge, there are only two published works that have applied this method to study pig gut microbiota: Konstantinov and co-workers (2004b), which used the method to quantify total bacteria, *Lactobacillus-enterococcus* group, *L. amylovorus* and *L. reuteri*-like in ileum and colonic digesta of weaning pigs; and recently, Tzortzis and co-workers (2005) applied FISH to study the effect of the addition of a novel galactooligosaccharide on *Bacteroides* spp, *Bifidobacterium* spp., *Clostridium hystoliticum* group, and *Lactobacillus/Enterococcus* group of the gastrointestinal tract of piglets.

In our case, the results obtained were more clarifying in distal sections of the gastrointestinal tract due to the higher coverage obtained with the probes used. The presence of bacteria belonging to different groups from those contained with the set of probes used, may explain the lack of coverage in the upper gastrointestinal tract. Of these groups, proteobacteria and also some other *Clostridium* groups that have been described as important habitants of the pig gut (Leser et al., 2002) may be behind differences obtained.

Regarding methodological aspects, a similar minimum level of detection was obtained with FISH and qPCR (10^5-10^6 cells /g of digesta). However, FISH has a main advantage compared to qPCR: DNA extraction and further DNA amplification is not required, thus avoiding the possibility of the previously mentioned bias.

Moreover, additional information regarding morphology of bacteria can be obtained with FISH. The application of flow cytometry (Wallner et al., 1997; Moter and Göbel, 2000) to count hybridized bacterial cells would make this method highly valuable in microbiological studies.

The bacterial profile based on polymorphism of the 16S rRNA gene was also studied. The T-RFLP methodology was implemented and used in **trial II,** following the method described by Höjberg and co-workers (2005). Results shown in **trial III** and **IV** included information obtained by RFLP method (Pérez de Rozas et al., 2003), that is substantially similar to T-RFLP. This method has been used to study pig gut microbiota by other research groups, to assess differences in microbial profiles after dietary changes as fiber composition content of the diet (Leser et al., 2000; Högberg et al., 2004), or the administration of different additives (Höjberg et al., 2005) providing valuable information of changes in microbiota due to these dietary modifications.

In addition to bacterial profile obtained by t-RFLP, fingerprinting methods also permit us to obtain the biodiversity of the samples (measured as number of bands) which seems to be a useful index to assess gut microbiota stability and health. In this regard, the robustness of a microbial ecosystem has been directly related to its biodiversity, a higher number of species being an indicator of a more stable ecosystem (Atlas, 1984) and thus a higher resistance against potential opportunistic pathogens (Hillman et al., 2001). However, literature regarding pig gut biodiversity is still scarce. In our case, results from **trial IV** showed an increase when different additives were used, supporting thus the hypothesis that additives improved gut health. The animals had achieved a more diverse ecosystem and therefore more difficult to be altered by opportunistic pathogens. Recently, other authors have been observed changes in biodiversity of pig gut microbiota. Konstantinov and co-workers (2003, 2004b) showed differences in biodiversity (measured as number of bands obtained by DGGE) after addition of fermentable carbohydrates to the diet of weaning pigs. The animals fed with fermentable carbohydrates in the diet showed a higher biodiversity. Similarly, Högberg and co-workers (2004) also found differences in pig microbial biodiversity obtained by t-RFLP in ileum of growing pigs after feeding diets with different types and quantities of non-starch polysaccharides. In this case, a lower biodiversity was obtained in animals fed insoluble non-starch polysaccharides. Recently, Inoue and co-workers (2005), using TGGE method

described the evolution of pig gut microbiota in the first weeksof life with a marked increase in biodiversity after weaning.

On the other hand, the theoretical inference of bands obtained by restriction of bacterial DNA in the samples with potential compatible species may be remarked. Although it is need to keep in mind that results are dependent on sequences deposited in the database, interesting results have been obtained (**trial II**). Other authors have also used similar procedures, but in this case only some particular bands were inferred to their potential compatibility using their own database obtained by cloning (Leser et al., 2000). In our case, we inferred bacterial groups of those bands that appeared at least in three animals, using public database software of RDP II (Cole et al., 2003). In doing so, we covered around 30% of the total peak area, the remaining 70 % being attributed to differences between animals, inherent background of the method, and also to peaks without theoretical correspondance in the database used.

In conclusion, molecular methods used in this thesis can be considered useful new tools for studying pig gut microbiota and for detecting changes in particular bacterial groups. As with other techniques, there are limitations; however, new information about the bacterial ecosystem structure is given by fingerprinting methods like t-RFLP, and practical advantages like not needing to work in fresh, make these methods especially attractive and complementary to traditional methodology and commonly used in intestinal microbiologial studies.

9.2. Weaning: a critical stage in the indigenous pig microbiota establishment

As described in the previous chapters, pig gut colonization by bacteria is a complex and successional process that takes several months to be completed (Swords et al., 1993) and undergoes a marked disruption when piglets are separated from the sow (Wallgren and Melin, 2001).

In nature, weaning is a transitional and long period of time in which mammals change from total nutritional and social dependence on the mother to total independence from her (Held and Mendl, 2001). However, the pig production system involves very early and abrupt piglet weaning at a time when the immune system is still immature (Bailey et al., 2001). As a consequence, animals refrain from eating, with several negative consequences for their health that are reflected in the post-

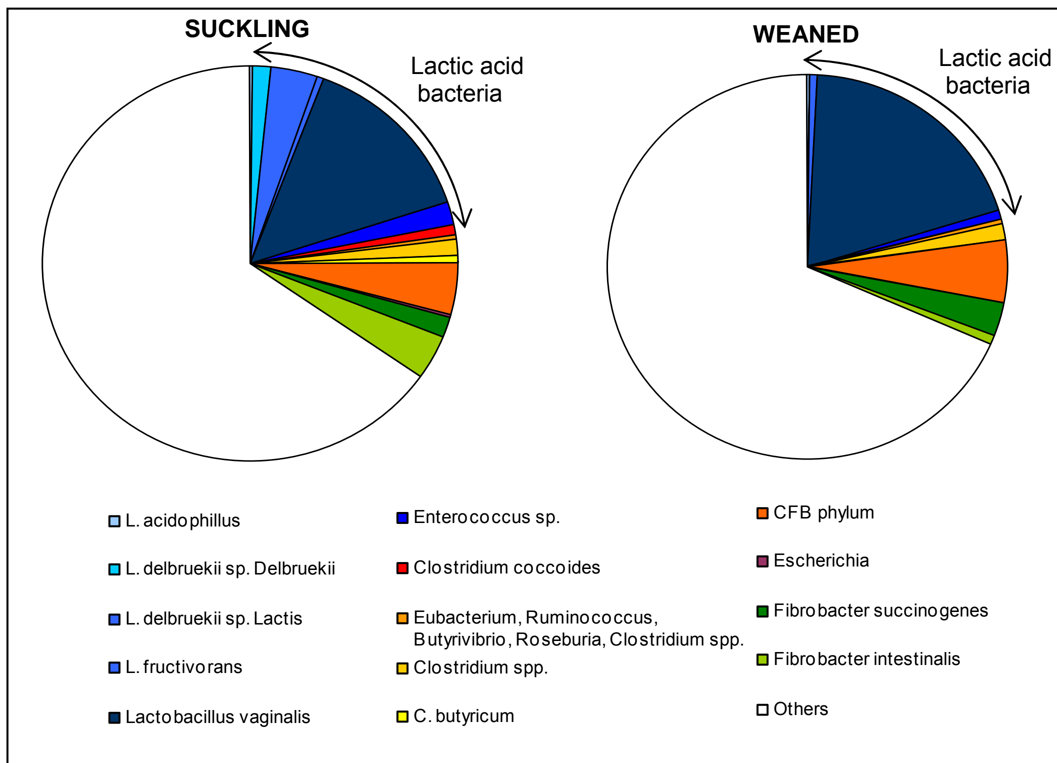
weaning syndrome (Pluske et al., 1997; see Figure 2.2, chapter 2). In this regard, the different trials completed with weaned pigs supported the stress described in pigs after weaning in different ways.

The shift in microbiota observed in **trial II** seems particularly remarkable**.** A marked change in the bacteria inhabiting the piglet caecum was observed one week after separation from the sow. This change was clearly reflected in the lactobacilli:enterobacteria ratio, with a significant decrease in weaned pigs. In this regard, previous works have described increases in coliform bacteria in parallel with decreases in lactobacilli after weaning (Jensen, 1998; Mathew et al., 1996; Franklin et al., 2002).

In addition, the ratio was lower than that obtained in animals slightly older from the rest of the trials (see Table 9.1). The short age of these piglets may explain the high enterobacteria counts, due to the fact that those bacteria are one of the main groups that colonize piglet gut after birth, coming from the mother feces and the environment (Swords et al., 1993; Ewing and Cole, 1994). The weaned pigs used in the other trials (**IV and V**) were sacrificed at an older age than those in **trial II,** a fact that may explain differences observed; those animals could have a more established microbial profile with lactic acid bacteria as one of the main bacterial groups (Leser et al., 2002).

The shift in microbiota profile was also demonstrated by t-RFLP profiles, which clustered animals separately. As expected, weaned pigs showed a lower similarity between them which again reflects the disbiosis suffered. The different compatible bacteria inferred from the results obtained in this trial seem particularly interesting. Although theoretical, and therefore restricted, results can be considered as an image of what is happening in this enormous ecosystem (Figure 9.2). Weaned pigs showed a lower compatibility with lactic acid bacteria and also an absence of compatible bands with bacteria such as *C. coccoides* and *C. butyricum* constantly present in suckling pigs.

Figure 9.2. Pie chart with the major 5'-terminal fragments expressed as the mean of the percentage of the total area in suckling (S) and weaned (W) group. Each portion represents the mean of the percentage of total area compatible with a potential bacteria.



Although a lower biodiversity in coliform bacteria has usually been described at weaning (Katouli et al., 1995; 1999), lactobacilli biodiversity have been less studied. These changes undoubtedly reflect the new situation where the piglets are, with a sudden change in the amount of type of substrate available for bacteria which results in a transitional decrease in biodiversity. This fact could help to explain the lower resistance of animals to potential pathogen colonization in the days following weaning. It has been recognized that diverse bacterial population plays a key role in the maintenance of the gastrointestinal health because it avoids potential colonization by pathogens (Van Kessel et al., 2004). Therefore, avoiding a marked decrease of this biodiversity can be especially important at weaning due to the fact that fluctuations may be an excellent opportunity for opportunistic bacteria that contribute to digestive disorders (Mathew et al., 1996). In this regard, the administration of probiotics,

mainly as lactic acid bacteria, might be a key strategy to avoid problems regularly associated with commercial weaning.

Another issue especially important in pig production, is the growth stasis described after weaning (Le Dividich and Herpin, 1994; McCracken et al., 1995; 1999). In our case, **trial II** confirmed the results, performance being clearly affected by weaning; weaned piglets showed a lower weight gain than their littermates that remainded with their dams. Similarly, in **trial V,** where daily evolution of feed intake of weaned pigs was assessed during the first 7 days after weaning, a marked drop was found in the first 2-3 days, which was not recovered until 7th day post-weaning. In this case, a peak of fecal inconsistence was detected on day 4; different causes could be involved in this fact. Among these, low intake and changes in gut wall arquitecture together with a microbiota unstabilization have been related with diarrhea outbreaks after weaning (Pluske et al., 1997).

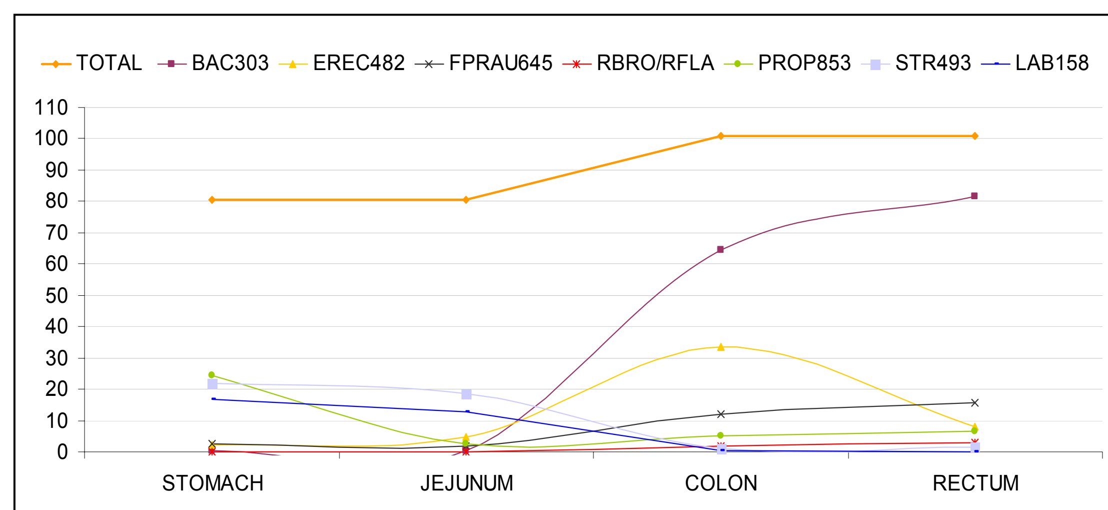
9.2.1. Establishment of adult gut bacteria

In **trial III**, an overall description of different bacteria inhabiting the growing-adult pig gastrointestinal tract was obtained. In agreement with literature, the total bacterial load measured showed a clear increase from small intestine to rectum of around 2 log units. Similarly to **trial IV,** with younger animals and qPCR 3 log units of difference between stomach and distal colon were found. The dissimilar environmental conditions mark the increase in population from proximal to distal parts of the gut. Whereas peristaltic movement and acidic conditions in the upper tract impairs bacterial colonization (Ewing and Cole, 1994), the high quantity of substrate and the lower rate passage improve colonization in caecum, colon and rectum (Stewart et al., 1999). Other authors using FISH to count total bacterial load have described similar values (Konstantinov et al., 2004b).

Moreover, gastrointestinal microbiota differs not only quantitatively, but also qualitatively throughout the gut. In this regard, **trial III** shows us an interesting description of main bacteria in the adult pig gut (stomach, jejunum, proximal colon and rectum; Figure 9.3). As expected, different bacteria were found as main groups in the upper and the lower gastrointestinal tract. However, the results obtained were more clarifying in distal sections of the gastrointestinal tract, due to the higher

coverage obtained with the probes used. Results obtained confirm the dominance of anaerobic bacteria related to clostridial clusters XIVa and to the clostridial cluster IV relatives in the large intestine, in comparison with stomach and jejunum where lactic acid bacteria appeared as the predominant group. Results agree with literature where lactic acid bacteria are described as the main bacteria in the upper gastrointestinal tract (Reid and Hillman, 1999; Hill et al., 2005), and obligate anaerobes such as eubacteria, clostridia and CFB phylum in the large intestine (Conway et al., 1994; Leser et al., 2002).

Figure 9.3. Total bacteria, *Bacteroides/Prevotella* group (probe Bac303), clostridia cluster XIVa (Erec482), *Faecalibacterium prausnitzii* (Fprau645), *Ruminococcus flavefaciens* and *R. bromii* (Rbro730 and Rfla729), clostridia cluster IX (Prop853), *Streptococcus/Lactococcus* sp. (Str493) and *Lactobacillus/ Enterococcus* sp. (Lab158) measured by FISH in gastrointestinal tract.



9.3. Are antibiotic-growth promoters a model to copy?

9.3.1. Mode of action of antibiotics: quantitative or qualitative effects on gut microbiota?

Until their total ban in January 2006, antibiotics as growth promoters were regularly used to improve feed utilization, growth, and to maintain piglet gut health. Although they were used widely in recent decades, their exact mechanism of action is

not completely known. The reduction of bacterial load in the upper gastrointestinal tract, and therefore in the energy potentially available for the host but consumed by normal microbiota, is one of the main hypothesis postulated (Anderson et al., 1999; Hardy et al., 2002).

However, results obtained in **trial IV** did not agree with this hypothesis, since the antibiotic did not reduce total bacteria either in the upper or in the lower gastrointestinal tract. Other authors have seen similar results when testing antibiotics. Collier and co-workers (2003) found a decrease in total bacteria on day 21 after feeding pigs with tylosin that was recovered one week after, probably due to a replacement of bacteria affected by other resistant strains. Moreover, an effect on lactobacilli was expected, due to the spectra of avilamycin against gram positive bacteria, however, this was not detected. In this regard, similar results have been found before with absence of effect of avilamycin on lactobacilli counts (Decuypere et al., 2002).

Despite the lack of effect of avilamycin on total bacterial load, the dendogram obtained by RFLP showed a clear separation of diets, a fact that could be behind a marked change in species bacteria composition with the antibiotic used. This fact might indicate that, contrary to thought, antibiotics could improve growth and gut health of the animals not by reducing total bacteria load, but by changing species composition becoming microbiota in a more favorable equilibrium for the host.

These results would suggest that the modulation of bacterial microbiota to achieve an optimal equilibrium would be the strategy to substitute antibiotics as a growth promotants instead of reducing total bacterial load as have been routinely proposed.

9.3.2. Other in feed-additives with antimicrobial properties

In response to the need for alternatives to in-feed antibiotics, research and development effort is being focused on the search for effective replacements. The different trials included in this thesis aimed to evaluate some of the additives used today in pig production (acidifiers, plant extracts, prebiotics and organic minerals), with special interest in their effects on gut microbiota (Table 9.2). Of these additives, organic acids and plant extracts are proposed as alternatives to antibiotic growth promoters due to their antimicrobial properties.

Results obtained from **trial IV** showed a modulation of gut bacteria with sodium butyrate and plant extract; the dendogram obtained by RFLP confirmed changes in the colonic bacterial ecosystem, although such as with antibiotics, when total bacterial load was measured it did not change.

The change in similarity showed by the dendogram was clearly reflected in changes in bacterial populations measured by qPCR with **plant extract.** It tended to reduce enterobacteria population in jejunum and increased lactobacilli in caecum digesta. Increases in lactobacilli population by this plant extract have been shown before (Manzanilla et al., 2004), although it is difficult to explain how this increase is produced. Previous works have demonstrated a broad antibacterial activity for plant extracts (Didry et al., 1994; Sen et al., 1998; Dorman and Deans, 2000); in particular, for two of the extracts included in the mixture used: carvacrol from oregano (Dorman and Deans, 2000) and cinamaldehyde from cinnamon (Mancini-filho et al., 1998). Indeed, a potential supplantation of specific bacteria inhibited by the plant extract (enterobacteria) by lactobacilli might be postulated. In this regard, recently, Si and co-workers (2006) showed specific antibacterial activity of carvacrol and cinnamon against *E. coli*. Also, modifications of gastrointestinal environment by a reduction of fermentative activity in the small intestine by the extracts directly or by bacterial shifts indirectly, could provide cecum and hindgut with a substrate with prebiotic effect for lactobacilli. However, we could not detect changes in microbial activity in the upper intestine measured as total bacteria, purine bases or microbial enzimatic activities.

Contrary to plant extract, **sodium butyrate** effects observed on dendogram were not reflected in lactobacilli or enterobacteria population, and presumibly this could have been reflected in other bacterial groups (**trial IV**). Previous works of other authors have found changes in ileal microbiota with decreases in coliform bacteria parallel with increases in lactobacilli after administration of this sodium butyrate to weaned pigs (Galfi and Bokori, 1990). Similarly, other works using formates have also shown reductions in coliform bacteria (Øverland et al., 2000) and in total, coliform and lactic acid bacteria (Canibe et al., 2005) throughout the gastrointestinal tract.

However, these previous works detected high amounts of the organic acid administered along the upper gastrointestinal tract that we could not confirm in our study with increases only detected in the stomach (Manzanilla et al., 2006). Taking

this into account, and that RFLP results indicate changes in proximal colon microbiota, some effect on stomach microbiota might somehow have modified the bacterial ecosystem in distal sections. In this regard, van Winsen and co-workers, after administration of fermented liquid feed to growing pigs found decreases in enterobacteria population in the stomach attributed to a higher population of lactobacilli that could have limited their growth. Surprisingly, lower levels of enterobacteria were maintained in feces where lactobacilli population were not different between diets. The authors attributed these results to some kind of carry over effect of microbiota of anterior sections over posterior ones (van Winsen et al., 2001).

In addition, this lack of butyrate detection in the small intestine could be related to the time of sampling, as Na-butyrate is readily absorbed in the gut starting in the stomach (Bugat and Bentajac, 1993). In our case, animals were sacrificed between 4-6.5 h after limiting their access to feed, being therefore a potential factor affecting lack of acid in the small intestine (ad libitum access to feed from 20h to 8h). A complete absorption of the acid at sacrifice time can therefore be behind the lack of butyrate.

On the other hand, some kind of systemic effect of the butyrate absorbed can not be excluded (see chapter 9.4).

9.3.3. Effects on microbiota by other mechanisms

Mode of action of some in-feed additives and feed strategies in gut microbiota seems to be due to some kind of indirect effects on bacteria rather than direct antibacterial activity. Indeed, modulation of gut environment, attachment sites and type/amount of substrate may be the key to the results found in some of the trials included in the thesis.

In this regard, in **trial V**, a clear effect of mannan-oligosaccharides on enterobacteria was observed. When piglets received the diet alone or in combination with organic zinc, qPCR results showed a selective reduction in enterobacteria counts, which was reflected in a higher lactobacilli:enterobacteria ratio. Similarly, White and co-workers (2002) found a lower concentration of coliforms in the feces of pigs fed diets with mannan-oligosaccharides. In this case, the effect as growth promotant of this compound is related to a modulation of the gastrointestinal ecosystem, reducing intestinal colonization by potentially pathogenic bacteria. This

modulation could be due to the fact that the oligosaccharide neutralizes binding proteins on the surface of some bacteria and thus prevents their further attachment to intestinal epithelium (Spring et al., 2000). However, a potential prebiotic effect of the oligosaccharide, can not be discarded, and could also explain the effects observed. However, in agreement with previous works (White et al., 2002), this hypothesis was not supported by changes in the fermentation patterns measured as short chain fatty acids.

Apart from the different additives evaluated, in **trial III**, administration of different types of fiber was also tested as a way to modulate the bacterial ecosystem. It is well known that the modification of substrate reaching the lower gastrointestinal tract is an effective tool to modify microbiota. However, controversies regarding fiber inclusion in the diet and apparition of some enteric diseases make this approach dubious (Hampson et al., 2001).

In our case, the administration of diets rich in fiber (in form of resistant starch and soluble and insoluble non-starch polysaccharides; **trial III**) did not result in marked changes in bacterial groups studied along the gastrointestinal tract. Also, differences in microbial activity measured as purine bases or enzymatic acitivities were not found (data not shown). However, differences were shown with RFLP analysis, showing that the animals fed with wheat bran had the lowest biodiversity in proximal colon content and the most homogenous ecosystem between animals. In agreement, Högberg and co-workers (2004) recently related administration of diets rich in insoluble non-starch polysaccharides with a lower microbial biodiversity, which might indicate a higher difficulty to digest this type of fiber resulting in a higher specialization of bacteria inhabiting the lower gut. This implies therefore a lower biodiversity that might be more easily interrupted by opportunistic pathogen colonization.

These effects promoted by the different types of fiber could be explained not only by increasing or changing the amount of substrate that arrives to be fermented but also by differences reported for the same diets in some parameters such as digesta viscosity, transit time and water binding capacity (Anguita et al., 2006). This might also modify gut environment and thus impair or improve colonization by different bacteria.

9.3.4. Other strategies to improve health and promote growth

Improvements in pig gut health and pig performance obtained when additives are added to pig diets can not be completely explained by their effect on microbiota. An improvement of the piglet immune response, gut barrier and digestive capacity are also considered as mechanisms by which some additives improve gut function and performance. Among the additives tested, mannan-oligosacharides and organic zinc (**trial V**) and sodium butyrate (**trial IV**) may be acting in these ways.

The administration of organic zinc (**trial V**) did not modify the bacterial groups studied, contrary to effects found when the mineral is added in inorganic form at much higher concentrations (Katouli et al., 1999; Höjberg et al. 2005). However, piglets showed a heavier empty ileum, considered as the section of the small intestine showing a continuous Peyer's Patch, which was also numerically longer. The higher weight observed may be reflecting a higher development of the Peyer's Patches. Also IgA and IgM measured in jejunum digesta were numerically higher when organic zinc was administered, although differences did not reach significance.

Therefore the organic mineral could act by a different mechanism of action than zinc oxide, with an immunoestimulatory effect, especially important at early's stages of piglet life when the immune system is still immature. In fact, it has been shown that Zn is crucial for the normal development and function of cells mediating nonspecific immunity, and also for T and B lymphocytes proliferation (Shankar and Prasad, 1998). Similarly, mannan-oligosaccharides have been reported to enhance the pig immune response through activation of different membrane receptors by their molecular similarity to different bacterial structures (Newman and Newman, 2001; O'Quinn et al., 2001; Davis et al., 2004b). However, immune measurements done in our study did not show differences.

Moreover, a synergic effect was observed in intestinal morphology when organic zinc and mannan-oligosaccharides were added together. Animals fed in both additives together showed the lowest crypt depth and this was reflected in the highest villus:crypt ratio which is considered an indicator of overall gut health (Zijlstra et al., 1994). Similar results have been obtained in poultry with mannan-oligosaccharides given alone (Iji et al., 2001; Ferket, 2002). This effect might be especially beneficial at piglet weaning when villus atrophy and crypt hyperplasia appear with the consequent impairment of gut function (Pluske et al., 1997). The reduction of enterobacteria counts, and also a more suitable immune response might be behind

these effects. Taking into account the relationship between the immune system and gut bacteria, results obtained with both these additives seem particularly interesting.

In the case of sodium butyrate (**trial IV**), we have seen before that their promoting effects on growth could be due to the observed effect on microbiota. However, a systemic effect on pig health due to absorption of sodium butyrate administered in the diet can not be discarded, specially taking into account the fact that acid was only detected in the stomach. It has been shown that sodium butyrate has a complex trophic effect on the gastrointestinal epithelium (Galfi and Bokori, 1990) by providing energy to epithelial cells (Bugat and Bentajac, 1993; Cummings1995), improving absorption of sodium and water (Bond and Levit., 1976), and also by stimulating proliferation index in crypts (Salminen et al., 1998). It is possible therefore that butyrate was also acting in these ways, improving pig health and thus explaining the improvement found in the gain:feed ratio and average daily gain (Manzanilla et al., 2006, In press).

9. 4. Summary

Results obtained in the different trials included in this thesis point to different modes of action of additives tested. Antibiotic growth promoter, regarded as a model to copy, did not act simply by reducing total bacterial load but probably by a more complex modification of bacterial profile. In this regard, antibiotic strategy, successfully and routinely used during the last fifty years, is not an easy strategy to mimic. Moreover, results obtained with the additives tested indicate different mechanisms of action: selective effect on microbial groups, modulation of host health by blocking adhesion of potential harmful bacteria, improvement of immune response, or other systemic effects. Further studies are therefore required to improve our knowledge regarding the exact mechanism of action of the different alternatives to antibiotics proposed, although the achievement of an optimal equilibrium of gut microbiota, and the improvement of gut function and immune response could be considered the key responses to improve pig health and performance.

Table 9.2. Summary of the main effects found for the different additives tested in the trials included in the thesis

Trial	Additive tested	Intestinal section	Microbial indexes[a] Total bacteria	Lactobacilli	Enterobacteria	Profile	Biodiversity	Gut morphology Villus: Crypt	Crypt depth	Immune status Igs
Trial IV	AB (avylamicin)	Jejunum	=	=	=					
	AC (sodium butyrate)		=	=	=					
	XT (plant extract)		=	=	↓					
	AB (avylamicin)	Caecum	=	=	=	≠	↑			
	AC (sodium butyrate)		=	=	=	≠	↑			
	XT (plant extract)		=	↑	=	≠	↑			
Trial V	BM (mannan-oligosacharides)	Jejunum		=	↓			=	=	=
	BP' (zinc-quelate)			=	=			=	=	↑
	BMP (mannan-oligosacharides plus zinc quelate)			=	=			=	↓	=

[a] A symbol has been assigned to classify effects observed on the different parameters evaluated (=, denotes abscence of changes; ↓, denotes a diminish and ↑ denotes an increase compared to control diet).

Chapter 10

CONCLUSIONS

The results obtained in this thesis allow us to conclude that in our experimental conditions:

1. Real-time PCR used to quantify gut bacterial groups, in terms of 16S rRNA gene copies, is a practical method to detect changes in microbiota equilibrium by the lactobacilli:enterobacteria index. However, for absolute quantification it generates higher counts than direct microscopy and selective culture.

2. Commercial weaning produces a marked shift in piglet cecum microbiota, with a significant decrease in the lactobacilli:enterobacteria ratio and changes in bacterial profiles assessed by terminal restriction fragment length polymorfism (t-RFLP). Specifically, a lower diversity in lactic acid bacteria and the absence of some particular species like *Lactobacillus delbruekii*, *Clostridium butyricum* and *C. perfringens* are related to microbiota disruption by weaning.

3. In the growing pig, the major bacterial groups quantified by fluorescent *in situ* hybridization (FISH) differ along the gastrointestinal tract. Streptococci and lactobacilli are the predominant in the upper tract, whereas *Bacteroides/Prevotella* group, clostrial cluster XIV, IV and ruminoccoci are the main groups in the lower tract.

4. The inclusion of coarse ground corn (4 mm), beet pulp (8%) or wheat bran (10%) in the diet of growing pigs does not affect the main bacterial groups of the intestinal tract. However, as observed by RFLP results, changes are produced in the diversity of species within each group. In particular, wheat bran, as a source of insoluble non-starch polysaccharides, promotes a decrease in microbial diversity with more similar profiles between animals.

5. Contrary to what we expected, effects of avilamicyn on microbiota are related to the modulation of its profile rather than a reduction in total bacterial load. In particular, an increase in microbial diversity was demonstrated by RFLP that could be behind the observed effects on performance.

6. Similarly to avilamicyn, sodium butyrate and plant extract are able to modify the microbial profile without modifying total microbial counts. However, each

additive promotes different changes. In particular, plant extract significantly increases lactobacilli in the cecum, and butyrate promoted the highest biodiversity.

7. The growth performance improvement with mannan oligosaccharides and organic zinc in weaning pigs are due to different modes of action. Whilst mannan-oligosaccharides show an inhibitory effect on enterobacteria population, organic zinc tends to improve development of the continuous Peyer's Patch. Synergy is manifested by a significant increase in villi:crypt ratio when both additives are included together.

Chapter 11

LITERATURE CITED

Abe, F., N. Ishibashi, and S. Shimamura. 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 78:2838-2846.

Abraham, S. N., J. D. Goguen, and E. H. Beachey. 1985. Hyperadhesive mutant of type-1 fimbriated E. coli associated with formation of FIMH organelles. Infect. Immun. 56:1023-1029.

Adami, A., and V. Cavazzoni. 1999. Occurrence of selected bacterial groups in faeces of piglets fed with *Bacillus coagulans* as probiotic. J. Basic Microbiol. 39:3-9.

Adams, C. A. 2001. Total Nutrition. Feeding animals for health and growth. Notthingham University Press, UK.

Aguirre, M., and M. D. Collins. 1993. Lactic acid bacteria and human clinical infection. J. Appl. Bacteriol. 75:95-107.

Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2:671-680.

Alam, M., T. Midtvedt, and A. Uribe. 1994. Differential cell kinetics in the ileum and colon of germ-free rats. Scand. J. Gastroenterol. 29:445-451.

Alander, M., R. Satokari, and R. Korpela. 1999. Persistence of colonisation of human colonic mucosa by a probiotic strain, Lactobacillus rhamnosus GG after oral consumption. Appl. Environ. Microbiol. 65:351-354.

Alexopoulus, C., I. E. Georgoulakis, A. Tzivara, S. K. Kritas, A. Siochu, and S. C. Kyriakis. 2004a. Field evaluation of the efficacy of a probiotic containing Bacillus licheniformis and Bacillus subtilus spores, on the health status and performance of sows and their litters. J. Anim. Phisiol. Anim. Nutr. 88 :381-392.

Alexopoulus, C., I. E. Georgoulakis, A. Tzivara, C. S. Kyriakis, A. Govaris, and S. C. Kyriakis. 2004b. Field evaluation of the effect of a probiotic-containing Bacillus licheniformis and Bacillus subtilis spores on the health status, performance, and carcass quality of grower and finisher pigs. J. Vet. Med. A. Physiol. Clin. Med.51 :306-312.

Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F. –O. Glöckner, A. Wille, and R. Psenner. 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. App. Environ. Microbiol. 62:2138-2144.

Allison, C., and G. T. MacFarlane. 1989. Influence of pH, nutrient availability, and growth rate on amine production by Bacteroides fragilis and Clostridium perfringens. Appl. Environ. Microbiol. 55:2894-2898.

Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin. 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. 62:3557-3559.

Alstchul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment tool. J. Mol. Biol. 215:403-410.

LITERATURE CITED

Amann, R. I., L. Krumholz, and D. A. Stahl. 1990a. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172:762-770.

Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl. 1990b. Combination of 16S RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919-1925.

Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59:143-169.

Amann, R., J. Snaidr, M. Wagner, W. Ludwig, and K. H. Schleifer. 1996. In situ visualization of high genetic diversity in a natural microbial community. J. Bacteriol. 178:3496-3500.

Amann, R., B. M. Fuchs, and S. Behrens. 2001. The identification of microoganisms by fluorescence in situ hybridization. Curr. Opin. Biotechnol. 12:231-236.

Ampe, F., A. Sirvent, and N. Zakhia. 2001. Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. Int. J. Food Microbiol.65:45-54.

Anderson, D. B., V. J. McCracken, R. I. Aminov, J. M. Simpson, R. I. Mackie, M. W. Verstegen, and H. R. Gaskins. 1999. Gut microbiology and growth promoting antibiotics in swine. Nutr. Abstr. Rev. B70:101-108.

Anderson, K. L., and S. Lebepe-Mazur, 2003. Comparison of rapid methods for the extraction of bacterial DNA from colonic and caecal lumen contents of the pig. J. Appl. Microbiol. 94:988-993.

Anguita, M.., J. Gasa, S. M. Martín-Orúe, M. Nofrarias and J. F. Pérez. 2006. Effect of coarse ground corn, sugar beet pulp, and wheat gran on the physicochemical characteristics of digesta in growing pigs. Submitted to the Livestock Science.

AOAC. 1990. Official methods of analysis. 15th edition. AOAC, Arlington, USA.

Apajalahti J. H., A. Kettunen, M. R. Bedford, and W. E. Holben. 2001. Percent G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. Appl. Environ. Microbiol. 67:5656-5667.

Apajalahti, J. H. A., A. Kettunen, P. H. Päivi, J. Jatila, and W. E. Holben, 2003. Selective plating underestimates abundance and shows differential recovery of bifidobacterial species from human feces. Appl. Environ. Microbiol. 69:5731-5735.

Ashwell, G. 1957. Colorimetric analysis of sugar. Methods in Enzymology 3:73-105.

Atlas, R. M. 1984. Diversity of microbial communities. In: Advances in microbial ecology. K. C. Marshall, ed. Plenum Press, New York.

Ayabe, T., D. P. Satchell, C. L. Wilson, W. C. Parks, M. E. Selsted, and A. J. Ouellette. 2000. Secretion of microbicidal alfa-defensins by intestinal Paneth cells in response to bacteria. Nature Immunol. 1:113-118.

Ayabe, T., T. Ashiba, Y. Kohgo, and T. Kono. 2004. The role of Paneth cells and their antimicrobial peptides in innate host defense. Trends Microbiol. 12:394-398.

Bach, H. J., J. Tomanova, M. Schloter, and J. C. Munch. 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. J. Microbiol. Methods 49:235-245.

Bach-Kudsen, K. E., and B. B. Jensen. 1991. Effect of source and level of dietary fibre on microbial fermentation in the large intestine of pigs. EAAP publication 54:389-394.

Bach Knudsen, K. E., and I. Hansen. 1991. Gastrointestinal implication in pigs of wheat and oat fractions. 1. Digestibility and bulking properties of polysaccharides and other major constituents. Br. J. Nutr. 65:217-232.

Bach Knudsen, K. E., B. B. Jensen, J. O. Andersen and I. Hansen. 1991. Gastrointestinal implications in pigs of wheat and oat fractions. 2. Microbial activity in the gastrointestinal tract. Br. J. Nutr. 65:233-248.

Bach-Knudsen, K. E., B. B. Jensen, and I. Hansen. 1993. Digestion of polysaccharides and other major components in the small and large intestine of pigs fed on diets consisting of oat fractions rich in beta-D-glucan. Br. J. Nutr. 70:537-556.

Bach Knudsen, K. E. 1997. In Proceedings of the International Symposium "Non-digestible oligosaccharides: Healthy food for the colon? R. Hartemink, ed. Wageningen Institute of Anim. Sciences, Wageningen, Netherlands.

Bach Knudsen, K. E. 2001. Carbohydrate and lignin contents of plan materials used in animals feeding. Anim. Feed Sci. Techol. 90:3-20.

Bailey, M., F. J. Plunkett, H. J. Rothkötter, M. A. Vega-López, K. Haverson, and C. R. Stokes. 2001. Regulation of mucosal immune responses in effector sites. Proceedings of the Nutrition Society 60:427-435.

Ballongue, J., C. Schumann, and P. Quignon. 1997. Effects of lactulose and lactitol on colonic microflora and enzymatic activity. Scand. J. Gastroenterol. 222:41-44.

Barcenilla, A., S. E. Pryde, J. C. Martin, S. H. Duncan, C. S Stewart, and H. J. Flint. 2000 Phylogenetic relationships of dominant butyrate producing bacteria from the human gut. Appl. Environ. Microbiol. 66:1654-1661.

Baron, S. F., and P. B. Hylemon. 1997. Biotransformation of bile acids, cholesterol, and steroid hormones. In Gastrointestinal Microbiology. R. I. Mackie, B. A. White and R. E. Isaacson, eds. Chapman and Hall, New York.

Barrow P. A., B. E. Brooker, R. Fuller, and M. J. Newport. 1980. The attachment of bacteria to the gastric epithelium of the pig and its importance in the microecology of the intestine. J. Appl. Bacteriol. 48:147-154.

Bedford, M. R. and H. Schulze. 1998. Exogenous enzymes for pigs and poultry. Nutr.Res. Rev. 11:91-114.

Berg, R. D. 1992. Translocation of enteric bacteria in health and disease. Curr. Stud. Hematol. Blood Transfus. 59:44-65.

Berg, R. D. 1996. The indigenous gastrointestinal microflora. Trends Microbiol 4:430-435.

Berg, R. D. 1999. Bacterial translocation from the gastrointestinal tract. Adv. Exp. Med. Biol. 473:11-30.

Bernet, M. F., D. Brassart, J. R. Neeser, and A. L. Servin. 1994. *Lactobacillus acidophillus* LA binds to cultured human intestinal cell lines and inhabits cell attachment and invasion by enterovirulent bacteria. Gut 35:483-489.

Bertschinger, H. U., and E. Eggenberger. 1978. Evaluation of low nutrient, high fibre diets for the prevention of porcine *Escherichia coli* enterotoxaemia. Vet. Microbiol. 3:281-290.

Bijlsma, I. G., and J. Bouw. 1987. Inheritance of K88-mediated adhesio of Escherichia coli to jejunal brush borders in pigs: a genetic analysis. Vet. Res. Commun. 11: 509-518.

Blackwood, C. B., T. Marsh, S. H. Kim, and E. A. Paul. 2003. Terminal Restriction Fragment Length Polymorphism data analysis for quantitative comparison of microbial communities. Appl. Environ. Microbiol. 69:926-932.

Blomberg, L., A. Henriksson, and P. L. Conway, 1993. Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. Appl. Environ. Microbiol 59:34-39.

Bokori, J., P. Galfi, and I. Boros. 1989. Swine experiment with a feed containing Na-n-butyrate. Magy. Allatory. Lapja. 44:501 (abs.).

Bolduan G., H. Jung, R. Schneider, J. Block, and B. Klenke. 1988. Influence of fumaric acid and propanediol formate on piglets. J. Anim. Physiol. Anim. Nutr. 59:143-149.

Bolduan, G., M. Beck, and C. Schubert. 1993. The effect of oligosaccharides on piglets. Arch. Tierernahr. 44:21-27.

Bollinger, R. R., M. L. Everett, D. Palestrant, S. D. Love, S. S. Lin, and W. Parker. 2003. Human secretory immunoglobulin A may contribute to biofilm formation in the gut. Immunology 109:580-587.

Bond, J. H., and M. D. Levit. 1976. Fate of soluble carbohydrate in the colon of rats and man. J. Clin. Invest. 57:1158-1164.

Bond, P. L., R. Erhart, M. Wagner, J. Keller, and L. L. Blackall. 1999. Identification of some of the major groups of bacteria in efficient and nonefficient biological phosphorus removal activated sludge systems. Appl. Environ. Microbiol. 65:4077-4084.

Bosi, P., H. J. Jung, I. K. Han, S. Perini, J. A. Cacciavillani, L. Casini, D. Creston, C. Gremokolini, and S. Mattuzzi. 1999. Effects of dietary buffering characteristics and protected or unprotected acids on piglet growth, digestibility and characteristics of gut content. Asian-Australasian J. Anim. Sci. 12:1104-1110.

Bovee-Oudenhoven, I. M. J., D. S. Termont, P. J. Heidt, and R. Van der Meer. 1997. Increasing the resistance of rats to the invasive pathogen *Salmonella enteritidis*: additive effects of dietary lactulose and calcium. Gut 40:497-504.

Boye, M., T. K. Jensen, K. Moller, T. D. Leser, and S. E. Jorsal. 1998. Specific detection of the genus *Serpulina, S. hyodysenteriae*, and *S. pilosicoli* in porcine intestines by fluorescent rRNA in situ hybridization. Mol. Cell. Probes 12:323-330.

Brandtzaeg, P. E. 2002. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. Ann. N. Y. Acad. Sci. 964:13-45.

Bray, T. M. and W. J. Bettger. 1990. The physiological role of zinc as an antioxidant. Free Radic. Biol. Med. 8:281-291.

Brook, I. 1999. Bacterial interference. Crit. Rev. Microbiol. 25:155-172.

Brooks, P. H., T. M. Geary, D. T. Morgan ad A. Campbell. 1996. New developments in liquid feeding. Pig Journal 36:43-63.

Brown, I., M. Warhurst, J. Arcot, M. Playne, R. J. Illman, and D. L. Topping. 1997. Fecal numbers of bificobacteria are higher in pigs fed *Bifidobacterium longum* with a high amylose cornstarch that with a low amylose cornstarch. J. Nutr.127:1822-1827.

Bry, L., P. G. Falk, T. Midvedt, and J. I. Gordon. 1996. A model for host-microbial cross-talk in an open mammanlian ecosystem. Science 273:1380-1383.

Buddington, R. K., C. H. Williams, S. –C. Chen, and S. A. Witherly. 1996. Dietary supplement of neosugar alters the fecal flora and decreases activities of some reductive enzymes in human subjects. Am. J. Clin. Nutr. 63:709-716.

Bugat, M., and M. Bentejac. 1993. Biological effect of short chain fatty acids in nonruminant mammals. Annu. Rev. Nutr. 13:217-241.

Bustin, S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25:169-193.

Canibe, N., S. H. Steien, M. Øverland, and B. B. Jensen. 2001. Effect of K-diformate in starter diets on acidity, microbiota, and the amount of organic acids in the digestive tract of piglets, and on gastric alterations. J. Anim. Sci. 79:2123-2133.

Canibe, N., O. Højberg, S. Højsgaard, and B. B. Jensen. 2005. Feed physical form and formic acid addtition to the feed affect the gastrointestinal ecology and growth performance of growing pigs. J. Anim. Sci.83:1287-1302.

Carlson, M. S., Hill, G. M., and J. E. Link. 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.

Carlson, M. S., C. A. Boren, C. Wu, C. E. Huntington, D. W. Bollinger, and T. L. Veum. 2004. Evaluation of various inclusion rates of organic zinc either as polysaccharide or chelate complex on growth performance, plasma, and excretion in nursery pigs. J. Anim. Sci. 82:1359-1366.

Carlstedt-Duke, B., T. Midvedt, C. E. Nord, and B. E. Gustafsson. 1986. Isolation and characterization of a mucin degrading strain of *Peptostreptococcus* from rat intestinal tract. Acta Pathol. Microbiol. Immunol. Scand. Sect. B. 94:292-300.

Case, C. L., and M. S. Carlson. 2002. Effect of feeding organic and inorganic sources of additional zinc on growth performance and zinc balance in nursery pigs. J. Anim. Sci. 80:1917-1924.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemotherapy 52:159-161.

Castillo, M., S. M. Martín-Orúe, E. G. Manzanilla, I. Badiola, M. Martín and J. Gasa. 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet. Microbiol. In Press.

Cebra, J. J. 1999. Influences of microbiota on intestinal immune system development. Am. J. Clin. Nutr. 69:1046S-1051S.

Charteris, W. P., P. M. Kelly, L. Morelli, and J. K. Collins. 1997. Review article:selective detection, enumeration and identification of potential probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. 35:1-27.

Chen X., B. Zehnbauer, A. Gnirke, and P. Y. Kwok. Fluorescence energy transfer detection as a homogeneous DNA diagnostic method. 1997. Proceedings of the Natl. Acad. Sci. USA. 94:10756-10761.

Cherbut, C., A. C. Aube, H. M. Blottiere, P. Pacaud, C. Scarpignato, and J. P. Galmiche. 1996. In vitro contractile effects of short chain fatty acids in the rat terminal ileum. Gut 38:53-58.

Cherbut, C. A. C. Aube, H. M. Blottiere, and J. P. Galmiche. 1997. Effects of short chain fatty acids on gastrointestinal motility. Scand. J. Gastroenterol. 222:58-61.

Cherrington, C. A., M. Hinton, G. C. Mead, and I. Chopra. 1991. Organic acids: chemistry, antibacterial activity and practical applications. Adv. Microb. Physiol. 32:87-108.

Chopra, S. L., Blackwood, A. C., and D. G. Dale. 1963. Intestinal microflora associated with enteritis of early-weaned pigs. Can. J. Comp. Med. Vet. Sci. 27:290-294.

Clegg, S., and G. F. Gerlach. 1987. Enterobacterial fimbriae. J. Bacteriol. 169:934-938.

Clement, B. G., L. E. Kehl, K. L. DeBord, and C. L. Kitts. 1998. Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J. Microbiol. Methods 31:135-142.

Clement, B., and Kitts, C. L. 2000. Isolation PCR quality DNA from human feces with a soil DNA kit. Biotechniques 28:640-646.

Close, W. H. 2000. Producing pigs without antibiotics growth promoters. Adv. Pork Prod.11:47-56.

Coates, M. E., R. Fuller, G. F. Harrison, M. Lev, and S. F. Suffolk. 1963. A comparison of the growth of xhicks in the Fustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. Br. J. Nutr. 17:141-151.

Coconnier, M. H., M. F. Bernet, S. Kernéis, G. Chauvière, J. Fourniat, and A. L. Servin. 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett.110:229-306.

Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje. 2003. The Ribosomal Database Project (RDB-II): previewing a new autoaligner that allows regular updates and the new prokariotic taxonomy. Nucleic Acids Res. 31:442-443.

Collier, C. T., M. R. Smiricky-Tjardes, D. M. Albin, J. E. Wubben, V. M. Gabert, B. Deplancke, D. Bane, D. B. Anderson, and H. R. Gaskins. 2003. Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. J. Anim. Sci. 81:3035-3045.

Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzbal, P. Garcia, J. Cai, H. Hippe and J. A. E. Farrow. 1994. The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44:812-826.

Collins, M. D., and G. R. Gibson. 1999. Probiotics, prebiotics, and symbiotics: approaches for modulating the microbial ecology of the gut. Am. J. Clin. Nutr. 69:1052S-1057S.

Contrepois, M. 1988. Les colibacilles pathogens: adherence et facteurs de colonisation des colibaciles entérotoxigènes. In: L'intestine Grêle. J. C. Rambaud, R. Modigliani, eds. Excerpta Medica, France.

Conway, P. L. 1994. Function and regulation of the gastrointestinal microbiota of the pig. EAPP publication 2:231-240.

Conway, P. L. 1997. Development of intestinal microbiota. Gastrointestinal microbes and host interactions. In: Mackie, R. I., Whyte, B. A. and Isaacson, R. E., eds. Gastrointestinal Microbiol., vol. 2. Chapman and Hall, London.

Costerton, J. W., R. T. Irvin, K. J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. 81:299-324.

Crittenden, R. G. 1999. Prebiotics. In: Probiotics: a critical review. G. W. Tannock, ed., Horizon Scientific Press, Wymondham, UK.

Cromwell, G. L., T. S. Stahly, and H. J. Monegue. 1985. Efficacy of sarsaponin for weanling and growing-finishing swine housed at two animal densities. J. Anim. Sci. 61:111 (abs).

Cromwell, G. L., T. S. Stahly, and H. J. Monegue. 1989. Effects of source and level of copper on performance and liver copper stores in weanling pigs. J. Anim. Sci. 67:2996-3002.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. 13:7-27.

Cummings J. H., and H. N. Englyst. 1987. Fermentation in the human large intestine and the available substrates.Am. J. Clin. Nutr. 45:1243S-1255S.

Cummings, J. H. 1995. Short chain fatty acids. In: Human Colonic Bacteria.: role in nutrition, physiology and pathology. G. R. Gibson and G. T. Macfarlane, eds. Boca Raton, FL: CRC Press.

Dahl, J. 1997. Feed related risk factoris for sub-clinical salmonella infections. Veterinæn information. 17-20 (abs).

Daims, H., J. L. Nielsen, P. H. Nielsen, K. H. Schleifer, and M. Wagner. 2001. In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. 67:5273-5284.

Danek, P., J. Novak, H. Semradova and E. Diblikova. 1991. Administration of the probiotics *Lactobacillus casei* CCM-4160 to sows –its effects on piglet efficiency. Zivocisna Vryoba 36:411-415 (abs).

Darveau, R. P., M. McFall-Ngai, E. Ruby, S. Miller, and D. F. Mangan. 2003. Host tissues may actively respond to beneficial microbes. ASM News 69:186-191.

Davis, M. E., C. V. Maxwell, E. B. Kegley, B. Z. de Rodas, K. G. Friesen, D. H. Hellwig, and R. A. Dvorak. 1999. Efficacy of mannan oligosaccharide addition at two leels of supplemental copper on performance and immunocompetence of early-weaned pigs. J. Anim. Sci.77(suppl.1):63(abs).

Davis, M. E., C. V. Maxwell, D. C. Brown, B. Z. de Rodas, Z. B. Johnson, E. B. Kegley, D. H. Hellwig and R. A. Dvorak. 2002. Effect of dietary mannan oligosaccharides and (or) pharmacological additions of copper sulfate on growth performance and inmunocompetence of weanling copper sulfate on growth performance and inmunocompetence of weanling and growing/finishing pigs. J. Anim. Sci. 80:2887-2894.

Davis, M. E., C. V. Maxwell, G. F. Erf, D. C. Brown and T. J. Wistuba. 2004a. Dietary supplementation with phosphorylated mannans improves growth response and modulates immune function of weanling pigs. J. Anim. Sci. 82:1882-1891.

Davis, M. E., D. C. Brown, C. V. Maxwell, Z. B. Johnson, E. B. Kegley and R. A. Dvorak. 2004b. Effect of phosphorylated mannans and pharmacological additions of zinc oxide on growth and inmunocompetence of weanling pigs. J. Anim. Sci. 82:581-587.

Dean, E. A. 1990. Comparison of receptors for 987P pili of enterotoxigenic *Escherichia coli* in the small intestines of neonatal and older pigs. Infect. Immun. 58:4030-4035.

Decuypere, C. J., N. Van Nevel, K. Dierick, and K. Molly. 2002. The influence of *Lentinus edodes* preparations on bacteriological and morphological aspects of the small intestine in piglets. Reprod. Nutr. Dev. 42:S18.

DeLong, E. D. 1992. Archaea in coastal marine environments. Proc. Natl Acad. Sci. USA. 89:5685-5689.

Didry, N., L. Dubreuil, and M. Pinkas. 1994. Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. Pharmaceutica Acta Helvetiae. 69:25-28 (abs).

Dierick, N. A., I. J. Vervaeke, D. I. Demeyer, J. A. Decuypere. 1989. Approach to the energetic importance of fibre digestion in pigs. 1. Importance of fermentation in the overall energy supply. Anim. Feed Sci. Techol. 23:141-167.

Doré, J., A. Sghir, G. Hannequart-Gramet, G. Corthier, and P. Pochart. 1998. Design and evaluation of a 16S rRNA-targeted oligonucleotide probe for specific detection and quantitation of human faecal Bacteroides populations. Syst. Appl. Microbiol. 21:65-71.

Dorman H. J., and S. G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88:308-316.

Dove, C. R., and K. D. Haydon. 1991. The effect of copper addition to diets with various iron levels on the performance and haematology of weanling swine. J. Anim. Sci. 69:2013-2019.

Dove, C. R. 1995. The effect of copper level on nutrient utilization of weanling pigs. J. Anim. Sci. 73:166-171.

Doyle, M. E. 2001. Alternatives to antibiotic use for growth promotion in animal husbandry. FRI Briefings.

Drasar, B. S., and P. A. Barrow. 1985. Intestinal Microbiology In: American Society of Microbiology. Washington D. C.

Dunbar, J., L. O. Ticknor, and C. R. Kuske. 2000. Assessment of microbial diversity in two southwestern U. S. soils by terminal restriction fragment analysis. Appl. Environ. Microbiol. 66:2943-2950.

Duncan, S. H., H. J. Flint and C. S. Stewart. 1998. Inhibitory activity of gut bacteria against *Escherichia coli* O157 mediated by dietary plant metabolites. FEMS Microbiol. Lett. 164: 283-288.

Durmic Z., D. W. Pethick, J. R. Pluske, D. J. Hampson. 1998. Changes in bacterial populations in the colon of pigs fed different sources of dietary fibre, and the development of swine dysentery after experimental infection. J. Appl. Microbiol. 85:574-582.

Durst, L., M. Feldner, B. Gedek, and B. Eckel. 1998. Bakterien als probiotikum sauenfütterng und der Ferkelaufzucht. Krauftfutter 9:356-364 (abs).

Dutta, S., A. Chatterjee, P. Dutta, K. Rajendran, S. Roy, K. C. Pramanik, and S. K. Bhattacharya. 2001. Sensitivity and performance characteristics of a direct PCR with stool samples in comparison to conventional techniques for diagnosis of *Shigella* and enteroinvasive *Escherichia coli* infection in children with acute diarrhoea in Calcutta, Indian J. Med. Microbiol. 50:667-674.

Dvorak, R., and K. A. Jacques. 1998. Mannanoligosaccharide, fructooligosaccharide and Carbadox for pigs days 0-21 post-weaning. J. Anim. Sci. 76(Suppl.2) :64(abs).

Eckburg, P.B., C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. Science 308:1635-1638.

Eckel, B., M. Kirckgessner, and F. W. Roth. 1992. Effect of formic acid on daily weight gain, feed intake, feed conversion rate and digestibility. 1. Communication: Investigations about the nutritive efficacy of organic acids in the rearing of piglets. J. Anim. Physiol. Anim. Nutr. 67:198-205.

Edwards C. A., and A. M. Parret. 2002. Intestinal flora during the first months of life: new prospective. Br. J. Nutr. 88 Suppl. 1:S11-S18.

Eidelsburger, U., M. Kirchgesner, M., and F. X. Roth. 1992. Zum Eingluβvon Fumarsäure. Salzsäure Natriumformiat, tylosin and toyocerin aug tägliache zunahmen, fulteraufnahme, futterverwertung, und verdaulichkeit. J. Anim. Physiol. Anim. Nutr. 68:82-92 (abs).

Elliot, S. E., A. Buret, W. McKnight, M. J. S. Miller, and J. L. Wallace. 1998. Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. Am. J. Physiol. 275:425-432.

Englyst, H. N., and J. H. Cummings. 1987. Digestion of polysaccharides of potato in the small intestine of man. Am. J. Clin. Nutr. 45:423-431.

Englyst, H. N. 1989. Classification and measurement of plant polysaccharides. Anim. Feed Sci. Techol. 23:27-42.

Englyst, H. N., S. M. Kingman, and J. H. Cummings. 1992. Classification and measurement of nutritionally important starch fractions. Eur. J. Clin. Nutr. 46:S33-S50.

Estrada, A., M. D. Drew, and A. van Kessel. 2001. Effect of the dietary supplementation of fructooligosaccharides and *Bifidobacterium longum* to early-weaned pigs on performance and fecal bacterial populations. Can. J. Anim. Sci. 82:607-609.

Ewing, W. N. and D. J. A. Cole. 1994. The microbiology of the gastrointestinal tract. Pages 45-65 in The living gut. An introduction to microorganisms in nutrition. W. N. Ewing and D. J. A. Cole, eds. Context, Ireland, UK.

Eyssen H. 1973. Role of the gut microflora in metabolism of lipids and sterols. Proceedings of Nutrition Society 32:59-63.

Falk, P. G., L. V. Hooper, T. Midtvedt, and J. I. Gordon. 1998. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. Microbiol. Mol. Biol. Rev. 62:1157-1170.

Farnworth, E. R., H. W. Modler, J. D. Jones, N. Cave, H. Yamazaki, and A. V. Rao. 1992. Feeding Jerusalem artichoke flour rich in fructooligosaccharides to weanling pigs. Can. J. Anim. Sci. 72:977-980.

Favier, C. F., E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans. 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. 68:219-226.

Ferket, P. R. 2002. Use of oligosaccharides and gut modifiers as replacements for dietary antibiotics. Proc. 63rd Minnesota Nutrition Conference, September 17-18, Eagan, MN, pp 169-182.

Février, C., G. Gotterbarm, U. Jaghelin-Peyraud, Y. Lebreton, F. Legouvec, and A. Aumaitre. 2001. Effects of adding potassium diformate and phytase excess for weaned piglet. Pages 136-138 in Digestive Physiology of Pigs. J. E. Lindberg and B. Ogle, eds. CABI Publishing, Oxon, UK.

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

Fioramonti, J., V. Theodorou, and L. Bueno. 2003. Probiotics: what are they? What are their effects on gut physiology? Best Pract. Res. Clin. Gastroenterol. 17:711-724.

Foegeding P. M., and F. F. Busta. 1991. Chemical food preservatives. In: Disinfection, Sterilization and Preservation. S. S. Block, ed., Lea & Febiger. Philadelphia.

Fogel, G. B., C. R. Collins, J. Li, and C. F. Brunk, 1999. Prokaryotic genome size and SSU rDNA copy number, estimation of microbial relative abundance from a mixed population. Microb. Ecol. 38, 93-113.

Fons, M., A. Gomez, and T. Karjalainene. 2000. Mechanisms of colonisation and colonisation resistance of the digestive tract. Microb. Ecol. Health Dis. 2:240S-246S.

Fonti, G., and P. Gouet. 1989. Fibre-degrading microorganisms in the monogastric digestive tract. Anim. Feed Sci. Techol. 23:91-107.

Forstner, J. F., and G. G. Forstner. 1994. Gastrointestinal mucus. In: Physiology of the Gastrointestinal Tract. Third Edition. L. R. Johnson, ed. Raven Press. New York.

Forsythe, S. J., and D. S. Parker. 1985. Nitrogen metabolism by the microbial flora of the rabbit caecum. J. Appl. Bacteriol. 58:363-369.

Franco, L. D., M. Fondevila, M. B. Lobera and C. Castrillo. 2005. Effect of combinations of organic acids in weaned pig diets on microbial species of digestive tract contents and their response on digestibility. J. Anim. Physiol. Anim. Nutr. 89:88-93.

Frank, K. 1994. Measures to preserve food and feeds from bacterial damage. Übersichten zur Tierernähr. 22:149-163

Franklin, M. A., A. G. Mathew, J. R. Vickers, and R. A. Clift. 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.

Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Shut, and G. W. Welling. 1998. Variations of bacterial populations in human feces measured by fluorescence in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 64:3336-3345.

Fraser, D., B. N. Milligan, E. A. Pajor, P. A. Philips, A. A. Taylor, and D. M. Weary. 1998. Behavioural perspectives on weaning in domestic pigs. Pages 121-138 in : Progress Pig

Science, J. Wiseman, M. A. Varley, and P. Chadwik, eds. Nottingham University Press, Nottingham, UK.

Freitas, M., L. G. Axelsson, C. Cayuela, T. Midtvedt, and G. Trugnan. 2002. Microbial-host interactions specifically control the glycosylation pattern in intestinal mouse mucosa. Histochemi. Cell Biol. 118:149-161.

Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. J. Food Prot. 65:1545-1560.

Fukushima, H., Y. Tsunomori, and R. Seki. 2003. Duplex real-time SYBR green PCR assays for detection of 17 species of food or waterborne pathogens in stools. J. Clin.Microbiol. 41:5134-5146.

Fuller, M. F., and P. J. Reeds. 1998. Nitrogen cycling in the gut. Ann. Rev. Nutr. 18:385.

Fuller, R., L. G. M. Newland, C. A. E. Briggs, R. Braude, and K. G. Mitchell. 1960. The normal intestinal flora of the pig. IV. The effect of dietary supplements of penicillin, chloratetracycline or copper sulphate on the faecal flora. J. Appl. Bacteriol. 23:195-205.

Furham, J. A., K. McCallum, and A. A. Davis. 1992. Novel major archebacterial group from marine plankton. Nature 356:148-149.

Galfi, P., and J. Bokori. 1990. Feeding trial in pigs with a diet containing sodium n-butyrate. Acta Vet. Hung. 38:3-17.

García-Lafuente, A., M. Antolín, F. Guarner, E. Crespo and J-R. Malagelada. 2001. Modulation of colonic barrier function by the composition of the commensal flora in the rat. Gut 48:503-507.

Gardiner, G., C. Stanton, P. B. Lynch, J. K. Collins, G. Fitzgerald, and R. P. Ross. 1999. Evaluation of cheddar cheese as a food carrier for delivery of a probiotic strain to the gastrointestinal tract. J. Dairy Sci. 82:1379-1387.

Gaskins, H. R. and K. W. Kelley. 1995. Immunology and neonatal mortality. In: The neonatal pig: development and survival. M. A. Varley, ed. CAB International, Wallingford, UK.

Gaskins, H. R. 2001. Intestinal bacteria and their influence on swine growth. In: Swine nutrition, 2nd edition. A. J. Lewis and L. Lee Southern, eds. CRC Press, USA.

Gaskins, H. R., C. T. Collier and D. B. Anderson. 2002. Antibiotics as growth promotants: mode of action. Anim. Biotechnol. 13:29-42.

Gaskins, H. R. 2003. Intestinal bacteria and their influence on swine growth. In: Swine Nutrition 2nd ed. A. J. Lewis and L. Lee Southern, eds. CRC Press. NY.

Geary, T. M., P. H. Brooks, T. Morgan, A. Campbell, and P. J. Russel. 1996. Performance of weaner pigs fed ad libitum with liquid feed at different dry matter concentrations. J. Sci. Food Agric. 72:17-24.

Gedek B, F. X. Roth, M. Kirchgessner, S. Wiehler, A. Bott, and U. Eidelsburger. 1992. Influence of fumaric acid, hydrochloric acid, sodium formate, tylosin and toyocerin on the microflora in different segments of the gastrointestinal tract. 14. Nutritive value of organic acids in piglet rearing. J. Anim. Physiol. Anim. Nutr.68:209-217.

Gedek B., M. Kirchgessner, S. Wiehler, A. Bott, U. Eidelsburger, and F. X. Roth. 1993. The nutritive effect of *Bacillus cereus* as a probiotic in the raising of piglets. 2. Effect and microbial count, composition and resistance determination of gastrointestinal and fecal microflora. Arch Tierernahr. 44:215-226 (abs).

Gherna, R., and C. R. Woese. 1992. A partial phylogenetic analysis of the "Flavobacter-Bacteroides" phylum: basis for taxonomic restructuring. Syst. Appl. Microbiol. 15:513-521.

Gianella, R. A. 1983. *Escherichia coli* best stable enterotoxin: Biochemical and physiological effects. Proc. Food Nutr. Sci. 7:147-153.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Giovanonni, S. J., E. F. DeLong, G. J. Olson, and N. R. Pace. 1988. Phylogenetic group-specific oligodeosynucleotide probes for identification of single microbial cells. J. Bacteriol. 170:720-726.

Gleed P. T., and B. F. Sansom. 1982. Ingestion of iron in sow's faeces by piglets reared in farrowing crates with slotted floors. Br. J. Nutr. 47:113-117.

Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K. H. Schleifer. 1996. An in situ hybridization protocol for detection and identification of planctonic bacteria. Syst. Appl. Microbiol. 19:403-406.

Gokarn, R. R., M. A. Eitnan, S. A. Martin, and K. E. Eriksson. 1997. Production of succinate from glucose, cellobiose, and various cellulosic materials by the ruminal anaerobic bacteria *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. Appl. Biochem. Biotechnol. 68:69-80.

Gong, J., R. J. Forster, H. Yu, J. R. Chambers, P. M. Sabour, R. Wheatcroft, S. Chen. 2003. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiol. Lett. 208:1-7

Graham H., K. Hesselman, P. Aman. 1986. The infuence of wheat bran and sugar-beet pulp on the digestibility of dietary components in a cereal-based pig diet. J. Nutr. 116:242-251.

Grela, E. R., R. Krusinska, and J. Matras. 1998. Efficacy of diets with antibiotic and herb mixture additives in feeding of growing-finishing pigs. J. Anim. Feed Science 7:171-175.

Griffiths, E., and R. S. Gupta. 2001. The use of signature sequences in different proteins to determine the relative branching order of bacterial divisions: evidence that Fibrobacter diverged at a similar time to Chlamydia and the Cytophaga-Flavobacterium-Bacteroides division. Microbiol. 147:2611-2622.

Guarner, F., and J-R. Malagelada. 2003. Gut flora in health and disease. Lancet 361:512-569.

Gubert, V. M., and W. C. Sauer. 1995. The effect of fumaric acid and sodium fumarate supplementation to diets for weanling pigs on amino acid digestibility and volatile fatty acid concentrations inileal digesta. Anim. Feed Sci. Technol. 53:243-254.

Gueimonde, M., S. Tölkkö, T. Korpimäki, and S. Salminen. 2004. New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. Appl. Environ. Microbiol. 70:4165-4169.

Gustaffson, B. E. 1959. Vitamin K deficiency in germfree rats. Ann. N. Y. Acad. Sci. 78:166-173.

Gustaffson, B. E., T. Midvedt, and K. Strandberg. 1970. Effects of microbial contamination on the cecum enlargement of germ-free rats. Scand. J Gastroenterol. 5:309-314.

Hahn, J. D., and D. H. Baker. 1993. Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. J. Anim. Sci. 71:3020-3024.

Haller D., P. Serrant, G. Peruisseau, C. Bode, W. P. Hammes, E. Schiffrin and S. Blum. 2002 IL-10 producing CD14low monocytes inhibit lymphocyte-dependent activation of intestinal epithelial cells by commensal bacteria. Microbiol. Immunol. 46:195-205.

Hammer, K. A. , C. F. Carson, T. V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86:985-990.

Hampson D. J. 1986. Attempts to modify changes in the piglet small intestine after weaning. Res.Vet. Sci. 40:313-317.

Hampson, D. J., J. R. Pluske and D. W. Pethick. 2001. Dietary manipulation of enteric disease. Pig News Inform. 22:21-28.

Han, I. K., S. C. Lee, J. H. Lee, J. D. Kim, P. K. Jung, and J. C. Lee. 1984. Studies on the growth promoting effects of probiotics II. The effect of *Clostridium butyricum* ID on the performance and changes in the microbial flora of the faeces and intestinal contents of the broiler chicks. Korean J. Anim. Sci. 26:159-165.

Han, Y. M., K. R. Roneker, W. G. Pond, and X. G. Lei. 1998. Adding wheat middlings, microbial phytase, and citric acid to corn-soybean meal diets for growing pigs may replace inorganic phosphorus supplementation. J. Anim. Sci. 76:2649-2656.

Hardy, B. The issue of antibiotic use in the livestock industry: what have we learned? 2002. Anim. Biotechnol. 13:129-147.

Hardy, D., D. Amsterdam, L. A. Mandell, and C. Rotstein. 2000. Comparative in vitro activities of ciprofloxacin, gemifloxacin, grepafloxacin, moxifloxacin, ofloxacin, sparfloxacin, trovafloxacin, and other antimicrobial agents against bloodstream isolates of gram-positive cocci. Antimicrob. Agents. Chemother. 44:802-805.

Harmsen, H. J., G. R. Gibson, P. Elfferich, G. C. Raangs, A. Wildeboer-Veloo, M. B. Roberfroid and G. W. Welling. 1999. Comparison of viable cell counts and fluorescence in

situ hybridization using specific rRNA-based robes for the quantification of human fecal bacterial. FEMS Microbiol. Lett. 183:125-129.

Harmsen, H. J., A. C. M. Wildeboer-Veloo, H. C. Raangs, A. A. Wagendorp, N. Klijn, J. G. Bindels, and G. W. Welling. 2000a. Analysis of intestinal flora development in breast-fed and formula-fed infants by sing molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. 30:61-67.

Harmsen, H. J. M., A. C. Wildeboer-Veloo, J. Grijpstra, J. Knol, J. E. Degener and G. W. Welling GW. 2000b. Development of 16S rRNA-based probes for the Coriobacterium group and the Atopobium cluster and their application for enumeration of Coriobacteriaceae in human feces from volunteers of different age groups. Appl. Environ. Microbiol. 66:4523-4527.

Harmsen, H. J. M., G. C. Raangs, T. He, J. E. Degener, and G. W. Welling. 2002. Extensive set of 16S rRNA-based probes for detection in human feces. Appl. Environ. Microbiol. 68:2982-2990.

Havenaar, R., and M. J. H. Huis In't Veld. 1992. Probiotics : a general view. In : Lactic Acid Bacteria in health and disease. Vol. 1. Elsevier Applied Science Publishers, Amsterdam.

Hebeler, S. D., S. Kulla, F. Winkenwerder, J. Kamphues, J. Zentek, and G. Amtsberg. 2000. Hannover 54th Proc. Soc. Nutr. Physiol. 7-3-2000. Gottingen, Germany.

Hein, I., A. Lehner, P. Rieck, K. Klein, E. Brandl, and M. Wagner, 2001. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol. 67:3122-3126.

Held, S., and M. Mendl. 2001. Behaviour of the young weaned pig. In: The weaner pig: nutrition and management. M. A. Varley and J. Wiseman, eds. CABI Publishing, NY, USA.

Hill, G. M., G. L. Cromwell, T. D. Crenshaw, R. C. Ewan, D. A. Knabe, A. J. Lewis, D. C. Mahan, G. C. Shurson, L. L. Southern, and T. L. Veum. 1996. Impact of pharmacological intakes of zinc and/or copper on performance of weanling pigs. J. Anim. Sci. 74:181 (abs.).

Hill, M. J. 1997. Intestinal flora and endogenous vitamin synthesis. Eur.J. Cancer Prev. 6:S43-S45.

Hill, G. M., G. L. Cromwell, T. D. Crenshaw, C. R. Dove, R. C. Ewan, D. A. Knabe, A. J. Lewis, G. W. Libal, D. C. Mahan, G. C. Shurson, L. L. Southern, and T. L. Veum. 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 J. E., S. M. Hemmingsen, B. G. Goldade, T. J. Dumonceaux, J. Klassen, R. T. Zijlstra, S. H. Goh, A. G. Van Kessel. 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.

Hillman K., R. J. Spencer, T. A. Murdoch, and C. S. Stewart. 1995. The effect of mixtures of *Lactobacillus* spp. on the survival of enterotoxigenic *Escherichia coli* in in vitro continuous culture of porcine intestinal bacteria. Lett. Appl. Microbiol. 20:130-133.

Hillman, K. 2001. Bacteriological aspects of the use of antibiotics and their alternatives in the feed of non-ruminant animals. In: Recent Advances in Animal Nutrition. P. C. Garnsworthy and J. Wiseman, eds. Notthingam University Press, Notthingam.

Hiyoshi M, and S. Hosoi. 1994. Assay of DNA denaturation by polymerase chain reaction-driven fluorescent label incorporation and fluorescence resonance energy transfer. Anal Biochem. 221:306-11.

Hobbie, J. E., R. J. Daley and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Högberg, A., J. E. Lindberg, T. Leser and P. Wallgren. 2004. Influence of cereal non-starch polysaccharides on ileo-caecal and rectal microbial populations in growing pigs. Acta Vet. Scand.45:87-98.

Höjberg, O., N. Canibe, H. D. Poulsen, M. S. Hedemann, and B. B. Jensen. 2005. Influence of dieary zinc oxide and copper sulphate on the gastrointestinal ecosystem in newly weaned piglets. Appl. Environ. Microbiol. 71:2267-2277.

Hold, G. L., A. Schwiertz, R. I. Aminov, M. Blaut, and H. Flint. 2003. Oligonucleotide probes that detect quantitatively significant groups of butyrate-producing bacteria in human feces. Appl. Environ. Microbiol. 69:4320-4324.

Holden, P. J., J. McDean, and E. Franzenburg. 1998. Botanicals for pigs- Garlic. 1998 ISU Swine Research Report. Iowa State University, Ames, USA.

Holden, P. J., and J. McKean. 2000. Botanicals for pigs- Garlic II. 2000 ISU Swine Research Report. Iowa State University, Ames, USA.

Holland, P. M., R. D. Abramson, R. Watson, D. H. Gelwand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceedings of the National Academy of Sciences USA 88:7276-7280.

Holm, A., and H. D. Poulsen. 1996. Zinc oxide in treating *E. coli* diarrhea in pigs after weaning. Compend. Contin. Edu. Pract. Vet. 18:26-29.

Hooper, L. V., L. Bry, P. G. Falk, and J. I. Gordon. 1998. Host-microbial symbiosis in the mammalian intestine: exploiring an intestinal ecosystem. BioEssays 20:336-343.

Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon. 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science 291:881-884.

Hooper, L. V., and J. I. Gordon. 2001. Commensal host bacterial relationships in the gut. Science 1115-1118

Hooper, L. V. 2004. Bacterial contributions to mammalian gut development. Trends Microbiol. 12:21-28.

Hopwood, D. E. and D. J. Hampson. 2003. Interactions between the intestinal microflora, diet and diarrhoea, and their influences on piglet health in the immediate post-weaning period. In: Weaning the pig. Concepts and consequences. J. R. Pluske, J. LeDividich and M. W. A. Verstegen, eds. Wageningen Academic Publishers, The Netherlands.

Hopwood, D. E., D. W. Pethick, and D. J. Hampson. 2002. Increasing the viscosity of the intestinal contents stimulates proliferation of enterotoxigenic *Escherichia coli* and *Brachyspira pilosicoli* in weaner pigs. Br. J. Nutr. 88:523-532.

Horton, G. M. J., D. B. Blethen, and B. M. Prasad. 1991. The effect of garlic (*Allium sativum*) on feed palatability of horses and feed consumption, selected performance and blood parameters in sheep and swine. Can. J. Anim. Sci. 71:607 (abs).

Hoskins, L. C., M. Agustines, W. B. McKee, E. T. Boulding, M. Driaris, and G. Niedermeyer. 1985. Mucin degradation in human colon ecosystems. J. Clin. Invest. 75:944-953.

Hoskins, L. C., E. T. Boulding, and T. A. Gerken. 1992. Mucin glycoprotein degradation by mucin oligosaccharide degrading strains of human fecal bacteria. Microb. Ecol. Health. Dis. 5:193-207.

Houdijk, J. G. M., R. Hartemink, K. M. J. Van Laere, B. A. Williams, M. W. Bosch, M. W. A. Verstegen, and S. Tamminga. 1997. Fructooligosaccharides and transgalacto-oligosaccharides in weaner pigs' diets. In: Proceedings of the International Symposium of Non-digestible Oligosaccharides "Healthy Food for the Colon"? Wageningen, Nederlands.

Houdijk, J. G. M., M. W. Bosch, M. W. A. Verstegen, and H. J. Berenpas. 1998. Effects of dietary oligosaccharides on the growth performance and faecal characteristics of young growing pigs. Anim. Feed Sci. Technol. 71:35-48.

Hozapfel, W. H., P. Haberer, R. Geisen, J. Bjorkroth, and U. Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J Clin. Nutr. 73(suppl):365-373.

Huijsdens, X. W., R. K. Linskens, M. Mak, S. G. M. Meuwissen, C. M. J. E. Vandenbroucke-Grauls, and P. H. M. Savelkoul. 2002. Quantification of bacteria adherent of gastrointestinal mucosa by real-time PCR. J. Clin. Microbiol. 40:4423-4427.

Huis in't Veld, J. H. J. and R. Havenaar. 1993. Selection criteria for microorganisms for probiotic use. In Prevention and control of potentially pathogenic microorganisms in poultry and poultry meat processing. J. F. Jensen, M. H. Hinton and R. W. A. W. Mulder, eds. The Netherlands.

Huseby, E., P. M. Hellstrom, and T. Midtvedt. 1994. Intestinal microflora stimulates myoelectric activity of rat intestinal wall by promoting cyclic initiation and aboral propagation of migrating myoelectric complexes. Dig. Dis. Sci. 39:946-956.

Ibevkwe, A. M., and C. M. Grieve. 2003. Detection and quantification of *E. coli* O157:H7 in environmental samples by real-time PCR. J. Appl. Microbiol. 94:421-431.

Iji, P. A., A. A. Saki, and D. R. Tivey. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. J. Sci. Food Agric. 81:1138-1192.

Ilver, D., A. Arnqvist, J. Pgren, I. M. Frick, D. Kersulyte, E. T. Incecik, D. E. Berg, A. Covacci, L. Engstrand, and T. Boren. 1998. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science 279:373-377.

Inoue, R., T. Tsukahara, N. Nakanishi, and K. Ushida. 2005. Development of the intestinal microbiota in the piglet. J. Gen. Appl. Microbiol. 51:257-265.

Jansen, G. J., A. C. M. Wildeboer-Veloo, R. H. J. Tonk, A. H. Franks, and G. W. Welling. 1999. Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. J. Microbiol. Methods 37:215-221.

Jaskari, J., P. Kontula, A. Siitonen, H. Jousimies-Somer, T. Mattila-Sandholm, and K. Poutanen. 1998. Oat beta-glucan and xylan hydrolysates as selective substrates for *Bifidobacterium* and *Lactobacillus* strains. Appl. Environ. Microbiol. Biotechnol. 49:175-181.

Jensen, B. B. and H. Jorgensen. 1994. Effect of dietary fibre on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. Appl. Environ. Microbiol. 60:1897-1904.

Jensen, M.T., R. P. Cox and B. B. Jensen. 1995. Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. Anim. Sci. 61:293-304.

Jensen, B. B. 1998. The impact of feed additives on the microbial ecology of the gut in young pigs. J. Anim. Feed Sci. Technol. 89:175-188.

Jensen, B. B., N. Agergaard, L. L. Hansen, L. L. Mikkelsen, M. T. Jensen and A. Laue. 1998. Effect of liquid feed on microbial production of skatole in the hind gut, skatole absorption to portal vein blood and skatole deposition in back fat. In: Skatole and Boar taint. W. K. Jensen, ed. Danish Meat Research Institute, Roskilde.

Jensen, B. B., and L. L. Mikkelsen. 1999. Feeding liquid diets to pigs. In: Recent Advances in Anim. Nutrition. P. C. Garnsworthy and J. Wiseman, eds. Nottingham University Press, Nottingham, UK.

Jensen, B. B. 2001. Possible ways of modifying type and amount of products from microbial fermentation in the gut. In: Gut Environment of Pigs. Piva, A., Bach Knudsen, K. E. and Lindberg J. E., eds. Nottingham University Press, United Kingdom.

Jensen, B. B., O.Höjberg, L. L. Mikkelsen, M. S. Hedemann, and N. Canibe. 2003. Enhancing intestinal function to threat and prevent intestinal disease. In: 9th International Symposium on Digestive Physiology of Pigs, Alberta, Canada.

Jensen, T. K., K. Moller, M. Boye, T. D. Leser, and S. E. Jorsal. 2000. Scanning electron microscopy and fluorescent in situ hybridization of experimental Brachyspira (Serpulina) pilosicoli infection in growing pigs. Vet.Pathol. 37:22-32.

Jensen-Waern M., L. Melin, R. Lindberg, A. Johannisson, L. Petersson, and P. Wallgren 1998. Dietary zinc oxide in weaned pigs -effects on performance, tissue concentrations, morphology, neutrophil functions and faecal microflora. Res. Vet. Sci. 64:225-231.

Johnson, R. 1992. Role of acidifiers and enzymes in assuring performance and health of pigs post-weaning. In Biotechnology in the Feed industry. Proc. Alltech's 8th annual symposium, Alltech Technical Publications, Nicholasville, KY (abs).

Jürgens, K., J. Pernthaler, S. Schalla, and R. Amann. 1999. Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. Appl. Environ. Microbiol. 65:1241-1250.

Kaplan, C. W., J. C. Astaire, M. E. Sanders, B. S., Reddy, and C. L. Kitts. 2001. 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. 67:1935-1939.

Kaplan, H., and R. W. Hutkins. 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl. Environ. Microbiol. 66:2682-2684.

Karlsson, K. A. 1989. Animal glycosphingolipids as membrane attachment sites for bacteria. Ann. Rev. Biochem. 58:309-350.

Katouli M., A. Lund A, P. Wallgren, I. Kuhn, O. Soderlind, and R. Mollby. 1995. Phenotypic characterization of intestinal *Escherichia coli* of pigs during suckling, postweaning, and fattening periods. Appl. Environ. Microbiol. 61:778-783.

Katouli M., A. Lund, P. Wallgren, I. Kuhn, O. Soderlind, and R. Mollby. 1997. Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. J. Appl. Microbiol. 83:147-154.

Katouli, M., L. Melin, M. Jensen-Waern, P. Wallgren and R. Möllby. 1999. The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. J. Appl. Microbiol. 87:564-573.

Kaufmann L. and P. J. Rousseeuw. 1990. Finding groups in data. An introduction to cluster analysis. John Wiley & Sons. New York.

Kelly, D., and T. P. King. 1991. The influence of lactation products on the temporal expression of histo-blood group antigens in the intestines of suckling pigs: lectin histochemical and immunohistochemical analysis. Histochem. J. 23:55-60

Kelly, D. and T. P. King. 1994. Luminal bacteria: regulation of gut function and immunity. In: Gut Environment of pigs. A. Piva, K. E. Bach Knudsen, and J. E. Lindberg, eds. Nottingham University Press, UK.

Kelly, D., R. Begbie, and T. P. King. 1994. Nutritional influences on interactions between bacteria and the small intestinal mucosa. Nutr. Res. Rev. 7:233-257.

Kelly, D., and T. P. King. 2001. Digestive physiology and development in pigs. In The Weaner Pig. Nutrition and Management. M. A. Varley and J. Wiseman, eds. CABI Publishing, U.K.

Kelly, D., S. Conway, and R. Aminov. 2005. Commensal gut bacteria mechanisms of immune modulation. Trends Immunol. 26:326-333.

Khaddour, R., C. A. Reid, and K. Hillman, 1998. Maintanance in vitro of the microflora and fermentation patterns of the porcine intestine. Pig News and Information 19:111N-114N.

Khan, A. A., M. S. Nawaz, L. Robertson, S. A. Khan, and C. E. Cerniglia. 2001. Identification of predominant human and animal intestinal bacterial species by terminal restriction fragments patterns (TRFPs): a rapid, PCR based method. Mol. Cell. Probes 15:349-355.

Kindon, H., C. Pothoulakis, L. Thim, K. Lynch-Devaney, and D. K. Podolski. 1995. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. Gastroenterology 109:516-523.

King, T. P., R. Begbie, R. Spencer and D. Kelly. 1993. Diversity of membrane sialo-glycoconugates in the developing porcine small intestine. Proceedings of the Nutrition Society 52:195.

King, T. P. 1995. Lectin cytochemistry and intestinal epithelial cell biology. In Lectins: Biomedical perspectives. A. Putzai, and S. Bardozc, eds. Taylor and Francis, London, UK.

Kirchgessner, M., R. X. Roth, U. Eidelsburger and B. Gedek. 1993. The nutritive efficiency of Bacillus cereus as a probiotic in the raising of piglets. 1. Effect on the growth parameters and gastrointestinal environment. Arch. Tierernahr. 44:111 (abs).

Kirkwood, R. N., S. X. Huang, M. McFall, and R. X. Aheren. 2000. Dietary factors do not influence the clinical expression of swine dysentery. Swine Health Prod. 8:73-76.

Kitts, C. L. 2001. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Curr. Issues Intest. Microbiol. 2:17-25.

Klein Gebbink, G. A. R., A. L. Sutton, B. A. Williams, J. A. Patterson, B. T. Richert, D. T. Kelly, and M. W. A. Verstegen. 2003. Effects of oligosaccharides in weanling pig diets on performance, microflora and intestinal health. 8th Digestive Physiology of Pigs, Alberta, Canada.

Knutton, S., D. R. Lloyd, D. C. A. Candy, and A. S. McNeish. 1985. Adhesion of enterotoxigenic Escherichia coli to human small intestinal enterocytes. Infect. Immun. 48:824-831.

Knutton, S., D. R. Lloyd, and A. S. McNeish. 1987. Identification of a new fimbrial structure in enterotoxigenic *Escherichia coli* (ETEC) serotypes 0148:HL 28 which adheres to human

intestinal mucosa: a potentially new human ETEC colonization factor. Infect. Immun. 55:86-92.

Konstantinov, S. R., W. Y. Zhu, B. A. Williams, S. Tamminga, W. M. de Vos, and A. D. L. Akkermans. 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.

Konstantinov, S. R., C. F. Favier, W. Y. Zhu, B. A. Williams, J. Klub, W. B. Souggrant, W. M. De Vos, A. D. L. Akkermans and H. Smidt. 2004a. Microbial diversity studies on the porcine gastrointestinal ecosystem during weaning transition. Anim. Res. 53:317-324.

Konstantinov, S. R., A. Awati, H. Smidt, B. A. Williams, A. D. L. Akkermans, and W. M. de Vos. 2004b. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotipe to dietary prebiotics in the guts of weaning piglets. Appl. Environ. Microbiol. 70:3821-3830.

Kopp E. B., and R. Medzhitov. 1999. The Toll-receptor family and control of innate immunity. Curr. Opin. Immunol. 11:13-18.

Kotarski S. F., and D. C. Savage. 1979. Models for study of the specificity by which indigenous lactobacilli adhere to murine gastric epithelia. Infect. Immun. 26:966-975.

Kowarz, M., F. Lettner, and W. Zollitsch. 1994. Use of a microbial growth promotor in feeding sows and piglets. Bodenkultur. 45:85 (abs).

Krause, D. O., R. A. Easter, B. A. White, and R. I. Mackie. 1995. Effect of weaning diet on the ecology of adherent lactobacilli in the gastrointestinal tract of the pig. J. Anim. Sci. 73:2347-2354.

Krogfelt K. A., L. K. Poulsen, and S. Molin. 1993. Identification of coccoid *Escherichia coli* BJ4 cells in the large intestine of streptomycin-treated mice. Infect. Immun. 61:5029-5034.

Kühn, I., M. Katouli, L. Melin, A. Lund, P. Wallgren, and R. Möllby. 1993. Phenotypic diversity and stability of the intestinal coliform flora in piglets during the first 3 months of age. Microb. Ecol. Health. Disease 6:101-107.

Kumprecht, I., and P. Zobac. 1998. The role of intestinal microflora in animal nutrition. In: Microbes and Biological Productivity, Society for General Microbiol. Symposium 21. D. E. Hughes and A. H. Rose, eds. Cambridge University Press, Cambridge, UK.

Kyriakis, S. C. 1989. New aspects of the prevention and/or treatment of the major stress induced diseases of the early weaned piglet. Pig News and Information 2:177-181.

Kyriakis, S. C., V. K. Tsiloyiannis, J. Vlemmas, K. Sarris, A. C. Tsinas, C. Alesopoulos, and L. Jansegers. 1999. The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. Res. Vet. Sci. 67:223-228.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. E. Stackebrandt and M. Goodfellow, eds. John Wiley & Sons, New York.

Langendijk, P. S., F. Schut, G. J. Jansen, G. C. Raangs, G. R. Kamphuis, M. H. F. Wilkinson, and G. W. Welling. 1995. Quantitative in situ hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in faecal samples. Appl. Environ. Microbiol. 61:3069-3075.

Lawson, P. A. 1999. Taxonomy and systematics of the predominant gut anaerobes. In: Colonic microbiota, nutrition and health. G. R. Gibson and M. R. Roberfroid, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Lay, C., L. Rigottier-Gois, K. Holmstrøm, M. Rajilic, E. E. Vaughan, W. M de Vos, M. D. Collins, R. Thiel, P. Namsolleck, M. Blaut, and J. Doré. 2005. Colonic microflora signatures across five northern european countries. Appl. Environ. Microbiol. 71:4153-4155.

Le Dividich, J., and P. Herpin. 1994. Effects of climatic conditions on the performance, metabolism and health status of weaned piglets: a review. Livest. Prod. Sci. 38:79-90.

Lee, A. 1984. Neglected Niches: the microbial ecology of the gastrointestinal tract. In: Advances in Microbial Ecology, K. Marshall, ed. Plenum Press. New York.

Lee, L. G., C. R. Connell, and W. Bloch. 1993. Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. 11:3761-3766.

Lee, Y. K., K. Nomoto, S. Salminen, S. L. Gorbach. 1999. Handbook of probiotics. New York. John Willey and Sons, Inc.

LeMieux, F. M., L. L. Southern and T. D. Bidner. 2003. Effect of mannan oligosaccharides on growth performance of weanling pigs. J. Anim. Sci. 81:2482-2487.

Lemke, M. J., C. J. McNamara, and L. G. Leff. 1997. Comparison of methods for the concentration of bacterioplancton for in situ hybridization. J. Microbiol. Methods 29:23-29.

Leser, T. D., R. H. Lindecrona, T. K. Jensen, B. B. Jensen, and K. Moller. 2000. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with *Brachyspira hyodisenteriae*. Appl. Environ. Microbiol. 66:3290-3296.

Leser, T. D., J. Z. Amenuvor, T. K. Jensen, R. H. Lindecrona, M. Boye, and K. Moller. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal microbiota revisited. Appl. Environ. Microbiol. 68:673-690.

Lievin, V., I. Peiffer, S. Hudault. 2000. Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut 47:646-652.

Lin, C., B. Flesher, W. C. Capman, R. I., and D. A. Stahl. 1994. Taxon specific hybridization probes for fiber-digesting bacteria suggest novel gut-associated Fibrobacter. Syst. Appl. Microbiol. 17:418-424.

Lindecrona, R. H., T. J. Jensen, B. B. Jensen, T. D. Leser, W. Jiufeng, and K, Moller. 2003. The influence of diet on the development of swine dysentery upon experimental infection. Anim. Sci.76:81-87.

Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining Terminal Restriction Fragment Length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516-4522.

Livak, K. J., S. J. Flood, J. Marmaro, W. Giusti, and K. Deetz. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4:357-362.

Lu, L., and W. A. Walker. 2001. Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. Am. J Clin. Nutr. 73:1124S-1130S.

Lueck, E. 1980. Antimicrobial Food Additives: Characteristics, uses, effects. Springer-Verlag Germany.

MacDonald, T. T. and P. B. Carter. 1979. Requirement for a bacterial flora before mice generates cells capable of mediating the delayed hypersensitivity reaction to sheep red blood cells. J. Immunol. 122:2426-2429.

MacFarlane, G. T., G. R. Gibson, E. Beatty and J. H. Cummings. 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched – chain fatty acid measurements. FEMS Microbiol. Ecol. 101:81-88.

MacFarlane, G. T., and J. H. Cummings. 1991. The colonic flora, fermentation, and large bowel digestive function. In: Phillips, S. F. Pemberton, J. H. Shorter, R. G., eds. The Large Intestine: Physiology, Patophysiology and Disease. Raven Press, New York.

MacFarlane, S., and G. T. MacFarlane. 1995. Proteolysis and amino acid fermentation. In: Human colonic bacteria: role in nutrition, physiology and pathology. G. R. Gibson and G. T. MacFarlane, eds. CRC Press, Boca Raton, FL.

MacFarlane, G. T. and A. J. McBain. 1999. The human colonic microflora. In: Colonic microflora, nutrition and health. G. R. Gibson and M. B. Roberfroid, eds. Kluwer Academic Publishers, The Netherlands.

MacFarlane, S., J. H. Cummings and G. T. MacFarlane. 1999. Bacterial colonisation of surfaces in the Large Intestine. In: Colonic microbiota, nutrition and health. G. R. Gibson and M. B. Roberfroid, eds. Kluwer Academic Publishers, UK.

Mackie R. I., A. Sghir, and H. R. Gaskins 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. 69:1035S-1045S.

Macpherson AJ, Geuking MB, McCoy KD. 2005. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology 115:153-162.

Mahan, D. C., S. D. Carter, G. C. Cromwell, G. M. Hill, R. L. Harrold, A. J. Lewis, and T. L. Veum. 2000. Efficacy of added zinc oxide levels with and without an antibacterial agent in the postweaning diets of pigs. J. Anim. Sci. 78:61 (abs.).

Makkar, H. P. S. and K. Becker. 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. Br. J. Nutr. 66:313-329.

Malinen, E., A. Kassinen, T. Rinttilä and A. Palva. 2003. Comparison of real-time PCR with SYBR Green I or 5' nuclease assays and dot-blot hybridization with rDNA targeted oligonucleotide probes in quantification of selected faecal bacteria. Microbiol. 149:269-277.

Mancini-Filho J., A. Van-Koiij, D. A. Mancini, F. F. Cozzolino, and R. P. Torres. 1998. Antioxidant activity of cinnamon (Cinnamomum Zeylanicum, Breyne) extracts. Boll. Chim. Farm. 137:443-447.

Mantle, M., and G. Stewart. 1989. Disulphide bonds and the 118,000 glycoprotein of human intestinal mucin. Symp. Soc. Exp. Biol.43:279-287.

Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Shleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiol. 142:1097-1106.

Manzanilla, E. G., J. F. Pérez, M. Martín, C. Kamel, F. Baucells, and J. Gasa. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. J. Anim. Sci. 82:3210-3218.

Manzanilla, E. G., M. Nofrarias, M. Anguita, M. Castillo, J. F. Pérez, S. M. Martín-Orúe, C. Kamel and J. Gasa. 2006. Effects of butyrate, avilamycin, and a combination of plant extracts on the intestinal equilibrium of early-weaned pigs. Submitted to the Journal of Animal Science.

Marchesi, J., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, D. Dymock, and W. G. Wade, 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64 :795-799.

Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. Curr.Opin. Microbiol. 2:323-327.

Martinez-Puig, D., J. F. Pérez, M. Castillo, A. Andaluz, M. Anguita, and J. Gasa. 2003. Consumption of Raw Potato Starch Increases Colon Length and Fecal Excretion of Purine Bases in Growing Pigs. J. Nutr. 133:134-139.

Martinez-Puig, D., M. Castillo, M. Nofrarias, E. Creus, and J. F. Pérez. 2006. Long -term effects of feeding large amounts of resistant starch on the digestive tract of growing pigs. Submitted to the J. Sci. Food Agric.

Mathew, A. G., A. L. Sutton, A. B. Scheidt, D. M. Forsyyth, J. A. Patterson, and D. T. Kelly. 1991. Effects of a propionic acid containing feed additive on performance and intestinal microbial fermentation of the weanling pig. In: Proc. 6th Int. Symp. on Digestive Physiology in Pigs. Wageningen, The Netherlands.

Mathew, A. G., A. L. Sutton, A. B. Scheidt, J. A. Patterson, D. T. Kelly and K. A. Meyerholtz. 1993. Effect of galactan on selected microbial populations in the ileum of the weanling pig. J. Anim. Sci. 71:1503-1509.

Mathew, A. G., M. A. Franklin, W. G. Upchurch, and S. E. Chattin. 1996. Effect of weaning on ileal short-chain fatty acid concentrations in pigs. Nutr. Res. 16:1689-1698.

Mathew, A. G., S. E. Chattin, C. M. Robbings, and D. A. Golden. 1998. Effects of a direct-fed yeast culture on enteric microbial populations, fermentation acids, and performance of weanling pigs. J. Anim. Sci.76:2138-2145.

Matsuki, T., K. Watanabe, and R. Tanaka. 2003. Genus- and species-specific PCR primers for the detection and identification of bifidobacteria. Curr. Issues Intest. Microbiol. 4:61-69.

Matsuo, K., H. Ota, T. Akamatsu, A. Sugiyama and T. Katsuyama. 1997. Histochemistry of the surface mucous gel layer of the human colon. Gut 40:782-789.

Matzinger P. 1998. An innate sense of danger. Semin. Immunol. 10:399-415.

Maxwell, C.V., and S.D. Carter. 2001. Feeding the weaned pig. In: Swine Nutrition. A.J.Lewis and L.L. Southern eds., CRC Press, Boca Raton, FL.

May, T., R. I. Mackie, G. C. Fahey, J. C. Cremin and K. A. Garleb. 1994. Effect of fiber soured on short-chain fatty acid production and on the growth and toxin production by *Clostridium difficile*. Scand. J. Gastroenterol. 29:916 (abs).

McBurney, M. I., and W. C. Sauer. 1993. Fiber and large bowel energy absorption: validation of the integrated ileostomy-fermentation using pigs. J. Nutr. 123:721-727.

McCartney, A. L. 2002. Application of molecular biological methods for studying probiotics and the gut flora. Br. J. Nutr. 88:S29-S37.

McCartney, E. 2005. Countdown to 2006. EU considers AGP alternatives. Feed International, April 2005:6-10.

McCracken B. A., H. R. Gaskins, P. J. Ruwe-Kaiser, K. C. Klasing, and D. E. Jewell. 1995. Diet-dependent and diet-independent metabolic responses underlie growth stasis of pigs at weaning. J. Nutr. 125:2838-2845.

McCracken B. A., M. E. Spurlock, M. A. Roos, F. A. Zuckermann, and H. R. Gaskins. 1999. Weaning anorexia may contribute to local inflammation in the piglet small intestine. J. Nutr. 129:613-619.

McCracken, V. J., J. M. Simpson, R. I. Mackie, and H. R. Gaskins. 2001. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J. Nutr. 131:1862-1870.

McDonald, D. E., J. R. Pluske, D. W. Pethick, and D. J. Hampson. 1997. Interactions of dietary nonstarch polysaccharides with weaner pig growth and post-weaning colibacillosis. In: Manipulating Pig Production VI. P. D. Cranwell, ed. Australasian Pig Science Association. Victoria, Australia.

McDonald, D. E., D. W. Pethick, J. R. Pluske, and D. J. Hampson. 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, D. E., D. W. Pethick, B. P. Mullan, and D. J. Hampson. 2001. Increasing viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulate proliferation of enterotoxigenic *Escherichia coli* in newly-weaned pigs. Br. J. Nutr. 86:487-498.

McFarland, S. P. 1998. The influence of dietary starches on the microflora and fermentation pattern of the porcine colon. M. Sc. Thesis, SAC/University of Aberdeen.

McFarlane, S., J. H. Cummings, and G. T. Marcfarlane. 1999. Bacterial colonisation of surfaces in the large intestine. In: Colonic microbiota, nutrition and health. G. R. Gibson and M. B. Roberfroid, eds. Kluwer Academic Publishers, The Netherlands.

McNamara, D. J., A. Prosa, and T. A. Miettinen. 1981. Thin layer and gas liquid chromatographic identification of neutral steroids in human and rat feces. J. Lipid Res. 22:474-484.

McOrist, A., M. Jackson, and A. L. Bird. 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods 50:131-139.

Melin L., M. Jensen-Waern, A. Johannisson A, M. Ederoth, M. Katouli, and P. Wallgren. 1997. Development of selected faecal microfloras and of phagocytic and killing capacity of neutrophils in young pigs. Vet. Microbiol. 54:287-300.

Melin L., M. Katouli, A. Lindberg, C. Fossum, and P. Wallgren. 2000. Weaning of piglets. Effects of an exposure to a pathogenic strain of *Escherichia coli*. J. Vet. Med. B Infect. Dis. Vet. Public Health 47:663-675.

Melin, L. 2001. Weaning of pigs with special focus on the intestinal health. Doctoral thesis. Acta Universitatis Agriculturae Sueciae Veterinaria. 112. Swedish University of Agricultural Sciences. Sweden.

Melin, L., S. Mattsson, M. Katouli, and P. Wallgren. 2004. Development of post-weaning diarrhoea in piglets. Relation to presence of *Escherichia coli* strains and rotavirus. J. Vet. Med. 51:12-22.

Mellor, S. 2000. Herbs and spices promote health and growth. Pig Progress, 16:27-30.

Metchinkoff, E. 1908. The prolongation of life. G. P. Putnam, ed. G. Putnam's Sons, New York.

Michalet-Doreau, B., I. Fernandez, C. Peyron, L. Millet and G. Fonty. 2001. Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. Repr. Nutr. Dev. 41:187-194.

Midvedt, T. 1974. Microbial bile acid transformation. Am. J. Clin. Nutr. 27:1341-1347.

Midvedt, T. 1989. Monitoring the functional state of the microflora. In Recent Advances in Microbial Ecology. T. Hattori, Y. Ishida, et al., eds. Japan Science Society Press. Tokyo.

Miguel J. C., S. L. Rodriguez-Zas, and J. E. Pettigrew. 2004. Efficacy of a mannan oligosaccharide (Bio-Mos) for improving nursery pig performance. J. Swine Health and Prod. 12:296-307.

Miguel, J. C., S. L. Rodríguez-Zas, and J. E. Pettigrew, J. E. 2003. Practical response to Bio-Mos® in nursery pigs: a meta-analysis. Pages 425-433 in: Nutritional Biotechnology in the Feed and Food Industries. Proceedings of Alltech's Eighteenth Annual Symposium. T. P. Lyons and K. A. Jacques, Eds., Nottingham University Press, U. K.

Mikkelsen, L. L., M. Jakobsen, and B. B. Jensen. 2003. Effects of dietary oligosaccharides on microbial diversity and fructo-oligosaccharide degrading bacteria in faeces of piglets post-weaning. Anim. Feed Sci. Tech. 109:133-150.

Miller, B. G., P. S. James, M. W. Smith and F. J. Bourne. 1986. Effect of weaning on the capacity of the pig intestinal villi to digest and absorb nutrients. Journal of Agricultural Science, Cambridge 10:579-589.

Molis, C., B. Flourié, F. Ouarne, M. Gailing, S. Lartigue, A. Guibert, F. Bornet, and J. P. Galmiche. 1996. Digestion, excretion, and energy value of fructooligosaccharides in healthy humans. Am. J. Clin. Nutr. 64:324-328.

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

Moore, R. J., E. T. Kornegay, R. L. Grayson and M. D. Lindemann. 1988. Growth, nutrient utilization and intestinal morphology of pigs fed high-fiber diets. J. Anim. Sci. 66:1570-1579.

Moore, W. E., and L. Holdeman. 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. 27:961-979.

Moore, W. E. C., L. V. H. Moore, E. P., Cato, T. D. Wilkins and E. T. Kornegay. 1987. Effect of high-fiber and high-oil diets on the fecal flora of swine. Appl. Environ. Microbiol. 53:1638-1644.

Morales, J., J. F. Pérez, S. M. Martín-Orúe, M. Fondevila, and J. Gasa. 2002. Large bowel fermentation of maize or sorghum-acorn diets fed as different source of carbohydrates to Landrace and Iberian pigs. Br. J. Nutr. 88:489-497.

Moran, C. A., G. Ward, J. D. Beal, A. Campbell, P. H. Brooks, and B. G. Miller. 2000. Influence of liquid feed, fermented liquid feed, dry feed and sow's milk fed ad libitum on the ecophysiology of the terminal ileum, caecum and colon of the potweaned piglet. In: 8th symposium Pig Digestive Physiology. Uppsala, Sweden.

Moran, E. T. 1982. Comparative nutrition of fowl and swine. In Gastrointestinal Systems. Office of Educcational Practice. University of Guelph, ON.

Moreau, M. C., R. Ducluzeau, and P. Raibaud. 1976. Hydrolysis of urea in the gastrointestinal tract of “monoxenic” rats: effect of immunization with strains of ureolytic bacteria. Infect. Immun. 13:9-15.

Morimoto, H., H. Noro, H. Ohtaki, and H. Yamazaki. 1984. Study on feeding fructooligosaccharides (Neosugar G) in suckling pigs. Japanese Science Feedstuffs. Association Report 59-88:1-17.

Morrison T.B., J. J. Weis, and C. T. Wittwer. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques. 246:954-962.

Moter, A., C. Hoenig, B. K. Choi, B. Riep, and U. B. Göbel. 1998a. Molecular epidemiology of oral treponemes associated with periodontal disease. J. Clin. Microbiol. 36:1399-1403.

Moter, A., G. Leist, R. Rudolph, K. Schrank, B. K. Choi, M. Wagner, and U. B. Göbel. 1998b. Fluorescence in siru hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. Microbiol. 144:2459-2467.

Moter, A., and U. B. Göbel. 2000. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods 41:85-112.

Mouricout, M. A., and R. A. Julien. 1987. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf small intestinal mucis. Infect.Immun. 55:1216-1223.

Mroz, Z. 2003. Organic acids of various origin and physicochemical forms as potential growth promoters for pigs. In: 9th Symposium Digestive Physiology in Pigs. Banff, Canada.

Mulder, R. W. A. W., R. Havenaar, J. H. J. Huis, and R. Fuller. 1997. Intervention strategies: the use of probiotics and competitive exclusion microfloras against contamination with pathogens in pigs and poulty. Probiotics-2. G. W. Tannock, ed. Horizon Scientific Press, UK.

Mullan, B. P., R. H. Wilson, D. Harris., J. G. Allen and A. Naylor. 2002. Supplementation of weaner pig diets with zinc oxide or Bioplex™ Zinc. Pages 419-424 in: Nutritional Biotechnology in the Feed and Food Industries. Proceedings of Alltech’s Eighteenth Annual Symposium. T. P. Lyons and K. A. Jacques, eds. Nottingham University Press, U. K.

Mullan, B. P., A. Hernandez, and J. R. Pluske. 2004. Influence of the form and rate of Cu and Zn supplementation on the performance of growing pigs. In: Biotechnology in the Feed Industry, Proceedings of the 20th Annual Symposium. T.P. Lyons and K.A. Jacques, eds. Nottingham University Press, UK.

Muralidhara, K. S., G. G. Sheggeby, P. R. Elliker, D. C. England, and W. E. Sandine, 1977. Effect of feeding lactobacilli on the coliform and lactobacillus flora of intestinal tissue and feces from piglets. J. Food Prot. 40, 288-295.

Muyzer, G., and E. C. de Waal. 1994. Determination of the genetic diversity of microbial communities using DGGE analysis of PCR amplified 16S rRNA. NATO ASI Series G35:207-214.

Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel elecrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek. 73:127-141.

Muyzer, G., E. C. de Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradients gel electrophoresis analysis of polymerase chain reaction-amplified genes encoding for 16S rDNA. Appl. Environ. Microbiol. 59:695-700.

Myers, R. M., S. G. Fischer, L. S. Lerman, and T. Maniatis. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Res. 13:3131-3145.

Nadkarni, M. A., E. F. Martin, N. A. Jacques, and N. Hunter. 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257-266.

Nagashima, K., T. Hisada, M. Sato, and J. Mochizuki. 2003. Application of new primer-enzyme combinations to Terminal Restriction Fragment Length Polymorphism profiling of bacterial population in human feces. Appl. Environ. Microbiol. 69:1251-1262.

National Research Council. 1998. Nutrient requirements of swine. 10th edition. National Academy Press, Washington, DC.

Nemcova, R. A., S. Boma, R. Gancarikova, R. Herich and P. Guba. 1999. Study of the effect of Lactobacillus paracasei and fructooligosaccharides on the faecal microflora in weanling pigs. Ber. Mucnch. Tierdrztl. Wschr. 112:225-228.

Netea M. G., R. Sutmuller, C. Hermann, C. A.Van der Graaf, J. W. Van der Meer, J. H. VanKrieken, T. Hartung, G. Adema, and B. J. Kullberg. 2004. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. J. Immunol. 15:3712-3718.

Newman, K. 1994. Mannan-oligosaccharides: natural polymers with significant impact on the gastrointestinal microflora and the immune system. Pages 167-174 in: Biotechnology in the Feed Industry. Proceedings of Alltech’s Tenth Annual Symposium. T. P. Lyons and K. A. Jacques, eds. Notthingham University Press, Nottingham, UK.

Newman, K. E., and M. C. Newman. 2001. Evaluation of mannan oligosaccharide on the microflora and immunoglobulin status of sows and piglet performance. J. Anim. Sci. 79. 189 (abs).

Nousiainen, J. T. 1991. Comparative observations on selected probiotics and olquindox as feed additives for piglets around weaning. 2. Effect on villus length and crypt depth in the jejunum, ileum, caecum and colon. J. Anim. Physiol. Anim. Nutr. 66:224-230.

Noverr, M. C., and G. B. Huffnagle. 2004. Does de microbiota regulate immune responses outside the gut? Trends Microbiol. 12:562-568.

O'Quinn, P. R., D. W. Funderburke, and G. W. Tibetts. 2001. Effects of dietary supplementation with mannan oligosaccharides on sow and litter performance in a commercial production system. J. Anim. Sci. 79 (Suppl. 1):212.

Orban, J. I., J. A. Patterson, O. Adeola., A. L. Sutton, and G. N. Richards. 1997. Growth performance and intestinal microbial populations of growing pigs fed diets containing sucrose thermal oligosaccharide caramel. J. Anim. Sci. 75:170-175.

Osborn, A. M., E. R. B. Moore, and K. N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (t-RFLP) analysis for the study of microbial community structure and dynamics. Environ. Microbiol. 2:39-50.

Ott, S. J., M. Musfeldt, D. F. Wenderoth, J. Hamper, O. Brant, U. R. Folsch, K. N. Timmis, S. Schreiber. 2005. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut 53:685-693.

Ott, S. J., M. Musfeldt, W. Ullmann, J. Hampe, and S. Schreiber. 2004. Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora. J. Clin. Microbiol. 42:2566-2572.

Øverland, M. and S. H. Stein. 1999. K-difromate (Formi™LHS) in diets for pigs. In : Manipulating pig production VII. P. D. Cranwell, ed. Australasian Pig Science Association. Victoria, Australia.

Øverland, M., S. H. Stein, G. Gotterbarm, and T. Granli. 1999. Formi™LHS- An alternative to antibiotic growth promoters. 50th annual meeting of the EAAP.

Øverland, M., T. Granli, N. Kjos, O. Fjetland, S. H. Steien, and M. Stokstad. 2000. Effect of dietary formats on growth performance, carcass traits, sensory quality, intestinal microflora and stomach alterations in growing-finishing pigs. J. Anim. Sci. 78:1875-1884.

Pabst, R., M. Geist, H. J. Rothkotter, and F. J. Fritz. 1988. Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs. Immunology 64:539-544.

Partanen, K. and Z. Mroz. 1999. Organic acids form performance enhancement in pig diets. Nutr. Res. Rev. 12:117-145.

Partanen, K. 2001. Organic acids- their efficacy and modes of action in pigs. Pages: 201-217 in: Gut environment of pigs. A. Piva, K. E. Bach Knudsen and J. E. Lindberg, eds. Notthingham University Press, UK.

Patterson, J. A., and K. M. Burkholder. 2003. Prebiotic feed additives: rationale and use in pigs. In: 9th Symposium on Digestive Physiology in Pigs. Banff, Canada.

Peet-Schwering, C. M. C. and J. W. G. M. Swinkels. 2000. Enteroguard as an alternative feed additive to antibiotics in weanling pig diets. J. Anim. Sci. 78:S184 (abs).

Penders, J., C. Vink, C. Driessen, N. London, C. Thijs, and E. E. Stobberingh. 2005. Quantification of *Bifidobacterium* spp., *Escherichia coli* and *Clostridium difficile* in faecal samples of breast-fed and formula-fed infants by real-time PCR. FEMS Microbiol. Lett. 243:141-147.

Perdigón, G., R. Fuller, and R. Raya. 2001. Lactic acid bacteria and their effect on the immune system. Curr. Issues Intest. Microbiol. 2:27-42.

Pérez de Rozas, A., M. Roca, R. Carabaño, C. de Blas, M. Francesch, J. Brufau, S. M. Martín-Orúe, J. Gasa, S. Campoy, J. Barbé, and I. Badiola. 2003. El estudio de la diversidad intestinal por RFLP. XIX Curso de especialización FEDNA. Madrid, Octubre de 2003.

Pettigrew, J. E. 2000. Mannan oligosaccharide's effects on performance reviewed. Feedstuffs. 12.

Pickard, K. M., A. R. Bremner, J. N. Gordon, and T. T. MacDonald. 2004. Immune responses. Best Pract. Res. Clin. Gastroenterol. 18:271-285.

Piva, A., A. Panciroli, E. Meola and A. Formigoni. 1995. Lactitol enhances short-chain fatty acid and gas production by swine cecal microflora to a greater extent when fermenting low rather than high fibre diets. J. Nutr. 126:280-289.

Platel. K. and K. Srinivasan. 2000. Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. Nahrung:44:42-46

Pluske, J. R. P. M. Siba, D. W. Pethick, Z. Durmic, B. P. Mullan, and D. J. Hampson. 1996a. The incidence of swine dysentery in pigs can be reduced by feeding diet that limit the amount of fermentable substrates entering the large intestine. J. Nutr. 126:2920-2933.

Pluske, J. R., I. H. Williams, and E. X. Aherne. 1996b. Villous height and crypt depth in piglets in response to increases in the cow's milk after weaning. Anim. Sci.62:145-158.

Pluske, J. R., D. J. Hampson and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. Livest. Prod. Sci. 51:215-236.

Pluske, J. R., Z. Durmic, D. Pethick, B. P. Mullan and D. J. Hampson. 1998. Confirmation of the role of readily fermentable carbohydrates in the expression of swine dysentery in pigs after experimental infection. J. Nutr. 128:1737-1744.

Pluske, J. R., D. W. Pethick, D. E. Hopwood, and D. J. Hampson. 2002. Nutritional influences on some major enteric bacterial diseases of pigs. Nutr. Res. Rev. 15:333-371.

Pluske, J. R., D. E. Hopwood, and D. J. Hampson. 2003. Relación entre la microbiótica intestinal, el pienso y la incidencia de diarreas, y su influencia sobre la salud del lechón tras el destete. XIX Curso de Especialización Fedna. Madrid.

Possemiers, S., K. Verthé, S. Uyttendaele, S. Bolca, T. Van de Wiele, N. Boon, and W. Verstraete. 2004. Molecular techniques for the structural analysis and quantification of an in vitro cultured intestinal microbial community. Reprod. Nutr. Dev. 44:S8.

Poulsen, H. D. 1995. Zinc oxide for weanling piglets. Acta Agric. Scand. Sect. A Anim. Sci. 45:159-167.

Poulsen, H. D. 1998. Zinc and copper as feed additives, growth factors or unwanted environmental factors. J. Anim. Feed Sci. 7:135-142

Poxton, I. R., and J. P. Arburhnott. 1990. Determinants of bacterial virulence. In: Topley and Wilson's principles of bacteriology, virology and immunity. 8th ed. A. H. Linton and H. M. Dick, eds. Edward Arnold, UK.

Prohaszka, L., 1986. Antibacterial mechanism of volatile fatty acids in the intestinal tract of pigs against *Escherichia coli*. Zentralbl. Veterinarmed. Reihe B 33:166 (abs).

Prohaszka, L., B. M. Jayarao, A. Fabian, ans S. Kovacs. 1990. The role of intestinal volatile fatty acids in the Salmonella shedding of pigs. Zentbl. Vetmed. Reihe B. 37:570 (abs.).

Pryde, S. E., A. J. Richardson, C. S. Stewart, and H. J. Flint. 1999. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl. Environ. Microbiol. 65:5372-5377.

Pullan, R. D., G. A. O. Thomas, M. Rhodes, R. G. Newcombe, G. T. Williams, A. Allen, and J. Rhodes. 1994. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. Gut 35:353-359.

Radcliffe, J. S., Z. Zhang, and E. T. Kornegay. 1998. The effects of microbial phytase, citric acid, and their interactions in a corn-soybean meal-based diet for weanling pigs. J. Anim. Sci. 76:1880-1886.

Radecki, S. V., and M. T. Yokohama. 1991. Intestinal bacteria and their influence on swine nutrition. In: Swine Nutrition. E. R. Miller, D. E. Ullrey, and A. J. Lewis, eds. Butterworth Heinemann, Boston, USA.

Rainey, F. A. and P. H. Janssen. 1995. Phylogenetic analysis by 16S rDNA sequence comparison reveals two unrelated groups of species within the genus Ruminococcus. FEMS Microbiol. Lett. 129:69-74.

Ramsing, N. B., H. Fossing, T. G. Ferdelmann, F. Andersen, and B. Thamdrup. 1996. Distribution of bacterial populations in a stratified fjord quantified by in situ hybridization and related to chemical gradients in the water column. Appl. Environ. Microbiol. 62:1391-1404.

Raskin, L., W. C. Capman, R. Sharp, L. K. Poulsen, and D. A. Stahl. 1999. Molecular ecology of the gastrointestinal ecosystems. In: Gastrointestinal Microbiology, R. I. Mackie and B. A. White, eds. Chapman and Hall Microbiology Series. New York.

Ratcliffe, B. 1991. The role of the microflora in digestion. In : In Vitro Digestion for Pigs and Poultry, Fuller, M. F., eds. CAB International, Edinburgh, U. K.

Ravindran, V., and E. T. Kornegay. 1993. Acidification of weaner pig diets. A review. J. Sci. Food Agric. 62:313-322.

Reid, C. A., and K. Hillman. 1999. The effects of retrogbradation and amylase/amylopectin ratio of starches on carbohydrate fermentation and microbial populations in the porcine colon. Anim. Sci. 68:503-510.

Reisner, D., G. Steger, U. Wiese, M. Wulfert, M. Heiby, and K. Henco. 1992. Temperature-gradient gel electrophoresis for the detection of polymorphic DNA and for quantitative polymerase chain reaction. Electrophoresis 13:632-636.

Requena, T., J. Burton, T. Matsuki, K. Munro, M. Simon, R. Tanaka, K. Watanabe, G. Tannock. 2002. Identification, detection, and enumeration of human Bifidobacterium species by PCR targeting the transaldolase gene. Appl. Environ. Microbiol. 68:2420-2427.

Richardson, A. J., A. G. Calder, C. S. Stewart, and A. Smith. 1989. Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. Lett. Appl. Microbiol. 9:5-8.

Rigottier-Gois, L., V. Rochet, N. Garrec, A. Suau, and J. Dore. 2003. Enumeration of Bacteroides species in human faces by fluorescent in situ hybridisation combined with flow cytometry using 16S rRNA probes. Syst. Appl. Microbiol. 26:110-118.

Ririe K. M., R. P. Rasmussen, and C. T. Wittwer. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction Anal. Biochem. 245:154-160.

Risley, C. R., E. T. Kornegay, M. D. Lindemann, and S. M. Weakland. 1991. Effects of organic acids with or without a microbial culture on the performance and gastrointestinal tract measurements of weanling pigs. Anim. Feed Sci. Technol. 35:259-270.

Risley, C. R., E. T. Kornegay, M. D. Lindemann, C. M. Wood, and W. N. Eigel. 1992. Effect of feeding organic acids on selected intestinal content measurements at various times postweaning in pigs. Journal of Animal Science 70:196-206.

Robertsson, J. 1994. Prohibited use of antibiotics as feed additive for growth promotion effects on piglet health and production parameters. Proceedings of the IPVS, Thailand.

Robinson, I. M., M. J. Allison, and J. A. Bucklin. 1981. Characterization of the cecal bacteria of normal pigs. Appl. Environ. Microbiol. 41:950-955.

Rodiger, W. E. W., and A. Moore, 1981. Effect of short chain fatty acids on sodium absorption in isolated human colon perturbed through the vascular bed. Digest. Dis. Sci. 26:100-106.

Rolfe, R. D. 1996. Colonization resistance. Gastrointestinal microbes and host interactions. In: Gastrointestinal Microbiol., vol. 2. R. I. Mackie, B. A. Whyte, and R. E. Isaacson, eds. Chapman and Hall, London. UK.

Rolfe, R. D. 2000. The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130:396S-402S.

Roth, F. X., B. Eckel, M. Kirchgessner, and U. Eidelsburger. 1992. Influence of formic acid on pH value, dry matter content, concentrations of volatile fatty acids and lactic acid in the

gastrointestinal tract. 3. Communication: Investigations about the nutritive efficacy of organic acids in the rearing of piglets. J. Anim. Physiol. Anim. Nutr. 67:148-156.

Roth, L. 2000. The battle of the bugs. Pig Progress 16:12-15.

Rotimi V. O., and B. I. Duerden. 1982. The bacterial flora of neonates with congenital abnormalities of the gastro-intestinal tract. J Hyg. 88:69-81.

Russell, E. G. 1979. Types and distribution of anaerobic bacteria in the large intestine of pigs. Appl. Environ. Microbiol. 37:187-193.

Russell, J. B. 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. 73:363-370.

Russell, J. B., and R. Diez-Gonzalez. 1998. The effects of fermentation acids on bacterial growth. Adv. Microbial Physiol. 39:205-234.

Russell, J. B., C. J. Sniffen, and P. J. Van Soest. 1983. Effect of carbohydrate limitation on degradation and utilization of casein by mixed rumen bacteria. J. Dairy Sci. 66:763-775.

Sadzikowski, M. R., J. F. Sperry, and T. D. Wilkins. 1977. Cholesterol reducing bacterium from human feces. Appl. Environ. Microbiol. 34:355-362.

Salanitro, J. P., I. G. Blake, and P. A. Muirhead. 1977. Isolation and identification of fecal bacteria from swine. Appl. Environ. Microbiol. 33:79-84.

Salminen, S., C. Bouley, M. C., and B. Boutron-Ruault. 1998. Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80:147-171.

Salter, D. N. 1984. Nitrogen metabolism. In: the germ-free animal in biomedical research. M. E. Coates and B. E. Bustafsson, eds. Laboratory Animals, Ltd., London, UK.

Salyers A. A., M. O'Brien, and S. F. Kotarski. 1982. Utilization of chondroitin sulfate by Bacteroides thetaiotaomicron growing in carbohydrate-limited continuous culture. J. Bacteriol. 150:1008-1015.

Salyers, A. A. 1979. Energy sources of major intestinal fermentative anaerobes. Am. J. Clin. Nutr. 32:158-163.

Salyers, A. A., J. R. Vercellotti, S. E. West, and T. D. Wilkins. 1977. Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. Appl. Environ. Microbiol. 33:319-322.

Sansom B. F., and P. T. Gleed. 1981. The ingestion of sow's faeces by suckling piglets. Br. J. Nutr. 46:451-456.

SAS Institute Inc. 1988. SAS User's Guide: Statistics. SAS Institute Ins., Cary, NC.

Sato, J., K. Mochizuki, and N. Homma. 1982. Affinity of the Bifidobacterium to intestinal mucosal epithelial cells. Bifidobacteria Microflora 1:51-54.

Savage, D. C. 1977. Microbial ecology of the gastrointestinal tract. Ann. Rev. Microbiol. 31:529-533.

Schiffrin, E. J., D. Brassart, A. L. Servin, F. Rochat, and A, Donnet-Hughes. 1997. Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. Am. J. Clin. Nutr. 66:515-520.

Schiffrin E. J., and S. Blum. 2002. Interactions between the microbiota and the intestinal mucosa. Eur. J. Clin. Nutr. 56:S60-S64.

Schmalenberger, A., F. Schwieger, and C. C. Tebbe. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. Appl. Environ. Microbiol. 67:3557-3563.

Scholten, R. H. J., C. M. C. Van der Peet-Schwering, M. W. A. Verstegen, L. A., den Hartog, J. W. Schrama and P. C. Vesseur. 1999. Fermented coproducts and fermented compounds diets for pigs: a review. Anim. Feed Sci. Techol. 82:1-19.

Schuppler, M., M. Wagner, G. Schön, and U. B. Göbel. 1998. In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes. Microbiology 144:249-259.

Seki, H., M. Shiohara, T. Matsumura, N. Miyagawa, M. Tanaka, A. Komiyama, and S. Kurata. 2003. Prevention of antibiotic-associated diarrhea in children by *Clostridium butyricum* MIYAIRI. Pediatr. Int. 45:86-90.

Selim, A. S., P. Boonkumklao, T. Sone, A. Assavanig, M. Wada, and A. Yokota. 2005. Development and assessment of a real-time PCR assay for rapid and sensitive detection of a novel thermotolerant bacterium, Lactobacillus thermotolerans, in chicken feces. Appl. Environ. Microbiol. 71:4214-4219.

Sen, S., H. P. S. Makkar, S. Muetzel and K. Becker. 1998. Effect of *Quillaja saponaria* saponins and *Yucca schidigera* plant extract on growth of *Escherichia coli*. Lett. Appl. Microbiol. 27:35-38.

Sghir, A., J. M. Chow, and R. I. Mackie. 1998. Continuous culture selection of bifidobacteria and lactobacilli from human faecal samples using fructooligosaccharides as selective substrates. J. Appl. Microbiol. 85:769-777.

Sghir, A., G. Gramet, A. Suau, V. Rochet, P. Pochart, and J. Doré. 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 66:2263-2266.

Shankar, A. H. and A. S. Prasad. 1998. Zinc and immune function: the biological basis of altered resistance to infection. Am. J. Clin. Nutr. 68 (Suppl. 2):447S-463S.

Sharma, R., and U. Schumacher. 1995. The influence of diets and gut microflora on lecting binding patterns of intestinal mucins in rats. Lab. Invest. 73:558-564.

Sharp, R. and C. J. Ziemer. 1999. Application of taxonomy and systematics to molecular techniques in intestinal microbiology. In:Colonic microbiota, nutrition and health. G. R.

Gibson and M. R. Roberfroid, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Shim, S. B., M. W. Verstegen, I. H. Kim, O. S. Kwon, and J. M. Verdonk. 2005. Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or symbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota. Arch. Anim. Nutr. 59:419-427.

Si, W., J. Gong, R. Tsao, T. Zhou, H. Yu, C. Poppe, R. Johnson, and Z. Du. 2006. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. J. Appl. Microbiol. 100:296-305.

Siljander-Rasi, H., T. Alaviuhkola, and K. Suomi. 1998. Carbadox, formic acid and potato fibre as feed additives for growing pigs. J. Anim. Feed Sci. 7:205-209.

Silva, A.T., R. J. Wallace, and E.R. Orskov. 1987. Use of particle-bound microbial enzyme activity to predict the rate and extent of fibre degradation in the rumen. Br. J. Nutr. 57:407-415.

Simon, O., W. Vahjen, and L. Scharek. 2003. Microorganisms as feed additives- probiotics. Pages 295-318 in 9th International Symposium on Digestive Physiology in Pigs, Canada.

Simpson, J. M., V. J. McCrcken, B. A. White, H. R. Gaskins, and R. I. Mackie. 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J. Microbiol. Methods. 36:167-179.

Simpson, J. M., V. J. McCrcken, H. R. Gaskins, and R. I. Mackie. 2000. Denaturing gradient gel electrophoresis analysis of 16S Ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microbiol. 66:4705-4714.

Smirnov A., R. Perez, M. Thein, and Z. Uni. 2005. Mucin dynamics in the chicken jejunum following dietary mannoligosaccharides (Bio-Mos) and antibiotic growth promoter (Virginiamycin) supplementation. Page 52 in: Avian gut function, health and disease. T Acamovic, ed. WPSA, Bristol, UK.

Smith, J. W., M. D. Tokach, R. D. Goodband, J. L. Nelssen, and B. T. Richert. 1997. Effects of the interrelationship between zinc oxide and copper sulphate on growth performance of early-weaned pigs. J. Anim. Sci. 75:1861-1866.

Smithe, L. D., I. L. Smith, G. A. Smith, M. F. Dohnt, M. L. Symonds, L. J. Barnett, and D. B. McKay. 2002. A quantitative PCR (Taqman) assay for pathogenic *Leptospira* spp. BMC Infect. Dis. 2:13.

Smits, R. J., and D. J. Henman. 2000. Practical experience with bioplexes in intensive pig production. In: Biotechnology in the Feed industry. T. P. Lyons and K. A. Jacques, eds. Nottingham University Press. Nottingham, UK.

Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer. 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. 63:2884-2896.

Sordeberg, T. A., B. Sunzel, S. Holm, T. Elmros, G. Hallman, and S. Sjoberg. 1990. Antibacterial effect of zinc oxide in vitro. Scand. J. Plast. Reconstr. Surg. Hand Surg. 24:193-197.

Southwick, P. L., L. A. Ernst, E. W. Tauriello, S. R. Parker, R. B. Mujumdar, S. R. Mujumdar, H. A. Clever and A. S. Waggoner. 1990. Cyanine dye labelling reagents.- carboxymethildocyanine succimidyl esters. Cytometry 11:418-430.

Specian R. D., and M. G. Oliver. 1991. Functional biology of intestinal goblet cells. Am. J. Physiol. 260:183-193.

Spring, P., C. Wenk, K. A. Dawson, and K. E. Newman. 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.

Spring, P. 2004. Impact of mannan oligosaccharide on gut health and pig performance. Pages 93-105 in Interfacing Immunity, Gut Health and Performance. L. A. Tucker and J. A. Taylor-Pickard , eds. Nottingham University Press, U. K.

Starling, J. R., and E. Balish. 1981. Lysosomal enzyme activity in pulmonary alveolar macrophages from conventional, germfree, monoassociated, and conventionalized rats. J. Reticuloendothelial Soc. 30:497 (abs).

Starvic, S., E. T. Kornegay, R. J. Wallace. A. Chesson. 1995. Microbial probiotics for pigs and poultry. Biotechnol. Anim. Feeds Anim. Feeding. Weinheim, Germany. Pp 205-231.

Stewart, C. S., A. Chesson. Making sense of probiotics. 1993. Pig Veterinary J. 31:11-33.

Stewart, C. S., K. Hillman, F. Maxwell, D. Kelly, and T. P. King. 1993. Recent advances in probiosis in pig: Observations on the microbiology of the pig gut. In: Recent Advances in Animal Nutrition. P. C. Garnsworthy and D. J. Cole, eds. Notthingam University Press, Notthingam, UK.

Stewart, C. S. 1997. Microorganisms in hindgut fermentors. In: Gastrointestinal Microbiology. R. I. Mackie and B. A. White, eds. Chapman and Hall Microbiology Series. New York.

Stewart, C. S. 1999. Microorganisms in hindgut fermentors. 2nd edition. In : Gastrointestinal Microbiol., R. I. Mackie and B. A. White, eds. Chapman and Hall Microbiol. Series. New York.

Stokes, C. R., M. A. Vega-López, M. Bailey, E. Termo, and B. G. Miller. 1992. Immune development in the gastrointestinal tract of the pigs. In: Neonatal survival and growth. M. A. Varley, P. E. V. Williams, and T. L. J. Lawrence, eds. Edinburgh, UK.

Stokes, C. R., M. Bailey, and H. Haverson. 2001. Development and function of the pig gastrointestinal immune system. In: Digestive physiology of pigs. J. E. Lindberg and B. Ogle, eds. CAB International, UK.

Suau A., R. Bonnet, M. Sutren, J. J. Godon, G. R. Gibson, M. D. Collins, and J. Dore. 1999. Direct analysis of genes encoding 16S rRNA from complex communities revealsmany novel molecular species within the human gut. Appl. Environ. Microbiol. 65:4799-807.

Suau, A., V. Rochet, A. Sghir, G. Gramet, S. Brewaeys, M. Sutren, L. Rigottier-Gois, and J. Doré. 2001. Fusobacterium prausnitzii and related species represents a dominant group within the human fecal flora. Syst. Appl. Microbiol. 24:139-145.

Suzuki, K., D. Meek, and Y. Dot. 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc. Natl. Acad. Sci. U.S.A. 101; 1981-1986.

Suzuki, M. T., And S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62:625-630.

Suzuki, M. T., L. T. Taylor and E. F. DeLong, 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5'-nuclease assays. Appl. Environ. Microbiol. 66, 4605-4614.

Swanson, B.F. and P. L. Gleed. 1981. The ingestion of sow's feces by peiglets. Br. J. Nutr. 46:451-456.

Swanson, K. S., C. M. Grieshop, E. A. Flickinger, L. L. Bauer, H. P. Healy, K. A. Dawson, N. R. Merchen and G. C. Fahey Jr. 2002. Supplemental fruuctooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. J. Nutr. 132:980-989.

Swords W. E., C. C. Wu, F. R. Champlin, and R. K. Buddington. 1993. Postnatal changes in selected bacterial groups of the pig colonic microflora. Biol. Neonate 63:191-200.

Tajima K., R. I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67:2766-74.

Takada, T., K. Matsumoto, and K. Nomoto. 2004. Development of multi-color FISH method for analysis of seven Bifidobacterium species in human feces. J. Microbiol. Methods 58:413-421.

Tannock, G. W., and J. D. B. Smith. 1970. The micro-flora of the pig stomach and its possible relationship to ulceration on the pars oesophagia. J. Comp. Path. 80:359-367.

Tannock, G. W., M. P. Dashkevicz, and S. D. Feigh Environm ner. 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl. Microbiol. 55:1848-1851.

Tannock, G. W. 1999. Modification of the normal microbiota by diet, stress, antimicrobial agents, and probiotics. In Gastrointestinal Microbiol., R. I. Mackie and B. A. White, eds. Chapman and Hall Microbiol. Series. New York.

Tannock, G. W., 2000. Molecular assessment of intestinal microflora. Am. J. Clin. Nutr. 73:410S-414S.

Taras, D., W. Vahjen, M. Macha, and O. Simon. 2005. Response of performance characteistics and fecal consistency to long-lasting dietary supplementation with the probiotic strain *Bacillus cereus* var-toyoi to sows and piglets. Arch. Anim. Nutr.9 :405-417.

Tedesco, D., S. Galletti, J. Turini, S. Stella. 2005. Effects of new natural feed additives on growth and intestinal microflora of weanling piglets. Ital. J. Anim. Sci. 4:494 (abs).

Thomlinson, J. R., and T. L. Lawrence. 1981. Dietary manipulation of gastric pH in the prophylaxis of enteric disease in weaned pigs: some field observations. Vet. Rec. 109:120-122.

Topping, D. L., and P. M. Clifton. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81:1031-1064.

Tortuero F., J. Rioperez, E. Fernandez, and M. L. Rodriguez. 1995. Response of piglets to oral administration of lactic acid bacteria. J. Food Protec. 58:1369–1374

Trebesius, K., D. Harmsen, A. Rakin, J. Chmelz, and J. Heesemann. 1998. Development of rRNA-Targeted PCR and in situ hybridization with fluorescently labelled oligonucleotides for detection of Yersinia species. J. Clin. Microbiol. 36:2557-2564.

Trowell, H., D. A. Southgate, T. M. Wolever, A. R. Leeds, M. A. Gassull, and D. J. Jenkins. 1976. Letter: dietary fibre refined. Lancet 1:967.

Tsiloyiannis V. K., S. C. Kyriakis, J. Vlemmas, and K. Sarris. 2001. The effect of organic acids on the control of porcine post-weaning diarrhoea. Res. Vet. Sci. 70:287-293.

Turck, D., A. S. Feste, and C. H. Lifschitz. 1993. Age and diet affect the composition of porcine colonia mucin. Pediatr. Res. 33:564-567.

Tzortzis, G., A. K. Goulas, J. M. Gee, and G. R. Gibson. 2005. A novel galactooligosaccharide misture increases the bifidobacerial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. J. Nutr. 135:1726-1731.

Umesaki, Y., H. Setoyama, S. Matsumoto, and Y. Okada. 1993. Expansion of a β T-cell receptor bearing intestinal intraepithelial lymphocytes alters microbial colonization in germ-free mice and its independence from thymus. Immunology 79:32-37.

Umesaki, Y., Y. Okada, S. Matsumoto, A. Imaoka, and H. Setoyama. 1995. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on

the samll intestinal epithelial cells in the ex – germ-free mouse. Microbiol. Immunol. 39:555-562.

Urlings, H. A., A. J. Mul, A. T. van't Klooster, P. G. Bijker, J. G. van Logstetijn, and L. G. van Gils. 1993. Microbial and nutritional aspects of feeding fermented feed (poultry-by-products) to pigs. Vet. Q. 15:146-151.

Valencia, Z., and E. R. Chavez. 1997. Lignin as a purified dietary fiber supplement for piglets. Nutr. Res. 17:1517-1527.

Van der Waaij, D. 1989. The ecology of the human intestine and its consequences for overgrowth by pathogens such as *Clostridium difficile*. Ann. Rev. Microbiol. 43:69-87.

Van Dijk, J. E., J. Huisman, and J. F. Koninkx. 2002. Structural and functional aspects of a healthy gastrointestinal tract. In: Nutrition and health of the gastrointestinal tract. M. C. Blok, et al., eds. Wageningen Academic Publishers, The Netherlands.

Van Immerseel, F., V. Fievez, J. de Buck, F. Pasmans, A. Martel, F. Haesebrouck, and R. Ducatelle. 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with *Salmonella enteritidis* in young chickens. Poult. Sci. 83:69-74.

Van Kessel, A., T. W. Shirkey, R. H. Siggers, M. D. Drew, and B. Laarveld. 2004. Commensal bacteria and intestinal development. Studies using gnotobiotic pigs. In: Interfacing immunity, gut health and performance. L. A. Tucker and J. A. Taylor-Pickard, eds. Nottingham University Press, Nottingham, UK.

Van Winsen, R. L., B. A. P. Urlings, L. J. A. Lipman, J. M. A. Snijders, D. Keuzenkamp, J. H. Verheijden, and R. V. Knapen. 2001. Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. Appl. Environ. Microbiol. 67:3071-3076.

Varel, V. H., W. G. Pond, J. C. Pekas, and Yen, J. T. 1982. Influence of high-fibre on bacterial populations in gastrointestinal tracts of obese and lean genotype pigs. Appl. Environ. Microbiol. 44:107-112.

Varel, V. H., S. J. Fryda, and I. M. Robinson. 1984. Cellulolytic bacteria from the large intestine. Appl. Environ. Microbiol. 47:219-221.

Varel, V. H., and W. G. Pond. 1985. Enumeration and activity of cellulytic bacteria from swine fed various levels of dietary fibre. Appl. Environ. Microbiol. 49:858-862.

Varel, V. H., I. M. Robinson, and H. J. Jung. 1987. Influence of dietary fiber on xylanolytic and cellulolytic bacteria on adult pigs. Appl. Environ. Microbiol. 53:22-26.

Varel, V. H., and J. T. Yen. 1997. Microbial perspective on fiber utilization by swine. J. Anim. Sci. 75:2715-2722.

Vaughan, E. E., H. G. H. J. Heilig, E. G. Zoetendal, W. M. de Vos, and A. D. L. Akkermans. 2000. A molecular view of intestinal ecosystem. Curr. Issues Intest. Microbiol. 1:1-12.

Verstegen, M. W. A. and B. A. Williams. 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Anim. Biotechnol. 13:113-127.

Visek, W. J. 1984. Ammonia: its effects on biological systems, metabolic hormones, and reproduction. J. Dairy Sci. 67:373-380.

Waar, K., J. E. Degener, M. J. van Luyn, and H. J. Harmsen. 2005. Fluorescent in situ hybridization with specific FNA probes offers adequate detection of *Enterococcus faecalis* and *Enterococcus faecium* in clinical samples. J. Med. Microbiol. 54:937-944.

Wagner, M., H. Matthias, and H. Daims. 2003. Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. Curr. Opin. Microbiol. 6:302-309.

Walker, A.W., S. H. Duncan, E. C. McWilliam Leitch, M. W. Child, and H. J. Flint. 2005. pH and peptide supply can radically alter bacterial populations and short chain fatty acid ratios within microbial communities from the human colon. Appl. Environ. Microbiol. 71:3692-3700.

Wallgren, P. and L. Melin. 2001. Weaning systems in relation to disease. In: The Weaner Pig. Nutrition and management. M. A. Varley and J. Wiseman, eds. CABI Publishing, UK.

Wallner, G., B. Fuchs, S. Spring, W. Beisker, and R. Amann. 1997. Flow sorting of microorganisms for molecular analysis. Appl. Environ. Microbiol. 63:4223-4231.

Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. Detection of *Lactobacillus, Pediococcus, Leuconostoc,* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 67:2578-2585.

Wang, J. F., Y. H. Zhu, D. F. Li, Z. Wang and B. B. Jensen. 2004. In vitro fermentation of various fiber and starch sources by pig fecal inocula. J. Anim. Sci. 82:2615-2622.

Wang, M., S. Ahrné, M. Antonsson, and G. Molin. 2004. T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different ages. J. Microbiol. Methods. 59:53-69.

Wang, R. F., W. W. Cao, and C. E. Cerniglia. 1996. PCR dectection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62:1242-1247.

Ward, T. L., G. A. Asche, G. F. Louis, and D. S. Pollman. 1996. Zinc-methionine improves growth performance of starter pigs. J. Anim. Sci. 74:S303.

White, L. A., M. C. Newman, G. L. Cromwell, and M. D. Lindemann. 2002. Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. J. Anim. Sci. 80:2619-2628.

Williams, B. A., M. W. A. Verstegen and S. Tamminga. 2001. Fermentation in the large intestine of single stomached Anim.s and its relationship to animal health. Nutr. Res. Rev. 14:207-227.

Wintzingerode, von, F., U. B. Göbel, and E. Stackenbrandt. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21:213-229.

LITERATURE CITED

Wise, M. G., and G. R. Siragusa. 2005. Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. Appl. Environ. Microbiol. 71:3911-3916.

Woese, C. R. 1987. Bacterial evolution. Microbiol. Rev. 51:221-271.

Woodall, P. F. 1989. The effects of increased dietary cellulose on the anatomy, physiology and behaviour of captive water voles, Arvicola terrestris (L.) (Rodentia:Microtianae). Comp. Biochem. Physiol. A. 94:615-621.

Wostmann, B. S. 1996. Germfree and gnotobiotic animal models: background and applications. Boca Raton, FL: CRC Press.

Wu, L. R., O. Zoborina, A. Zaborin, E. B. Chang, M. Musch, C. Holbrook, J. R. Turner, and J. C. Alverdy. 2005. Surgical injury and metabolic stress enhance the virulence of the human opportunistic pathogen *Pseudomonas aeruginosa*. Surg. Infect. 6:185-195.

Yajima, T. 1985. Contractile effect of short-chain fatty acids on the isolated colon of the rat. J. Physiol. 368:667-678.

Yang, W. Z., K. A. Beauchemin and L. M. Rode, 2001. Effect of dietary factors on distribution and chemical composition of liquid- or solid-associated bacterial populations in the rumen of dairy cows. J. Anim. Science. 79:2736-2746.

Yen, J. T. 2001. Anatomy of the digestive system and nutritional physiology. In: Swine Nutrition, 2nd ed. A. J. Lewis and L. Lee Southern, eds. CRC Press, NY, USA.

Yen, J. T., J. A. Nienaber, D. A. Hill, and W. G. Pond. 1991. Potential contribution of absorbed volatile fatty acids to whole-animal energy requirement in conscious swine. J. Anim. Sci. 69:2001-2012.

Zani, J. L., F. Weykamp da Cruz, A. Freitas dos Santos, and C. Gil-Turnes. 1998. Effect of probiotic CenBiot on the control of diarrhoea and feed efficiency in pigs. J. Appl. Microbiol. 84:68-71.

Zhang, X. B., and Y. Ohta. 1993. Microorganisms in the gastrointestinal tract of the rat prevent absorption of the mutagen-carcinogen 3-amino-1,4-dimethyl-5H-pyrido(4,3-b) indole. Can. J. Microbiol. 39:841-845.

Zhou, W., E. T. Kornegay, M. D. Lindemann, J. W. G. M. Swinkels, M. K. Welton, and E. A. Wong. 1994a. Stimulation of growth by intravenous injection of copper in weanling pigs. J. Anim. Sci. 72:2395-2403.

Zhou, W., E. T. Kornegay, M. D. Lindemann. 1994b. The role of feed consumption and feed efficiency in copper-stimulated growth. J. Anim. Sci. 72:2385-2394

Zhu, W. Y., B. A. Williams, S. R. Konstantivnov, S. Tamminga, W. M. de Vos, and A. D. L. Akkermans, 2003. Analysis of 16S rDNA revelas bacterial shift during in vitro fermentation of fermentable carbohydrate using piglet faeces as inoculum. Anerobe 9:175-180.

Zijlstra, R. T., J. Odle, W. F. Hall, B. W. Petschow, H. B. Gelberg, and R. E. Litov. 1994. Effect of orally administered epidermal growth fator on intestinal recovery of neonatal pigs infected with rotavirus. J. Pediatr. Gastroentero. Nutr. 19:382-390.

Zoetendal, E. G., A. D. L. Akkermans, and W. M. de Vos. 1998. Temperature gradient gel electrophoresis analysis of the 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. 64:3854-3859.

Zoetendal, E. G., A. D. L. Akkermans, W. M. Akkermans-van Vliet, J. A. Arjan, G. M. de Visser, and W. M. de Vos. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb. Ecol. Health Disease 13:129-134.

Zoetendal, E. G., K. Ben-Amor, H. J. M. Harmsen, F. Schut, A. D. L. Akkermans, and W. M. de Vos. 2002a. Quantification of uncultured Ruminococcus obeum-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16 rRNA-targeted probes. Appl. Environ. Microbiol. 68:4225-4232.

Zoetendal, E. G., A. von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A. D. L. Akkermans, and W. M. de Vos. 2002b. Mucosa-Associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. 68:3401-3407.

Zoetendal, E.G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins. 2004. Molecular ecological analysis of the gastrointestinal microbiota: A review. J. Nutr. 134:465-472.