Lactobacilli in the Porcine Intestine: From Composition to Functionality

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Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

Lactobacilli in the Porcine Intestine: From Composition to Functionality

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman,
in het openbaar te verdedigen
op maandag 27 juni 2005
des namiddags te 13:30 in de Aula

Konstantinov, S.R., 2005.
Lactobacilli in the Porcine Intestine: From Composition to Functionality
PhD Thesis Wageningen University, Wageningen, the Netherlands
With references-With summary in Dutch and Bulgarian

ISBN: 90-8504-233-X

to my wife Pavlina
на моята съпруга Павлина

ABSTRACT

Monogastric animals including pigs coexist with a diverse and dense commensal microbiota in their gastrointestinal (GI) tract. Most of these microbes are beneficial and provide the necessary nutrients or protection against harmful pathogens for the host. The microbial colonization of the porcine intestine begins after birth and follows a rapid succession during the neonatal and weaning period. In the immediate post-weaning period, the balance between the development of beneficial microbiota or the establishment of bacterial intestinal pathogens can be easily tipped toward disease expression. In order to enhance the animal growth and suppress the activity of the gut microbiota, antimicrobial compounds have been fed to weaning piglets for more than four decades. Nowadays, however, the emergence of antibiotic resistance in the human commensal bacteria has raised concerns about the impact of antimicrobial compounds for agricultural use, and accelerated the search for alternative nutritional strategies, such as the addition of probiotics and prebiotics. The aim of the current study was to understand the interactions between the intestinal microbiota, feed components and the host in the porcine GI tract and to validate some novel dietary strategies *in vivo* and *in vitro*.

The porcine intestinal microbiota development was analysed by 16S ribosomal RNA gene (rRNA) targeted approaches in relation to differences in nutritional strategies, age and pathogenic challenge. The GI tract microbial diversity was assessed using PCR-analysis of 16S rRNA genes by cloning and denaturing gradient gel electrophoresis. In addition, methods such as real-time PCR and fluorescent *in situ* hybridisation were applied to quantify the predominant bacterial community and the major *Lactobacillus* populations. Furthermore, we developed a novel strain detection system based on isolation of specific genomic fragments by representative difference analysis and their further quantification by real-time PCR. The data obtained during the course of the study indicated that a stable and complex commensal bacterial community might be considered as prerequisite of a healthy intestinal ecosystem. Moreover, the addition of fermentable carbohydrates to the diet of weaning piglets was found to enhance the stability and diversity of the microbiota in the large intestine of piglets, and to stimulate the growth of strains closely related to *Lactobacillus amylovorus*. The phenotypic and molecular taxonomic characterization of these strains, however, revealed significant differences with the type strain of *L. amylovorus* (DSMZ 20531^T) and the name *Lactobacillus sobrius* sp. nov. was proposed. *L. sobrius* strain 001^T was found to exert a significant protective effect against the enterotoxigenic *Escherichia coli* K88 promoted intestinal damages *in vitro* using porcine intestinal cell cultures. Furthermore, when *L. sobrius* was dietary fed to piglets subjected to *E. coli* K88 challenge, improved daily weight gain (+ 74%, $P<0.05$), modulation of the total secretory IgA, and reduced *E. coli* K88 intestinal levels were observed. The data indicate that *L. sobrius* exerts a protective effect against specific intestinal challenges while improving the weight gain and immunity of weaning piglets. Hence, the finding of this beneficial member of the porcine *Lactobacillus* community may have further implications for new dietary strategies aiming to improve the animal health during weaning.

Keywords: lactobacilli, *Lactobacillus sobrius*, porcine gastro intestinal tract, prebiotics, probiotics, 16S rRNA, DGGE, RDA, real-time PCR

OUTLINE OF THE THESIS

Considerable efforts have been devoted to the understanding of infectious diseases, including the biology of pathogens, host resistance and antimicrobial therapy in pigs. By contrast, very little is known of the prevention of diseases through dietary strategies in piglets during the neonatal and weaning period. Thus far, the gastrointestinal (GI) tract disorders during the weaning period have been overcome by adding sub-therapeutic doses of antibiotics and elevated levels of metal trace elements (zinc and copper) in feed. Nowadays, European-wide directives are in place to establish a sustainable pig production without using production enhancers and chemotherapeutics. Hence, novel dietary strategies need to be directed toward the maintenance of GI tract homeostasis and improvement of animal performance during weaning. The development of these dietary approaches is only possible when the composition and activity of the indigenous microbiota are evaluated. Specifically, the key members of the beneficial porcine microbiota need to be identified and further characterized. Selected alternative dietary strategies require also a careful *in vitro* and *in vivo* evaluation. To gain this knowledge, a study was initiated that aimed to (i) characterize the changes in the intestinal porcine microbiota composition and metabolic activities during the neonatal and weaning period and after certain dietary interventions (**Chapters 2, 3, 4** & **8**), (ii) isolate a novel member of the porcine commensal microbiota defined as *Lactobacillus sobrius* sp. nov., and to further elucidate its beneficial role *in vitro* (**Chapters 5** & **6**), (iii) develop molecular tools for further exploitation of the successive changes in the *Lactobacillus sobrius* population during the neonatal period and weaning. Furthermore, the effect of dietary addition *L. sobrius* against pathogenic challenge *in vivo* was examined (**Chapters 7 & 8**).

The porcine intestinal microbiota diversity is predominated by uncultivated bacteria. Thus, the data about the development of the intestinal microbiota in neonatal and weaning piglets based on selective media cultivation and microscopic analysis need to be complemented with new studies where molecular microbial ecological approaches are applied. A combination of polymerase chain reaction (PCR) and DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), have successfully been applied in some studies to monitor the changes in the porcine microbiota during the weaning period and after certain dietary treatments. **Chapter 1** summarizes the advantages and the drawbacks of these techniques.

In **Chapter 2** the development of the microbiota and its metabolic activities in the guts of neonatal and weaning piglets was examined. In agreement with earlier cultivation-based approaches, *E. coli* related 16S rRNA gene amplicons were detected in the samples of two days old piglets. When the samples were examined by sequence analysis of 16S rRNA genes and real-time PCR, the

colonization of the neonatal porcine gut by three predominant lactobacilli was evidenced. Following the weaning process, however, these populations were significantly diminished. Emergence of bacteria phylogenetically related to *E. coli* and clostridia, and a significant shift in the predominant microbiota was detected. The concentrations of some key metabolites differed also significantly between the intestinal samples of the unweaned when compared to weaned piglets.

The global concern about the replacement of antibiotics as growth promoters accelerated the search for alternative nutritional strategies, such as the addition of probiotics and prebiotics. These approaches have become an increasingly important consideration in swine nutrition, because of accumulating evidences to their potential benefits in animals and humans. The hypothesis that the dietary addition of specific fermentable carbohydrates may enhance the stability and diversity of the microbiota and stimulate the growth of some beneficial microbes was tested in **Chapters 3** and **4** of the current thesis. The results showed that careful design of the diet can indeed stimulate some anaerobic bacteria in the colon while lactobacilli related to *Lactobacillus amylovorus* were enriched in the porcine ileum. Further studies aiming to cultivate and characterize the *L. amylovorus*-related population revealed that strains isolated from the porcine intestine differ significant from the type strain of *L. amylovorus* (DSMZ 20531T) and the name *Lactobacillus sobrius* sp. nov. was proposed (**Chapter 5**).

Piglets are sensitive to infections and one must emphasize the high mortality around weaning. During that period piglets are particularly susceptible to diarrhoea that can be largely attributed to outgrowth of enterotoxigenic *Escherichia coli* K88. In close collaboration with other research groups, the protective effect of the newly isolated *L. sobrius* against epithelial damages promoted by *E. coli* K88 was demonstrated *in vitro* (**Chapter 6**).

To enable the study of the *L. sobrius* persistence *in vivo*, a comprehensive set of strain- and species-specific DNA oligonucleotide probes were designed and validated. **Chapter 7** describes the development of an efficient strain-specific system based on representative difference analysis (RDA) applied to the genomes of closely related *L. sobrius* isolates. Using this approach, we first identified strain-specific fragments that were subsequently targeted in a real-time PCR assay. In addition to the strain-specific detection, a species-specific quantification based on 16S rRNA gene-targeted real-time PCR amplification was also validated using pure cultures and intestinal samples.

Chapter 8 focuses on the effect of dietary addition of a live *L. sobrius* culture on the immune system and pathogenic prevalence in piglets challenged with *E. coli* K88. Lower levels of *E. coli* K88 in the ileum, modulation of the secretory IgA development, and an improved daily weight gain were evidenced after the administration of *L. sobrius*. The data indicate that it may provide

the first line of defence against harmful pathogens and exert a positive effect on the piglets'body growth. Finally, **Chapter 9** summarizes our current knowledge on the porcine microbiota composition and activities that has been gained in this and other studies. Specific emphasis is given to the *in vivo* and *in vitro* properties of the newly isolated *L. sobrius*. Furthermore, possibilities for the development of future dietary strategies aiming to replace in-feed antibiotics are discussed.

CHAPTER 1

From Composition to Functionality of the Intestinal Microbial Communities

This chapter has in part been published in:

Sergey R. Konstantinov, Nora Fitzsimons, Elaine E. Vaughan, & Antoon D. L. Akkermans. *Probiotics and Prebiotics: Where are we going?* (2002) Caister Academic Press, London, G. W. Tannock (*ed*.), From composition to functionality of the intestinal microbial communities, p. 59-84,

Sergey R. Konstantinov, Christine F. Favier, Wei-Yun Zhu, Barbara A. Williams, Jeannette Klüß, Wolfgang-Bernhard Souffrant, Willem M. de Vos, Antoon D. L. Akkermans, & Hauke Smidt. *Animal Research* (2004) Microbial diversity study of the porcine GI tract during the weaning transition, **53:**317–324.

ABSTRACT

The mammalian gastrointestinal (GI) tract harbours a large bacterial community that has an essential role in creating optimum health conditions for the host. This chapter focuses on the use of molecular fingerprinting tools to describe the taxonomic and functional diversity of the microbial community in the GI tract. Special attention is given to the composition analysis of microbial communities based on 16S rRNA sequence diversity. Basic principles and new developments of several PCR-based methods, such as denaturing gradient gel electrophoresis (DGGE) and related fingerprint methods as well as methods to analyse these fingerprints are described. Advantages and drawbacks of DGGE are described and compared with the terminal restriction fragment length polymorphism (T-RFLP) method. In addition to methods investigating the taxonomic diversity of microbial communities in the GI tract, we also address the recent progress to describe the functional diversity of bacterial communities in the GI tract.

INTRODUCTION

The mammalian gastrointestinal (GI) tract represents a dynamic ecosystem containing a complex community of microaerophilic and anaerobic microbes that are involved in the fermentative conversion of ingested food and the components secreted by the host into the intestinal tract. Insight into the structure and function of the GI tract microbial communities and into the activity of a specific microbial species within this ecosystem is necessary for the development of functional foods such as probiotics and prebiotics. It is now well accepted that many microbes from natural environments including the GI tract have yet to be isolated and characterised (5, 94, 95). Comparison between microscopic and plate counts, group-specific dot-blot hybridisation and Fluorescent *In Situ* Hybridisation (FISH) has indicated that a significant fraction of the microbiota can escape cultivation (25, 46, 81, 87). Similar results have been reported for bacterial inhabitants in a variety of animal models including pigs (47, 67). This is understandable considering the challenges for the microbiologist studying the GI tract. Firstly, the anaerobic conditions in colonic ecosystems require extra facilities and care in handling samples. Secondly, the substantial diversity of GI microbes necessitates the use of suitable selective media and/or sufficiently high-throughout approaches, and incubation times can be significantly longer for anaerobes. Realising the limitation of the traditional methods, such as microscopy and cultivation to gain insight into the structure and activity of the bacterial communities, new molecular microbial techniques have been developed. For the most part these novel methods are based on particular molecular markers, such as the 16S ribosomal RNA (rRNA) or its encoding gene, and are being increasingly used to explore the microbial diversity of bacterial communities (33, 95, 111). A summary of the uses and drawbacks of various techniques for analysis of the mammalian intestinal ecosystem is given in Table 1.

Denaturing Gradient Gel Electrophoresis (DGGE) is a fingerprinting technique used to bypass cultivation and visualise complex bacterial communities without prior knowledge of the composition. In this review DGGE will be described with special focus on the application to the intestinal tract bacterial communities. Other novel fingerprinting techniques including Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and DNA arrays for intestinal diversity will also be briefly

described and compared to DGGE. The potential of further molecular methods for analysing diversity and functionality will also be addressed.

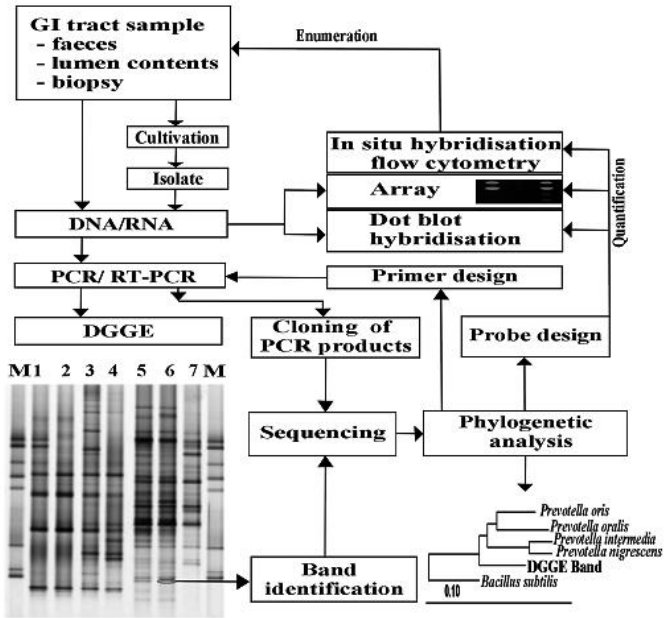
TABLE 1. Overview of the uses and drawbacks of various techniques for analysis of intestinal microbiota.

Methods	Uses	Drawbacks
Cultivation	"Gold standard"; recovery of isolates for further analysis	Not representative; laborious
Direct molecular approaches	Rapid assessment of the bacterial communities composition; uncultured bacteria can be detected; pre-treated samples can be stored for later analysis	Isolates not recovered
● DGGE/ TGGE/TTGE	Rapid and reproducible diversity assessment; easy comparative analysis; allows band extraction from the gel; Southern blot hybridisation	Subject to PCR biases; semi-quantitative; cloning and sequencing is required for bands identification
●T- RFLP	Fast and sensitive fingerprinting method with a potential for high throughput; standardised comparison between gels; size fragment analysis	Subject to PCR biases; Southern blot hybridisation is not possible,
●FISH	Detection; enumeration; possibilities for an automated comparative analysis	Laborious without automation; requires sequence information; probe design and validation
●Dot-blot hybridisation	Detection; quantification of the relative abundance of rRNA	Probe design and validation; relative abundance difficult to relate to cell numbers; laborious at species level
●Real-Time PCR	Rapid detection and quantification	PCR-biases
●16S rRNA gene sequencing	Phylogenetic identification	Large scale monitoring is laborious; PCR-biases
●Non 16S rRNA gene based diversity analysis	Rapid comparative analysis	Requires the use of additional 16S rRNA- based analysis
●Diversity DNA arrays	High throughput detection; identification; estimates the relative abundance	Expensive; under development; not quantitative

ESTIMATION OF DIVERSITY BY MOLECULAR FINGERPRINTING TECHNIQUES

Gradient gel electrophoresis. Several types of gradient gel electrophoresis have been proposed to describe microbial diversity. DGGE was first applied in microbial ecology to study the bacterial diversity in a marine ecosystem (60). Since the first publication a cascade of studies in microbial ecology have used DGGE and Temperature Gradient Gel Electrophoresis (TGGE), and only occasionally Temporal Temperature Gradient Gel Electrophoresis (TTGE). These techniques are particularly suitable for rapidly comparing bacterial communities from different environments and/or monitoring changes in the composition of abundant and/or metabolically active members of a specific community over time. A general strategy for use of these techniques is presented in Fig. 1.

Figure 1. Scheme for analysis of GI tract samples using 16S rRNA-targeted approaches.



Initially, nucleic acids (DNA or RNA) are extracted followed by Polymerase Chain Reaction (PCR) amplification of genes encoding the 16S rRNA and subsequent separation of the PCR products by DGGE, TGGE or TTGE. In case that RNA is analysed, PCR is coupled to a preceeding reverse transcription (RT-PCR). The band identification in TGGE and DGGE may be done either by screening of 16S rRNA clone libraries, or by directly excising the bands from the gel followed by reamplification and sequence analysis. These techniques are presently used for a variety of applications: describing the bacterial community complexity and stability over time in numerous ecosystems, monitoring the enrichment and isolation of bacteria, comparing different DNA/RNA extracting protocols, screening of clone libraries, cloning biases and heterogeneity in rRNA genes (58, 59).

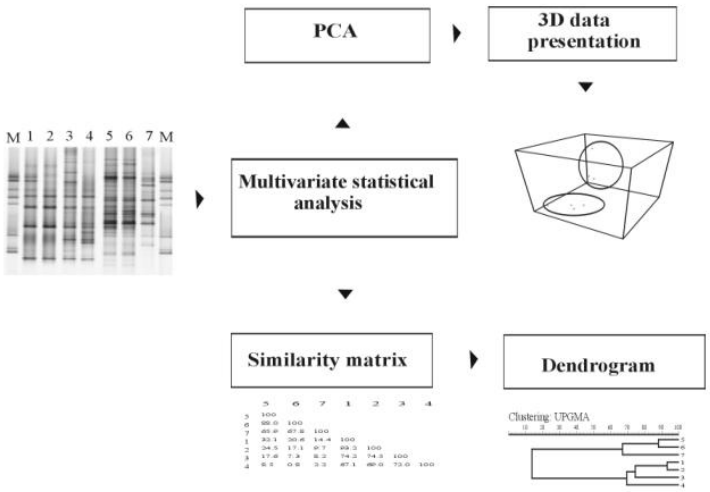
Principal of denaturing electrophoresis techniques. DGGE is a technique that allows the separation of DNA molecules differing by single base changes (62, 63). The separation of DNA fragments of the same length is based on the sequence-specific melting behaviour in a polyacrylamide gel containing either a linear gradient of chemical denaturant during DGGE (26), or a linear temperature gradient as in TGGE (71, 74). Alternatively, the separation is based on a temporal temperature gradient that increases gradually in a linear fashion over the length of the electrophoresis time during TTGE (106). During electrophoresis, each of the DNA molecules (mostly 16S rRNA-targeted PCR amplicons in case of diversity fingerprinting) starts to melt in so-called melting domains. Sequence variations within such domains cause the melting temperatures of the amplicons to differ. The incorporation of a GC-clamp, i.e. a 30 to 50 basepair (bp) GC-rich domain, into the amplicons during PCR by adding it at the 5'-end of one of the primers, prevents complete melting of the DNA molecules. In principle, all single base differences at each position can be separated for PCR products of up to 500 bp (62, 83). Therefore, a mix of fragments with different sequences will essentially stop migrating at different positions in the denaturing gradient. To obtain the best possible separation of different DNA fragments, it is necessary to experimentally optimise either chemical or temperature gradient, and the duration of the electrophoresis (60).

TTGE differs from TGGE in that TGGE has a fixed temperature gradient from the top to the bottom of the gel. In TTGE the temperature at any location in the gel is the same at any particular point in time, but changes with the progression of time

(temporal temperature). TTGE, a modified parallel form of DGGE, does not require the preparation of a chemical denaturant gradient gel and can be performed without a GC clamp (12).

Analysis and interpretation of complex fingerprints. Electrophoretic patterns obtained by DGGE, TGGE and TTGE are often highly complex. Therefore, numerical analysis of banding profiles is essential to obtain an objective interpretation, which by visual evaluation is very difficult to achieve. Interpretation is greatly facilitated by the use of computer assisted pattern analysis using specialised software packages (28). Fig. 2 shows a general strategy of multivariate statistical analysis of fingerprints.

Figure 2. Cluster analysis of the DGGE fingerprints can be achieved by different ways: for example by a dendrogram using the similarity matrix based on Pearson product-moment correlation coefficient between DGGE fingerprints, or by 3 dimensional (3D) data presentation as in Principle Component Analysis (PCA).



DGGE/TGGE patterns can be analysed based on distinct bands or using densimetric curves. Using a band-based method, a collection of fingerprints, irrespective of their complexity, can be transformed into a matrix of binary variables; bands present in a profile at a particular position are designated as 1, and those absent designated 0 (68).

This binary system is representative only of the number of the bands and their position in the fingerprint (richness); consequently, it is not sufficient to express the more complex attributes of profiles such as band intensities, the band area under the peaks or different ratios in the peak heights (evenness).

The analysis of fingerprint patterns generally requires a simplification of the original data via the generation of a proximity matrix based on dissimilarity or similarity criteria. Such proximity matrices can be generated using a wide range of coefficients (68). The Sorenson's coefficient, sometimes refereed to as the Dice coefficient (17), is a pairwise similarity coefficient used to compare species compositions of different ecosystems (30, 48, 52, 57, 84). The Sorenson's coefficient is defined as: $Cs=[2nAB/(nA+nB)]\times100$, where A is the number of bands in lane 1, B represents the number of bands in lane 2, and nAB is the number of common DGGE bands (30, 57, 84).

DGGE or TGGE fingerprints can also be compared using the Pearson product-moment correlation coefficient (66, 108) that is directly applied to the array of densimetric values forming the fingerprint. The product-moment correlation coefficient is insensitive to the relative concentrations of bands between fingerprints, to the background and it is also insensitive to differences in overall intensity of profiles (68). In general, the Pearson product-moment coefficient is better suited for identification of DNA fingerprints profiles than band matching algorithms (31).

Cluster analysis of the DGGE/TGGE fingerprints can be achieved by different approaches, usually by a dendrogram (40) or 3D data presentation as in Principle Component Analysis (PCA) (14, 73, 105) and Canonical Correspondence Analysis (CCA) (77, 90). Several algorithms are available for clustering analysis leading to generation of dendrograms. The unweighted pair group method using arithmetic averages (UPGMA) (85) and the method of Ward (100) are the most widely used clustering algorithms to process DGGE/TGGE profiles. The selection of clustering methods depends on the nature of the original data and the purpose of the analysis. Moreover, while the clustering methods are useful to describe and explain the data extracted from the fingerprints, they do not constitute statistical tests to prove or reject a hypothesis (68).

The complexity of the DGGE/TGGE profiles can also be expressed by diversity indices (Shannon's, Simpson's and Hill's) that account for richness and evenness of a fingerprint, and are calculated from peak number and area or relative intensities of

bands in an individual lane (20, 52, 82). Thus, a distinct diversity value for each sample is obtained and changes in community diversity over time may be observed.

Insight into GI tract microbiota using PCR and DGGE/TGGE. D/TGGE analysis of amplified 16S rRNA gene fragments retrieved from the mammalian GI tract is now recognised as a fast and reliable method to access profiles from the intestinal microbial communities. The ability to analyse a large set of samples in a short time allows for the monitoring of establishment, persistence and composition of the GI tract bacteria over time (2, 88, 95). This has already resulted in substantial knowledge concerning factors that affect the community structure such as environmental disturbances, physiological conditions and the genetic background of the host as described below. Comparison of the TGGE fingerprints from faecal samples of adult humans has demonstrated that the composition of the predominant microbiota is host specific and stable over time (108, 109). By detailed analysis of PCR amplicons generated from the V6 to V8 regions of the 16S rRNA gene separated by TGGE, it was shown that each individual harbours a unique faecal bacterial population suggesting a strong host influence. A similar observation has been made for the DGGE banding profiles of weaning piglets (84). The hypothesis that the genetic background strongly influences the composition of the GI tract bacterial community has been recently tested in a comparative study of DGGE profiles from adults with a different genetic relatedness varying from unrelated persons to monozygotic twins (108). Statistical analysis based on the Pearson product-moment coefficient of pairwise comparison showed that the similarity between DGGE profiles of monozygotic twins was significantly higher than that for genetically unrelated individuals, indicating that the genetic background indeed affects the composition of the predominant microbiota. These findings could partly explain why in controlled trials a probiotic strain has little or no effect on the dominant microbial community in adults relative to the control subjects (16, 95).

PCR-DGGE has also been used to investigate the contribution of non-cultivable bacteria to gut disorders such as necrotizing enterocolitis for the specific case of pre-term infants (55, 56) Moreover, DGGE combined with 16S rRNA sequence analysis have been especially valuable to monitor the establishment of the bacterial community in the new-born intestinal ecosystem (22, 23). After birth and during the first few days thereafter, often only one phylotype is enriched and dominant in the faeces. This was

visualized by DGGE fingerprints of 16S rRNA gene PCR amplicons derived from faecal samples of human babies that were taken at regular intervals after birth. After cloning and sequencing of 16S rRNA genes, it appeared that the first colonizers often belong to *Escherichia coli* or *Clostridium* spp. After a few days, the first signals of *Bifidobacterium* spp. appeared in the DGGE fingerprint. These remained prominent during the time of breast-feeding. After weaning, the DGGE profiles became more complex and several groups of amplicons were replaced by other ribotypes of *Clostridium*, *Ruminococcus*, *Enterococcus* and *Enterobacter* spp. As children grew older, the profiles became more complex and more constant in time. The percentage of similarity with known sequences in databases was usually below 97%, indicating that many of those sequences belong to as yet undescribed species. This conclusion is in agreement with studies of adult humans (109). Earlier studies based on plate counting often reported the occurrence of almost all genera that have been described. The molecular detection of amplicons belonging to *Ruminococcus* spp. that were not detected by plate counting, demonstrated the advantage of the 16S rRNA gene approach. In contrast, it must be admitted that *Bacteroides* spp. that were often found in faeces by plate counting and in clone libraries, were seldom found in a DGGE profile (109). Following molecular analysis of the GI tract microbiota it is likely that similar results will be found in other mammals including pigs.

Another important application of DGGE to the GI tract microbial ecology is the analysis of the response of bacterial community structure to changes in the diet, weaning, antibiotic treatment or the introduction of an exogenous strain to the bacterial community (78, 84, 89). DGGE in combination with FISH and flow cytometry was also used to describe the microbiota attached to human intestinal biopsies, and to identify the genetic diversity of viable, injured and dead bacteria in faeces (7, 112).

Group specific PCR – DGGE profiling. It has been reported that DGGE or TGGE are sensitive enough to visualise populations that constitute up to 1% of the total bacterial community (60, 109). A combination of flow cytometry and PCR-DGGE of dilution series of pure cultures estimated a detection limit of 10^5 cells.ml^{-1}, which was influenced by the DNA isolation protocols (110). The sensitivity of these techniques may be vastly improved by combining specific primers for groups of interest. An

overview of frequently used 16S rRNA gene-targeted primers in gradient gel electrophoresis studies of the GI tract microbiota is presented in Table 2.

Table 2. 16S rRNA-targeted primers used for DGGE, TGGE and TTGE of domain Bacteria, *Lactobacillus* group and *Bifidobacterium* species found in the human and animal intestine

Primer name	Specificity	Primer sequence (5'-3')	References
F 341-GC	Bacteria	(GC clamp)-CCT ACG GGA GGC AGC AG	(60)
R 534	Universal	ATT ACC GCG GCT GCT GG	(60)
R 907	Universal	CCG TCA ATT CCT TTR (A/G)GT TT	(59)
F 968-GC	Bacteria	(GC clamp)-AAC GCG AAG AAC CTT AC	(24)
R 1401	Bacteria	CGG TGT GTA CAA GAC CC	(24)
HDA1-GC	Bacteria	(GC clamp)- AC TCC TAC GGG AGG CAG CAG T	(89)
HDA2	Bacteria	GTA TTA CCG CGG CTG CTG GCA C	(89)
[a]S-G-Lab-0677-a-A-17	*Lactobacillus* group*	CAC CGC TAC ACA TGG AG	(34)
[a]S-G-Lab-0159-a-S-20	*Lactobacillus* group*	GGA AAC AG(A/G) TGC TAA TAC CG	(34)
F Lac1	*Lactobacillus* group*	AGC AGT AGG GAA TCT TCC A	(98)
R Lac 2-GC	*Lactobacillus* group*	(GC clamp)-ATT (C/T)CA CCG CTA CAC ATG	(98)
F Lacto #1-GC	*Lactobacillus reuteri* group	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGT CGA (A/G)CG (A/C)AC TGG CCC	(84)
R Lacto #2	*Lactobacillus reuteri* group	GCT GCC TCC CG(A/G) AGG AGT	(84)
F Bif164-GC	*Bifidobacterium*	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-GGG TGG TAA TGC CGG ATG	(78)
R Bif662-GC	*Bifidobacterium*	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-CCA CCG TTA CAC CGG GAA	(78)
F Bif164	*Bifidobacterium*	GGG TGG TAA TGC CGG ATG	(46, 78)
R Bif662	*Bifidobacterium*	CCA CCG TTA CAC CGG GAA	(46, 78)

[a]Nomenclature according to (4); *Includes genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Weissella*;
(GC clamp)- 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3' (39);
F- forward primer; R- reverse primer

Bifidobacteria, which constitute approximately 3% of the total microbiota of human adults, and up to 90 % in breast-fed babies, usually appear as a single band towards the end of a denaturing gel, presumably due to the high G+C content of their DNA (110). Bifidobacterial specific primers were used to PCR-amplify 16S rRNA gene sequences from human bifidobacterial populations in faeces, and following optimisation of the denaturing gradient, their diversity could be visualised by DGGE

profiling (79). This method has recently been used to monitor faecal bifidobacterial populations in a prebiotic and probiotic feeding trial (78, 79).

However, other important species are present in even lower numbers in the hindgut of adult mammals, such as *Lactobacillus* spp., which have been shown to constitute less than 1% of the total bacterial community in humans (81). Therefore, using universal bacterial primers, it is nearly impossible to monitor this group. Recently, two sets of *Lactobacillus* group-specific PCR primers in combination with DGGE have been used to monitor the molecular diversity of *Lactobacillus* spp. and related lactic acid bacteria in the human intestine (34, 98). The approach has also been used to study the persistence in faecal samples of an emerging *Lactobacillus paracasei* strain F19 administered to children during a clinical trial. In addition to tracking of the F19 strain, the DGGE profiles supported the natural presence of this strain within the intestinal community of a proportion of individuals (34). The authors concluded that DGGE in combination with group-specific PCR analysis of 16S rRNA genes allows for the characterisation of bacteria present in low numbers in the human GI tract. Furthermore, the dietary addition of an exogenous *Lactobacillus* strain was found to exert antagonistic relationship with another indigenous *Lactobacillus* spp. (84). The application of *Lactobacillus*-specific PCR-DGGE analysis also unveiled lactobacilli as part of the porcine predominant microbiota, which decreased during the actual weaning process, but re-established to pre-weaning values soon after, independently of dietary regimes (44). The majority of lactobacilli found in that study were related to *L. amylovorus* or phylotype (OTU) 171 (47). This phylotype has also recently been characterized as one of the most abundant *Lactobacillus* phylotypes in the GI tract of Danish pigs (47).

Recently, additional group-specific PCR-DGGE assays have been reported for other microbial groups relevant to the intestinal ecosystem (93), strengthening the notion that this is indeed a viable approach to visualize functionally important taxonomic groups that are numerically less abundant.

Developments and improvements in denaturing gradient techniques. PCR-DGGE or TGGE are well-established methods to study the bacterial diversity and the community structure in the GI tract. They are fast, reliable and sensitive tools to demonstrate differences between bacterial communities from different individuals and to monitor changes in time. By using universal bacterial primers, the spatial and

temporal distribution of yet to be cultivated species could be analysed. However, bands with an identical position within a DGGE or TGGE profile do not always represent the same 16S rRNA and further analysis is required. The potential drawbacks can be overcome and the sensitivity of the techniques can be improved by a combination of group- or species-specific PCR or by subsequent hybridisation analysis (58). The possibility of identification of existing bands by sequencing or by hybridisation with a specific probe is a strong advantage compared to other fingerprinting methods. Moreover, following cloning and sequencing, probes may be designed and used in hybridisation analysis precluding the need for phylogenetic information (36). In addition to the general limitations of all PCR based methods (80, 96), DGGE and TGGE have their own specific restrictions (59, 61). Heteroduplex molecules introduced during the PCR may affect DGGE analyses (91); DNA fragments produced from different rRNA operons from one organism (64), the comigration of closely related sequences, and only relatively small DNA fragments can be separated (~500 bp). Difficulties in standardising the casting of DGGE and TGGE gels, and maintenance of constant electrophoresis conditions over the long running period (10-16 h) makes comparison of different gels difficult, and requires reasonable experience on the part of the researcher. Nevertheless, the use of a DNA standard ladder on each gel has allowed effective comparison between samples run on different gels (84).

While DGGE/TGGE are considered to be semi quantitative methods, the absolute quantification of band intensity by competitive PCR as already applied in environmental studies (24), still remains to be done for the complex bacterial communities in the GI tract. Recently, a new development of the DGGE technique called constant-denaturant capillary electrophoresis (CDCE) in combination with a novel quantitative PCR (QPCR) approach, accurately enumerated microbial cells at abundance ranging from approximately 10 to 10^4 cells.ml^{-1} (49). To our knowledge, however, the method has not been vastly applied in the analysis of the GI tract bacterial diversity.

Terminal-restriction fragment length polymorphism. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is a molecular method for rapid analysis of microbial community diversity. The technique is a further development of RFLP where one of the 16S rRNA specific PCR primers is labelled with a fluorescent dye to

allow detection of the amplified product. The PCR products are then digested with a restriction enzyme and the DNA sequencer is employed to analyse the size of the terminal restriction fragments (6). Different fluorescently labelled terminal restriction fragments (TRF) in theory may correspond to different bacterial species based on the nucleotide sequence diversity of the 16S rRNA gene. The raw data are automatically converted to a digitised form that can be analysed with a variety of multivariate statistical tools. A variety of tools have now been developed for the *in silico* identification of specific TRFs by comparison to entries in on- and offline sequence databases, such as TAP-TRFP for RDP-II provided by Michigan State University, Center for Microbial Ecology (13, 54)(http://rdp.cme.msu.edu) and TRF-CUT for the ARB project (70), or by comparison to a clone library. The method is used for characterisation of complex microbial communities and assessing community dynamics in a variety of ecosystems (43, 50, 53). TRF patterns are recognised as having better resolution than other DNA based fingerprinting techniques and have also been applied in a variety of studies to investigate complex microbial communities in intestines (32, 37, 41, 42, 48, 75, 76). Potential biases and artefacts associated with this technique have been reported that can be avoided by adapted experimental protocols (18, 19).

PERSPECTIVES

Compositional analysis. Developing PCR primer sets suitable for DGGE/TGGE analyses targeting additional relevant bacterial groups of the intestinal microbiota could provide further insight into this complex ecosystem. Direct analysis of 16S rRNA genes from human faecal samples and porcine intestine revealed that the majority of sequences have low homology with sequences in the databases (47, 87), and strongly suggests that novel and more universal bacteria primers would benefit fingerprinting techniques. Furthermore, functional genes as molecular markers can be used to separate or differentiate species within a genus, or ecologically different populations, where the 16S rRNA sequences are not sufficiently diverse to allow for resolution. This concert of methods will enable us to address fundamental questions on the intestinal microbial community structure at very different levels of complexity, from single cells to major phylogenetic groups. It is clear that analysis of the composition of the microbiota in faeces does not tell us what happens in the small

intestine. Although taxonomic diversity of the microbiota reveals the identity of microorganisms present, it provides only limited information on their potential functional role. Furthermore, a stable and complex community certainly is a prerequisite of a healthy ecosystem!

Diversity and functional DNA microarrays. More recently, microbial function and diversity can also be determined by the design and application of DNA arrays (51, 69, 86, 107). Microarray technology allows a fast analysis of RNA abundance and DNA homology of genes in a single experiment. Two major factors have facilitated the rapid development of the DNA array technology during the past decade – the large scale genome sequencing and the ability to immobilise thousands of DNA fragments on a surface, such as coated glass slides or membrane. An entire microbial genome can be easily represented in a single array, allowing genome-wide analysis. By using genome fragments and DNA microarray technology the similarity or differences in genetic contents between species can be also estimated. Currently, a number of bacterial species ranging from pathogens, laboratory strains and environmental isolates are being studied by DNA chip technology, including commensal inhabitants of the mammalian intestine (15, 103, 104).

DNA microarrays are basically a form of dot blot, but in a high-throughput format. According to the size of the fragments spotted on the array there are two major types of DNA microarrays. One is the oligonucleotide-based array and the other is the PCR product-based array. A DNA microarray experiment includes array fabrication, probe preparation, hybridization and data analysis (69). DNA microarrays are becoming a common tool in many areas, including microbial physiology, pathogenesis, phylogeny and ecology. A drawback of the current DNA microarrays is, however, the low degree of the signals quantification. The recent development of general as well as ecosystem-tailored diversity microarrays has brought forward a novel generation of high-throughput microbial ecological tools, bearing a large potential for composition analysis and phylogenetic studies, including the complex microbiota residing in the GI tract (8, 21, 99, 101). To address functional diversity in microbial ecosystems, microarrays have also been developed that target different functional genes for monitoring relevant environmental processes (51, 102). In conclusion, the future design of a functional–chip containing probes targeting unique 16S rRNA and/or 23S rRNA sequences for most of the representative GI bacteria and also genotype markers

of the major biochemical pathways known from the intestine will reveal the relationships between the members of the intestinal microbiota and the host microbial interactions in the gut.

Functional analysis. Although the aforementioned methods have been developed to determine the composition and diversity of the GI tract microbiota (47, 87, 109), the functionality of its constituent populations also needs to be investigated. The functional efficacy of a particular organism in the complex microbial environment of the GI tract is dependent on its numerical abundance, survival, competitiveness and metabolic activity. Assessment of the functionality of a particular microorganism in the gastrointestinal milieu necessitates the use of molecular approaches to specifically detect expression of a particular set of genes. Although the development of techniques to detect *in vivo* bacterial gene expression is underway, such as the use of *in vivo* expression technology (IVET) (9), or green fluorescent protein (gfp) gene from *Aequoria victoria* as a marker to e.g. detect in vivo expression and location of a *Lactobacillus* strain in the mouse GI tract (29), there is a need to develop additional methods to detect prokaryotic gene expression in complex ecosystems.

Most of the currently available methodology for transcriptional analysis is applicable only to poly (A)-tailed messenger RNA (mRNA), which is rarely present in prokaryotes. Since only 4-5% of total bacterial RNA is comprised of mRNA, detecting transcription of genes, particularly those that are poorly expressed or that have unstable messengers, remains technically challenging. Specific prokaryotic gene expression has mainly been monitored in pure cultures using techniques such as Reverse Transcriptase-PCR (RT-PCR) (35), *in situ* RT-PCR (ISRT-PCR) coupled with *in situ* hybridisation (ISH) and flow cytometry (10), ISRT-PCR (38, 45) or ISH using labelled mRNA probes (97) coupled with epifluorescence or phase contrast microscopy, or customised amplification libraries (3). Assessment of bacterial gene expression has been achieved within complex ecosystems such as the expression pattern of *Helicobacter pylori* during its infection of the gastric mucosae using real time RT-PCR (72), lignin-degrading communities by ISRT with microscopy (11), in low biomass sediments with RT-PCR (65), and soils by Northern hybridisation (92). However, in terms of microbial density, such environments are less complex relative to faeces by several orders of magnitude (92), and its varying composition and presence of inhibitors present a challenge to the extraction of mRNA at sufficient

quality and purity for downstream analyses. A molecular protocol has been developed in our laboratory to assess bacterial activity by detection of mRNA by reverse transcription-PCR (RT-PCR) in GI tract samples (27). This methodology has potential applicability to the assessment of the *in vivo* gene expression/activity of a specific microbe in a complex ecosystem, such as a probiotic culture in the GI tract, where its efficacy in conferring health-promoting/immunomodulating effects to the host is a function of its competitiveness and metabolic activity in the microbial environment.

Monitoring differential gene expression in micro- and higher organisms has been greatly facilitated by the development of DNA microarrays. This technology was exploited to monitor global intestinal transcriptional responses to colonisation of germ-free mice with *Bacteroides thetaiotamicron*, a prominent member of the normal murine and human intestinal microbiota (39). The cellular location of selected responses was determined by laser-capture microdissection. This study showed that this commensal was able to modulate expression of host genes participating in diverse and fundamental physiological functions, including nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis and postnatal intestinal maturation. To determine if other members of the GI tract microbiota elicited similar host responses, the mice were colonised with *Bifidobacterium infantis* and *E. coli*. The resultant species selectivity for some of the colonisation-associated changes in gene expression highlights how changes in host physiology can be affected by changes in the composition of the indigenous microbiota.

The development of ultra-fast genome sequencing techniques which could allow a human genome to be sequenced in days (1), and advances in data mining technology imply that detection of differences in global gene expression is within our grasp and will be the ultimate means of determining the efficacy and functionality of our intestinal microbes and probiotics *in vivo*. In particular this will aid in the unravelling of the mechanisms underlying the postulated health-promoting effects of probiotics and uncover novel functionalities of our commensal microbiota.

ACKNOWLEDGEMENTS

This work has been carried out with financial support of the European Community specific RTD programme "Quality of Life and Management of Living Resources" research projects: HEALTHYPIGUT (QLK5-LT2000-00522) and Microbe Diagnostics (QLK1-2000-00108). Thomas D. Leser is gratefully acknowledged for his hospitality to S. R. Konstantinov in Danish Veterinary Laboratory, Department of Microbiology, Copenhagen V, Denmark and for excellent discussions on T-RFLP analysis.

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CHAPTER 2

Postnatal Development of the Porcine Microbiota Composition and Activities: Implications for the Weaning Process

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ABSTRACT

The current study describes the development of the porcine microbiota and its metabolic activities during the neonatal and weaning period. Using 16S rRNA-based approaches, we analyzed the ileal and colonic microbiota of neonatal piglets at days 2, 5, and 12 after birth. Furthermore, to investigate the effect of weaning at three weeks of age, nineteen days old piglets (n=64) were randomly allocated into two groups. Half of the piglets remained with their sows throughout the study, whilst the remaining piglets were weaned. Piglets from each group were sacrificed at 19, 23, 27 and 32 days of age. As revealed by sequence analysis of 16S rRNA amplicons, the samples of two days old piglets harbored a consortium of *Escherichia coli*, *Shigella flexneri*, *Lactobacillus amylovorus*-like, *L. reuteri,* and *L. acidophilus* related sequences. Moreover, species-specific real-time PCR assays unveiled that *L. amylovorus*-like and *L. reuteri* predominated in the ileal samples of the neonatal and unweaned piglets with population levels up to 7×10^8 cells/g of lumen content. Following weaning, however, the two lactobacilli were detected at significantly lower levels ($<10^3$) in the ileal samples. A shift in composition and metabolic activities of the predominant microbiota, and emergence of clostridia and *E. coli*, were encountered in the intestinal samples of the piglets after the early post-weaning period. The data indicate that the populations of specific lactobacilli are significantly suppressed during the weaning transition, leaving the animal host vulnerable to proliferation of potential pathogens.

manuscript in preparation

INTRODUCTION

The contribution of the commensal gastrointestinal (GI) tract microbiota to mammalian host health and performance, including provision of nutrients, stimulation of immune response, and protection from pathogens, is becoming increasingly apparent during the neonatal and weaning period of monogastric animals (10). Starting at birth, the microbiota must ultimately develop from a simple, unstable into a complex and climax community, a process that can be influenced by diet, environmental factors, and the host itself (14). In contrast to the slow weaning process of human infants, piglets in the commercial animal production setting experience an early and critical transition from sow's milk onto a solid diet, rich in plant polysaccharides, between 21 and 28 days of life. As weaning progresses, piglets become vulnerable to a higher incidence of gastrointestinal and respiratory diseases (10). Often, the presence of a pathogen alone is not sufficient to cause disease since the host's defence mechanisms are able to resist the effects of the pathogen. However, some factors can adversely influence the host and thus lead to disease. The changes in the composition and activity of the GI tract microbial community after weaning have been suggested among the factors that predispose the animals to pathogenic infections (10). Qualitative and quantitative analysis of the predominant microbiota and its biochemical activities around weaning is, therefore, essential for the understanding of the porcine GI tract colonization by commensal bacteria. Moreover, the role of specific bacteria in the creation and the maintenance of so-called healthy porcine microbiota needs a further examination.

In addition to the host cell non-immune system of defense, bacteria of the resident gut microbiota exert a barrier effect against pathogens (18). It has been reported that *Escherichia coli*, one of the first bacterial genera that colonize the intestine of humans and piglets, displays antimicrobial activity against *Salmonella* infection (12). Other species of the endogenous human and porcine microbiota such as *Bifidobacterium* and *Lactobacillus* spp., exert antimicrobial activity (18, 23, 30). Furthermore, the intestinal commensal microbiota interacts with a wide range of physiological functions of the host, as evidenced by the maldevelopment of the intestinal functions of germ-free animals (7). Germ-free rodents require a higher calorific intake to maintain their weight than those with intestinal microbiota (34). Moreover, commensal microbes are essential for the development of the innate and adaptive immune system in the intestine (2, 7, 29). Since the intestinal microbiota interacts ultimately with the animal host, insight in its temporal, spatial, and functional development is needed. This information is required for the development of nutritional strategies aiming to prevent GI tract infections diseases, while improving the host performance during and beyond weaning.

The knowledge of the intestinal microbiota development in suckling and/or weaned piglets is limited and derived mainly from cultivation-based studies (28). *E. coli*, lactobacilli, and streptococci have been detected in the stomach and small intestine of new born piglets three hours after birth. While the initial low numbers of these microaerophilic bacteria increased to up to 10^6 to 10^8 cell/ml (27), anaerobes like *Bacteroides* spp. have not been found in the small intestine in the first 24 hours after birth. It has been reported that they appeared two days after birth in the colon (28). In the majority of cultivation-based studies, however, identification to species level has been achieved for less than 10% of all isolates (28). Furthermore, the plating in selective media relies on the assumption that all bacterial groups show equal plating efficiency or viability (16). However, according to recent estimates, a significant fraction of the entire mammalian intestinal microbiota may be uncultivable by conventional cultivation techniques (35). These limitations inherent to culture-dependent approaches can partly be overcome by molecular techniques such as those based the sequence diversity of the 16S rRNA gene. A combination of polymerase chain reaction (PCR) and DNA fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), has successfully been applied to monitor the changes in the porcine microbiota during the weaning and after certain dietary treatments (13, 26). However, it is important to examine the changes in the porcine intestinal microbiota occur in all early phases of the weaning, and to assess whether these are affected by diet or host. Recent studies of human infants showed that the microbiota diversifies significantly before weaning, suggesting that the effect of weaning needs to be studied carefully, taking all dietary, environmental, and host factors into consideration (5, 6).

This paper describes the changes in structure and metabolic activity of the predominant porcine ileal and colonic bacterial communities after birth and during the weaning transition. Weaning at three weeks of age was observed to have a profound effect on the diversity and the metabolic activities of the porcine microbiota. Moreover, the abundance of several key populations, notably *L. amylovorus*-like and *L. reuteri* species, was significantly lower during the weaning, while the same bacteria remained stable over time in the intestine of the neonatal and unweaned piglets.

MATERIALS AND METODS

Experimental design. For each replicate of the experiment, two sows from a local commercial pig farm were allocated to the experiment. The sows were allowed to farrow under standard commercial conditions and the piglets were reared to 21 days on the sow with no creep feed. The piglets were ear-tagged within 24 hours of birth and piglets were randomly allocated to weaned or

unweaned treatment groups. At 21 days of age, the weaned group animals were removed from the sow and transferred to the pig facility at the University of Bristol's School of Veterinary Science where they had *ad libidum* access to the standard HealthyPigut EU project diet (Table 1) and fresh water. Piglets from each group were sacrificed at 19 (pre-weaning), 23, 27 and 32 days of age, and tissue and digesta samples were collected. The animal experiment was conducted in four replicates including in total sixty-four piglets.

Independent of the experiment above, samples of two piglets, sacrificed at day 2, 5, 12 of age, were also collected and used for comparing the microbiota of the neonatal, unweaned and weaned piglets.

TABLE 1. Diet composition.

Ingredients	g/100g
Wheat seeds	24,31
Barley seeds	24,50
Soybean meal	16,00
Maltodextrin	4,00
Dehydrated whey	15,00
Soluble fish protein concentrate	8,25
Sunflower oil	2,80
Calcium carbonate	1,50
Monocalcium phosphate	2,00
Trace elements and vitamin premix	0,50
L-lysine HCl	0,13
DL-methionine	0,85
L-threonine	0,13
L-tryptophan	100,00
Crude protein	21,3
Digestible lysine	1,2
Digestible methionine	0,46
Digestible sulphur amino acids	0,75
Digestible threonine	0,81
Digestible tryptophan	0,23
Net energy , Mcal/kg	2,4

Sample collection and DNA isolation. Samples of gut content from the second half of the small intestine and the colon of all piglets were divided into aliquots. One of the aliquots was used for genomic DNA extraction by using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA) as described (13). In parallel, aliquots from the same samples were fixed for determination of lactic acid, volatile fatty acids (VFA) and ammonia concentrations. Dry matter content of the ilea and the colonic lumen samples was also analysed.

DGGE analysis. The amplicons obtained from the ileal and colonic lumen-extracted DNA were separated by DGGE according to the protocol published earlier (13). All gels were scanned at 400 dpi and analysed using the Bionumerics software package version 3.0. (Applied Maths, Kortrijk, Belgium). After normalization, bands were defined for each sample using the bands searching algorithm within the program. A manual check was done using the according densitometric curves, and the DGGE bands constituting less than 1% of the total area of all bands were omitted from further analysis (16). Clustering was done using the Dice similarity coefficient and the UPGMA method. The correlation between the DGGE profiles were plotted for the samples from day 2, 5, 12, 19, 23, 27, and 32 to evaluate the bacterial community stability using the principle of moving window correlation (22).

TABLE 2. List of DNA oligonucleotides used in this study.

Oligonucleotides	Sequence 5'-3'	Target	Reference
S-D-Bact-0011-a-A-17	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	16S rRNA	(17)
S-D-Bact-1492-a-A-19	GGTTACCTTGTTACGACTT	16S rRNA	(17)
S-D-Bact-0968-a-S-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGG-GGGCACGGGGGGAACGCGAAGAACCTTAC	16S rRNA	(21)
S-D-Bact-1401-a-A-17	CGGTGTGTACAAGACCC	16S rRNA	(21)
S-G-Lab-0159-a-A-2	CGGTATTAGCACCTGTTTCC	16S rRNA	Chapter 7
L-*-OTU171-0077-a-S-2	ACTTCGTAATGACGTTG	16S rRNA	Chapter 7
L. reuteri-91_FOR	TGAATTGACGATGGATCACCAGTG	16S rRNA	(3)
L. acidophilus-70_FOR	AGCTGAACCAACAGATTCAC	16S rRNA	(32)
K88AD_F	GGCACTAAAGTTGGTTCA	*E. coli* (ETEC)	(1)
K88AD_R	CACCCTTGAGTTCAGAATT	*E. coli* (ETEC)	(1)
T7	TAATACGACTCACTATAGG	Promega	
Sp6	GATTTAGGTGACACTATAG	Promega	

PCR amplification. All PCR primers used in this study are listed in Table 2. Primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 (21) were used to amplify the V6–V8 region of the 16S rRNA gene with the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures (50 µl) contained 0.5 µl of *Taq* polymerase (1.25 U), 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 3.0 mM $MgCl_2$, 200 µM of each dNTP, 5 pmol of the primers, 1 µl of DNA diluted to approximately 1 ng/µl and UV sterile water. The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and the cycling consisted of 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40 s, and 68°C for 7 min final extension. Aliquots of 5µl were analyzed by electrophoresis on 1.2% agarose gels (w/v) containing ethidium bromide to check for product size and quantity.

Generation and screening of 16S rRNA gene clone libraries. Using primers S-D-Bact-0011-a-S-17 and S-D-Bact-1492-a-A-19 (Table 2), 16S rRNA gene-targeted PCR was performed on DNA isolated from selected porcine ileal samples with a *Taq* DNA polymerase kit from Life Technologies as described previously (13). After amplification, the PCR product was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) and cloned into pGEM-T (Promega, Madison, WI) using competent *E. coli* JM109 as a host, and as previously described (13). Plasmid DNA was isolated using the Wizard Plus purification system (Promega), and used for sequence analysis of the cloned 16S rRNA genes by using a Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, UK) according to the manufacturer's specifications and using either the T7 or SP6 primers labelled with IRD-800. Sequences with appoximate lenght of 500 bp were automatically analysed on a LI-COR DNA Sequencer 4000L (LiCor, Lincoln, NE), and compared to those available in public databases by using BLAST analysis (20).

Reference strains used for real-time PCR analysis. *L. amylovorus*-like strain 001^T (Chapter 5, this thesis), *L. reuteri* DSMZ 20016^T, and *L. acidophilus* $DSMZ20079^T$ were propagated at 37°C anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Difco, Le Point de Claix, France). *E. coli* strain K88 was grown in Luria-Bertani broth containing 1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0. The cultures (2ml) were harvested after 24h by centrifugation at 5,000 x g for 10 min and washed with 0.2 µm pore size filtered PBS (per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4; pH 7.2). The bacterial pellet was finally resuspended in 1 ml PBS. Ten µl of each culture was used for total cell counts, using 4', 6-diamino-2-phenylindole (DAPI) staining coupled to microscopy analysis as described (13). Isolation of genomic DNA from the remaining 990 µl bacterial culture was done using the Fast DNA Spin Kit (Qbiogene). Finally,

genomic DNAs were diluted to concentrations corresponding to 10^8 to 10 cells per real-time PCR reaction and used for generation of real-time PCR standard curves.

Real-time PCR assay for quantification of *L. amylovorus*-like strain 001^T, *L. reuteri* DSMZ 20016^T, *L. acidophilus* DSMZ 20079^T, and enterotoxigenic *E. coli* strain K88. Species-specific PCR primers targeting the four different species are listed in Table 2. Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, The Netherlands). A reaction mixture (25µl) consisted of 12.5 µl of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set, and 5 µl of the template DNA. The PCR conditions for the quantification of the three lactobacilli were: an initial DNA denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 62.5°C for 45 s. Enterotoxigenic *E. coli* strain K88 was quantified using the following PCR program: 40 cycles: 92°C for 45 s, 50°C for 45 s, 72°C for 45 s (1). For the determination of the number of *L. amylovorus*-like strain 001^T, *L. reuteri* DSMZ 20016^T, *L. acidophilus* DSMZ 20079^T, and enterotoxigenic *E. coli* strain K88 in porcine samples, fluorescent signals detected from two serial dilutions were compared to a standard curve generated with the respective bacterium in the same experiment.

Lactic acid, volatile fatty acid and ammonia analysis. The lactic acid concentrations in ileal lumen samples were analyzed by High Performance Liquid Chromatography (Jasco instruments) using a column (Supelcogel, C-610H, 30cm*7.8mm ID) and precolumn (Supelcoguard, C-610H, 5cm*4.6mm ID) with 1% H_2SO_4 as the mobile phase. The concentrations were determined by UV detection at 210 nm. Volatile fatty acid (VFA) concentration in the samples were analysed by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy), using a glass column fitted with Chromosorb 101, as carrier gas N_2 saturated with methanoic acid, at 190 °C and using *iso*-caproic acid as internal standard. The ammonia concentrations were determined according to the method described (11). Supernatant was deproteinized using 10% trichloro-acetic acid. Ammonia and phenol were oxidized by sodium hypochlorite in the presence of sodium nitro-prusside to form a blue complex, which was measured colorimetrically at a wavelength of 623 nm.

Statistical analysis. Differences between the groups (weaned and unweaned), site of GI tract and slaughtering age, and the interactions between them were tested for significance by ANOVA using the repeated measurement procedure with the following model:

$$Y_{ijkl} = \mu + W_i + G_j + S_k + (W*G)_{ij} + (W*S)_{ik} + (G*S)_{jk} + (W*G*S)_{ijk} + \varepsilon_{ijkl}$$

Where Y is the parameter to be tested, μ is the overall mean, W_i effect of the group i; G_j effect of site of GIT j; S_k effect of slaughter day k; ε_{ijkl} is the error term. The effect of replicate and litter was tested separately, and was not significant for any of the parameters. It was therefore removed from the statistical model. Differences between treatment means were evaluated using Tukey test of multiple comparisons. Differences were considered significant, when $p < 0.05$.

The observations on day 19 were not included in statistical analysis as those piglets were not yet divided into weaned and un-weaned group. The means of the observations on D19 are presented separately within Table 5.

All statistical analyses were performed using the PROC GLM procedure of the statistical program SAS (24).

RESULTS

Outline of the experiment and general observations. To investigate the effect of weaning at three weeks of age on the diversity and activity of the GI tract microbiota, 64 piglets (19 days of age) were randomly allocated into two groups. Half of the piglets remained with their sow throughout the experiment (unweaned group), while the rest were weaned at 21 days of age (weaned group). Piglets from each group were sacrificed at 19, 23, 27 and 32 days of age. The bacterial colonization in ileum and colon of the weaned and the unweaned group was compared with samples from six normally developing and healthy neonatal piglets.

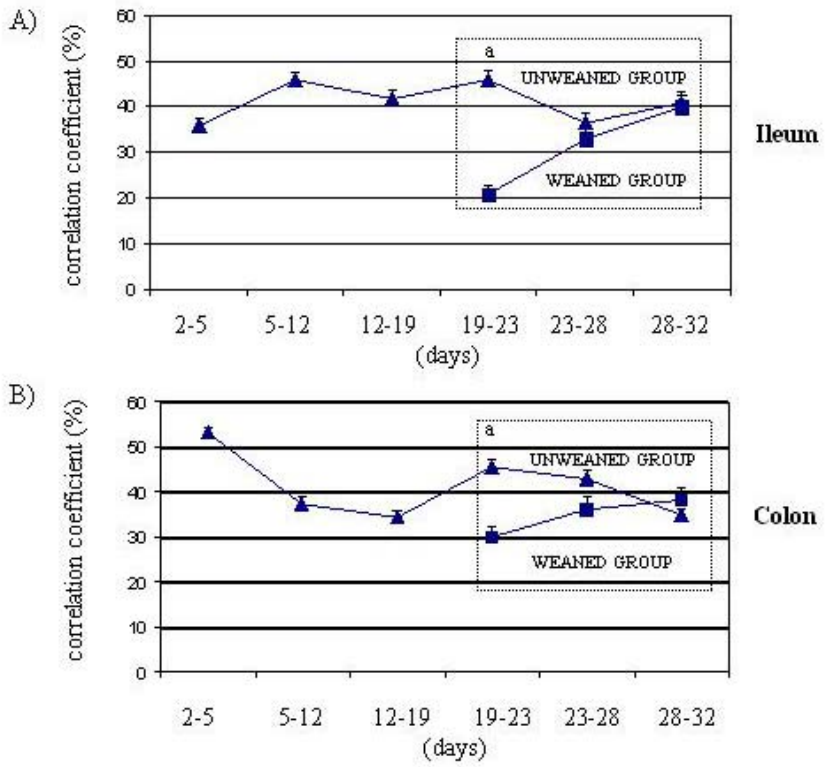
PCR-DGGE analysis of the bacterial community in the ileum and colon of neonatal, unweaned and weaned piglets. 16S rRNA gene-targeted DGGE fingerprinting analysis was performed on ileal and colonic lumen samples of piglets sacrificed at 2, 5, 12, 19, 23, 27 and 32 days of age. For all samples analysed, we first assessed the number of amplicons detected in the two GI tract locations across all time periods. A significantly higher number of amplicons (average ± STDEV, 16.4 ± 3.7, $p<0.05$) in the colonic compared to the ileal lumen samples (9.1 ± 3.1) was evident for all piglets at all time points reflecting the differences in spatial colonization along the gut. The number of DGGE bands in the samples from the ileum content of suckling piglets at day 2, 5, 12, and 19 days of age was not statistically different, and the same was found for the samples from the colon (data not shown). A slightly higher number of amplicons, although not statistically significant, was observed, when ileal samples of weaned piglets were compared with unweaned piglets on day 23 of age (12 ± 3.8 versus 9.5 ± 3.6 amplicons, respectively). Furthermore, no

significant differences were found for the number of amplicons occurring in the colon of piglets at day 23, and in the ileal and colonic lumen samples of the weaned and the unweaned piglets at day 27 and day 32 of age (data not shown).

The stability of the microbial community structure was assessed by measuring similarities between DGGE profiles over time using the moving window correlation approach. When the correlation coefficients between days 2-5, 5-12, 12-19, 19-23, 23-27, and 27-32 for the ileal and colonic samples were plotted against time, a graphical representation of the development of microbiota stability during the neonatal and weaning periods was obtained (Fig. 1). The highest variability was seen for the ileal and colonic bacterial community after weaning (days 19 to 23). Within that period, the DGGE profiles of the ileal samples of the unweaned group showed 45% similarity, whereas that similarity in the weaned group was a significantly lower (21%, $p<0.05$) (Fig. 1A). Similar changes were seen in the colonic samples (Fig. 1B). The comparison between the similarities of the gut samples from earlier or later time points were not statistically different. These results indicate a significant fluctuation in the porcine bacterial community composition in the early period after weaning.

16S rRNA gene clone library analysis of ileal lumen microbiota in neonatal, unweaned and weaned piglets. The 16S rRNA genes from the ileal lumen samples of two newborn piglets (2 days old), two weaned and two unweaned piglets (23 days of age) were amplified and cloned into *E. coli*, and 10 clones per sample were partially sequenced (Table 3). Approximately 30 % of all clones (n=12 out of 40) analysed from the samples of the neonatal and the weaned piglets were found to have high similarities (>98%) to 16S rRNA of *E. coli* and *Shigella flexneri*. Furthermore, in the samples from the neonatal and unweaned piglets, clones (n=12, representing 30% of all sequenced amplicons) were detected that were matching (>98%) with the 16S rRNA of *L. amylovorus*-like, *L. acidophilus johnsonii*, and *L. reuteri* (DSMZ 20016^T). *L acidophilus johnsonii*, *L. reuteri* (DSMZ 20016^T), but not *L. amylovorus*-like 16S rRNA gene amplicons were also detected among the identified clones from the weaned group samples. The samples from the weaned piglets were also found to harbor more sequences (n=8, 40%) with distinct similarities to 16S rRNA of *E. coli* and *Clostridium* spp., compared with the age matching piglets from the unweaned group (n=1, 5%).

FIgure. 1. Moving window correlation for the neonatal, weaned, and unweaned piglets, representing the ileal (A) and colonic (B) samples, respectively. Variability between consecutive sampling days was calculated from DGGE patterns of the general bacterial community using the Bionumerics software and correlation coefficients (Dice) for pairwise comparison. Bars indicate the standard error. Significant difference is highlighted by (**a**), $p<0.05$



Quantitative real-time PCR analysis of the predominant microbiota in ileal lumen samples of neonatal, unweaned and weaned piglets. Selected ileal lumen samples, including those used for the generation of 16S rRNA gene clone libraries, were examined by real-time PCR. The populations of the predominant lactobacilli detected by the sequencing analysis, *L. amylovorus*-like, *L acidophilus johnsonii*, and *L. reuteri* (DSMZ 20016^{T}) were quantified using species-specific assays (Table 4).

In addition, the occurrence of enterotoxigenic *E coli* strain K88 was assessed by strain-specific real-time PCR. The calculated numbers were compared between the groups and across the different time points. This analysis revealed that the populations of *L. amylovorus*-like and *L.*

reuteri were significantly higher in all samples of neonatal and unweaned piglets compared with the weaned piglets. *L. acidophilus* was detected at a significantly lower level throughout the neonatal and the weaning period. Enterotoxigenic *E. coli* strain K88 was detected in only one sample from a piglet at 12 days of age and in two samples of weaned piglets at 23 days of age (data not shown).

TABLE 3. Clones that were retrieved from ileal lumen samples of two neonatal piglets of two days of age (Neo), two weaned piglets (W) and two unweaned piglets (UW). The clones are listed according to their abundance in the six different 16S rRNA cloning libraries. The percentage of identity to known sequences in Genbank.

No	Closest relative in Genbank (accession no.)	Piglets Neo	W	UW	Average similarity (%)
1.	*Escherichia coli* (AF527827)	4	3	0	98
2.	*Shigella flexneri* (AE016989)	5	1	0	99
3.	*L. amylovorus*-like (AF371469)	4	0	3	99
4.	*L. acidophilus johnsonii* (M99704)	2	2	3	99
5.	*L. reuteri* (DSM 20016^{T})(X76328)	2	1	4	98
6.	*Clostridium* sp. (AF390549)	0	5	1	97
7.	*Streptococcus bovis* (AF396922)	0	2	3	96
8.	*Bacillus* sp. (16973341)	0	1	2	97
9.	*Pasteurella aerogenes* (U66492)	1	2	0	98
10.	*Acinetobacter* sp. (2326328)	1	1	0	99
11.	*Megasphaera elsdenii* (AY196917)	0	1	2	99
12.	*Vagococcus carniphilus* (AY179329)	0	1	1	97
13.	*Neisseria* sp. (AJ7868091)	1	0	0	93
14.	*Selenomonas* sp. oral clone (AY349410)	0	0	1	97
Total number of clones		20	20	20	

Fermentation end-products and dry matter. The least square means of the dry matter and the fermentation end-products such as VFA, lactic acid, and ammonia are shown in Table 5. These parameters were significantly different between the two GI tract locations over time. Furthermore, the dry matter content of all ileal and colonic lumen samples decreased in both weaned and unweaned piglets over the time course of the experiment ($p<0.05$).

TABLE 4. Real-time PCR results for *L. amylovorus*-like, *L. reuteri*, and *L. acidophilus* in porcine ileal samples. Two samples were examined for the samples of 2, 5, and 12 days old piglets. Four samples were quantified for all 23, 27, and 32 day old piglets. UW, unweaned piglets; W, weaned piglets. Counts are expressed as mean ± SD cells g^{-1}.

DAY-treatment	*L. amylovorus*-like	*L. reuteri*	*L. acidophilus*
2	$4.72 \pm 3.9 \times 10^7$	$1.34 \pm 0.5 \times 10^7$	$4.84 \pm 0.1 \times 10^4$
5	$9.63 \pm 1.2 \times 10^8$	$2.13 \pm 2.17 \times 10^7$	$7.61 \pm 0.9 \times 10^4$
12	$1.01 \pm 1.05 \times 10^8$	$3.08 \pm 3.97 \times 10^7$	$7.22 \pm 1.1 \times 10^4$
19	$6.85 \pm 0.1 \times 10^8$	$1.48 \pm 1.3 \times 10^7$	$1.04 \pm 0.1 \times 10^4$
23-UW	$4.72 \pm 3.9 \times 10^7$	$1.34 \pm 0.5 \times 10^7$	$4.84 \pm 0.1 \times 10^4$
23-W	$<10^3$	$<10^3$	$<10^3$
27-UW	$2.46 \pm 0.9 \times 10^8$	$1.07 \pm 0.8 \times 10^7$	$4.91 \pm 0.1 \times 10^4$
27-W	$<10^3$	$<10^3$	$<10^3$
32-UW	$1.18 \pm 0.9 \times 10^7$	$6.13 \pm 1.3 \times 10^7$	$<10^3$
32-W	$<10^3$	$<10^3$	$<10^3$

Ammonia concentration was significantly lower ($p<0.05$) for the weaned, when compared with the unweaned group, for both ileum and colon. Moreover, a significantly higher lactic acid concentration was detected in the ileal samples of weaned compared with the unweaned piglets over the period of the study. The total VFA concentration in the ileal and colonic contents of the piglets was not significantly affected throughout the weaning period. Except for the branched chain fatty acids, no effect of weaning was found for the concentrations of individual VFA in the GI tract of the piglets.

DISCUSSION

The current study describes compositional and functional aspects the bacterial colonization process in the GI tract of neonatal and weaning piglets. Using 16S rRNA-based approaches, *L. amylovorus*-like and *L. reuteri* were found to predominate in the ileal samples of two days old piglets. During the course of the study, the two lactobacilli were able to colonize and persist in the GI tract of the unweaned piglets, while they were detected at significantly lower levels in the weaned group. Moreover, significant changes in the bacterial community composition and the products of its metabolic activities were evidenced in the intestinal samples of the piglets weaned at three weeks of age.

TABLE 5. The least square means of the dry matter and the fermentation end-products such as VFA, lactic acid, and ammonia; ns, not significantly different ($p>0.05$); *significant effect ($p<0.05$); when the effects of slaughtering day (19, 23, 27, and 32), group (UW, unweaned; W, weaned group), GI tract site (ileum, colon), and the interactions between them were examined.

Age (days)	GIT Site	Group	Dry matter (g/Kg chyme)	Ammonia ($mmol.L^{-1}$)	Lactic acid ($mmol.L^{-1}$)	Total VFA ($mmol.L^{-1}$)	Molar proportions (% of total VFA)			
							Acetic	Propionic	Butyric	Branched Chain
19	Colon	UW	605.01	131.62	-	95.49	66.41	18.30	7.28	5.34
	Ileum	UW	549.41	50.10	47.84	26.42	87.40	9.24	1.08	1.42
21	Colon	UW	674.52	359.42	-	93.92	64.32	19.72	7.56	5.44
		W	465.98	40.33	-	65.16	67.68	19.05	6.69	4.29
	Ileum	UW	453.42	43.22	42.83	52.49	79.51	13.51	2.77	2.54
		W	485.98	17.32	94.92	42.08	83.78	10.79	1.55	1.77
27	Colon	UW	624.02	362.19	-	136.77	70.26	17.64	5.53	4.66
		W	407.97	55.53	-	120.25	62.16	22.35	8.84	4.08
	Ileum	UW	403.46	35.76	37.17	72.21	86.75	8.79	1.55	2.04
		W	361.83	13.59	87.36	15.46	88.31	7.17	0.56	1.74
32	Colon	UW	411.68	88.64	-	76.31	63.33	20.82	8.45	5.21
		W	367.95	67.23	-	123.4	61.32	23.27	8.74	3.93
	Ileum	UW	273.34	27.25	48.17	14.6	87.05	8.59	1.28	1.99
		W	258.13	19.69	64.78	28.32	84.2	8.87	2.39	1.89
Slaughtering day			*	ns	ns	ns	ns	ns	ns	ns
Group			ns	*	*	ns	ns	ns	ns	*
Slaughtering day*Group			ns	ns	ns	ns	ns	ns	ns	ns
GIT site			*	*	-	*	*	*	*	*
Slaughtering day*GITsite			ns	ns	-	ns	ns	ns	ns	ns
Group*GITsite			ns	ns	-	ns	ns	ns	ns	ns
Slaughtering day*Group*GIT site			ns	ns	-	ns	ns	ns	ns	ns

The developmental process of the intestinal microbiota is similar for humans and most animals (19). Initial sources for colonization are formed from maternal and environmental microbes, which are transferred to the neonatal intestine by several processes, such as suckling and nursing just after birth. Intestines of infant piglets are initially colonized by large numbers of *Escherichia coli* and *Streptococcus* spp., often reaching 10^8-10^{10} g in faeces (4, 28). It has been proposed that the first bacterial colonizers are responsible for the creation of an environment favorable for the establishment of the anaerobic phyla *Bacteroides, Bifidobacterium,* and *Clostridium* (4, 28). In agreement with these cultivation-based studies, we found that 45% of all clones analyzed (n=20) from the samples of two-day old piglets harbored *E. coli* and *S. flexneri* related 16S rRNA sequences (Table 3). While *E. coli* is phylogenetically indistinguishable from *Shigella*, strains of the two species have been characterized as etiological agents of human and animal GI tract diseases. It is estimated that *Shigella* species cause >1 million human deaths per year from dysentery and diarrhea worldwide (33). Moreover, diarrhoea in suckling and weaned piglets is one of the most important sources of large economic losses in production farms and it has been generally believed to be caused by proliferation of pathogens, including enterotoxigenic strains of *E. coli* K88. Although this particular strain was detected by real-time PCR in three out of ten samples (12 and 23 days old piglets), which was probably due to the fact that this was not a challenge trial and the piglets were reared under experimental conditions and in comparatively clean facilities. Moreover, we found that the emergence of some potentially pathogenic species, namely *E. coli* and *S. flexneri,* in the ileal samples of two days old piglets, was accompanied by the proliferation of three particular *Lactobacillus* spp. As demonstrated by real-time PCR, the populations of *L. amylovorus*-like and *L. reuteri* were above 10^7 cells/g ileal lumen samples, while *L. acidophilus* was detected at a significantly lower level (Table 4). Although with quantitative differences, the populations of the three lactobacilli were found to persist in all ileal samples from the piglets at 2, 5, 12 and 19 days of age. Specifically, the lactobacilli related to *L. amylovorus* have been identified as a common inhabitant of the human and animal intestine (13). The taxonomic characterization of *L. amylovorus*-like strains isolated from the intestine of piglets, including the ones from the current experiment will be further discussed in Chapter 5 of the current thesis. Lactobacilli are known for their potential to prevent the infection or colonization of pathogens by competition for nutrients and epithelial-binding sites, and the production of anti-microbial factors, such as lactic acid and bacteriocins (8). Given the fact that *L. amylovorus*-like, *L. reuteri*, and to a lesser extent *L. acidophilus*, were abundant in the intestine of the neonatal piglets, we anticipate that these particular *Lactobacillus* species may play a crucial role in the establishment and the maintenance of the GI tract bacterial homeostasis after birth.

During the weaning process, the three species of lactobacilli were detected at significantly lower levels ($<10^3$ cells/g) in the ileal samples of the weaned compared with the unweaned piglets (Table 4). Previous studies have demonstrated that the porcine *Lactobacillus* community can decrease in time (25), or after the exposure of the animal host to stress factors such as mixing with other piglets, sub-optimal feed intake and transportation (16). However, in many experiments, further characterization beyond the genus level after plating on a selective media, FISH or dot-blot analysis with group specific DNA probes, has not been achieved (9, 25). Using a species-specific real-time PCR approach, we demonstrated that the populations of *L. amylovorus*-like, *L. reuteri* and *L. acidophilus,* colonizing the porcine intestine in the early postnatal period, were significantly diminished during weaning.

In practice, the two-week period following early weaning is often characterised by profuse, watery diarrhoea and weight loss. Although the aetiology of the disease is not fully understood, pathogenic challenges were suggested as a primary cause of post-weaning diarrhoea in piglets. In addition, accumulation of lactate or succinate in the intestine (31), and an insufficiently developed intestinal microbiota and mucosal immune system were also among the factor predisposing weaning piglets to GI tract disturbances (14, 29).

Next to the quantitative analysis of selected key populations of some abundant *Lactobacillus* species by real-time PCR, our study intended to unveil the changes in the composition and metabolic activities of the predominant bacterial communities throughout the weaning process. Here we used the principle of moving window correlation as a quantitative measure to evaluate the extend of bacterial diversity shifts over time (22). Using pairwise comparisons of DGGE fingerprints, the stability of profiles for the different samples were quantitatively evaluated and compared between the groups. A significant change in the correlation coefficients of the DGGE profiles was found between day 19 and 23 in the ileal and colonic samples of most piglets used in our study. The changes were probably due to the establishment of a new bacterial community after weaning, following the dietary changes from maternal milk onto a solid feed rich in plant polysaccharides. Furthermore, after a direct sequencing analysis, abundance of clones showing a high similarity to the 16S rRNA genes of *Clostridium* spp. and *E. coli* were found in the ileal samples of the weaned piglets (23 days old). Moreover, higher lactate and lower ammonia concentrations were measured in these samples. Lactate is one of the principal metabolic products of the fermentation of carbohydrates by many microorganisms in the mammalian intestine, including clostridia (15). Ammonia can be utilized during the bacterial growth and thus its concentration was decreased likely due to the rapid proliferation of bacteria colonizing the porcine ileal and colonic lumen during the post-weaning period. The roles of individual members of the

genus *Clostridium* in the fermentative conversion of ingested solid feed after weaning are yet to be revealed. Moreover, further studies analyzing the disturbances in the microbial ecological equilibrium in relation to the animal host immune system and physiological development still need to be performed.

In conclusion, it has been demonstrated that the weaning in piglets is accompanied with a significant change in the bacterial composition and its metabolites. The observed reduction in the number of previously abundant lactobacilli during weaning may potentially leave the animal host vulnerable to pathogenic challenges. Hence, we anticipate that dietary supplementations of *L. amylovorus*-like and/ or *L. reuteri* may be further used in order to improve the intestinal microbial balance in the piglets during the critical weaning transition.

ACKNOWLEDGEMENTS

This work has been carried out with financial support of the European Community specific RTD programme "Quality of Life and Management of Living Resources" research project: HEALTHYPIGUT (QLK5-LT2000-00522). We thank Wilma Akkermans - van Vliet for her exellent assistance with the sequencing analyses.

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CHAPTER 3

Effect of Fermentable Carbohydrates on Piglet Faecal Bacterial Communities as Revealed by DGGE Analysis of 16S Ribosomal RNA

This chapter has been published in:

FEMS Microbiology Ecology (2003) **43**:225-235

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ABSTRACT

The effect of fermentable carbohydrates (sugar beet pulp and fructooligosaccharides) on the faecal bacterial communities of weaning piglets was analysed using 16S rRNA-based approaches. Amplicons of the V6-V8 variable regions of bacterial 16S rRNA were analysed by denaturing gradient gel electrophoresis (DGGE), cloning and sequencing. Differences in piglets faecal bacterial community structure were determined based on the Dice coefficients for pairwise comparison of the DGGE fingerprints and revealed significant changes in the faecal microbiota immediately after weaning. Piglets fed with fermentable carbohydrates showed a higher bacterial diversity and a more rapid stabilisation of the bacterial community compared with that of the animals fed with the control diet. Thirteen dominant DGGE bands were matched with sequences that showed 91 to 97% similarity to those derived from the *Clostridium coccoides* group and the *Clostridium leptum* subgroup. Amplicons related to *Ruminococcus*-like species were found in all DGGE fingerprints derived from pigs on the diet containing sugar beet pulp and fructooligosaccharides, but not in pigs on the control diet. These results indicate that these bacteria may play a role in the utilisation of dietary fibres.

INTRODUCTION

The large intestine of pigs and other production animals is densely colonised with bacteria, but little is understood about their activity, which affects animal performance, health and food safety. There is considerable interest in understanding and influencing the intestinal microbiota, notably because of the urgent need to replace antibiotics as growth promoters in animal production. Moreover, the pig's intestinal tract is considered to be an appropriate model system for the human intestinal tract (36). Stimulating the fermentation of specific carbohydrates in the intestine has been suggested to influence the development of the bacterial community. Such fermentation may promote the production of volatile fatty acids that have been shown to inhibit the growth of certain pathogens, e.g. *Salmonella* (37, 39)

Using traditional culture techniques, it has been shown that the majority of the faecal and colon microbiota isolated from adult swine were Gram-positive obligate anaerobes (33). Most of the isolates were found to belong to the genera *Streptococcus*, *Lactobacillus*, *Fusobacterium*, *Eubacterium*, and *Peptostreptococcus.* The Gram-negative organisms comprise about 10% of the total culturable bacteria belonging to the *Bacteroides* and *Prevotella* groups. Such culture-dependent methods are highly sensitive and accurate to monitor those viable bacteria, of which the growth requirements are known. However, one of the limitations of these methods is that plating in selective media relies on the assumption that all bacterial groups show equal plating efficiency or viability. These limitations of conventional culture-dependent detection techniques have led to the development of rRNA-based approaches by which the presence and identification of the bacteria is based on the sequence diversity of the 16S rRNA gene (1, 2), although this approach may be biased as well (25, 38). Recent phylogenetic analysis of the small subunit (16S) rRNA from pig intestine has revealed that the intestinal microbial community is very complex and that the majority of the bacterial species colonising the intestine have not been characterised (13, 24). A combination of polymerase chain reaction (PCR) and DNA fingerprinting techniques, such as temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) have been used successfully to describe the microbial diversity in complex ecosystems (21, 22), including the mammalian gastrointestinal (GI) tract (30, 31, 40).

Both quantitative and qualitative aspects of the faecal bacterial community may be sensitive to changes in environmental factors. Stress factors such as weaning, dietary change and transportation can cause a decrease in lactobacilli and an increase of coliforms in the GI tract (35). The introduction of high levels of fibres in the diets of pigs has been found to stimulate growth of bacteria with cellulolytic and xylanolytic activities (4, 19). Moreover, changes in porcine colon and

faecal bacterial population have been demonstrated when the animals were fed different diets and after introduction of an exogenous *Lactobacillus* strain (14).

Here we describe the changes in time of the predominant faecal bacterial community in weaning piglets that were fed diets containing fructooligosaccharides (FOS) and/or sugar beet pulp (SBP). The results indicated that addition of fermentable carbohydrates to the diet results in a rapid stabilisation of the bacterial community and the emergence of notably *Ruminococcus obeum*- like species.

MATERIALS AND METHODS

Experimental approach. To describe bacterial diversity in the piglet GI tract, total DNA was isolated from faecal samples and used as a template for PCR amplification. Amplicons of the V6 to V8 regions of bacterial 16S rRNA were analysed using DGGE. The band position in different gels was compared by using a marker, which consisted of amplified V6 to V8 regions from nine clones with different mobility. Additionally, a clone library of 16S rRNA amplicons (*Escherichia coli* positions 8 to 1510) was prepared from a faecal sample of a piglet, which had been fed for 13 days with a diet containing SBP and FOS. Cloned amplicons that showed the same mobility upon DGGE, as specific bands in the DNA-derived profile were further characterised by nucleotide sequence analysis.

Animals, diets, and sampling. Three identical, but independent feeding trials with healthy conventionally raised piglets (crossbred Hypor X Pietrain) were started immediately at the time of weaning (25-28 days old). To prevent cross-contamination between litters, as well as the stress which mixing litters would cause, piglets from the same litter were kept together and therefore fed the same diet. However, it was for this reason that the experiment was repeated three times, involving nine litters in total (three litters per diet). At the beginning of each experiment, three litters containing 4 piglets each were offered one of the three diets with different amounts of non-digestible (fermentable) carbohydrates (control, SBP or SBP/FOS) (Table 1). The diets were composed in such way that total energy and protein content were comparable. The set-up of the trials and the number of the piglets and their codes are given in Table 2. All piglets from one litter were separated into two respiration chambers and maintained at an initial temperature of 24 ^{o}C from day 1 to day 6. At 6 days, one half of the piglets were exposed to a temperature of 15 ^{o}C for a week, while the other half were kept at 24 ^{o}C for another week. Faecal samples were collected per rectum from days 1, 2, 5, 6, 7, 8, 9, 13 and stored at – 20 °C. The faecal samples from the first

feeding trial were analysed within six months after the experiment was finished. The faecal samples from the other two feeding trials were kept at –20^{o}C for one year.

TABLE 1. Formulation of the diets used for the *in vivo* experiment.

Ingredients (% [wt/wt]) [a]	Control Diet	SBP	SBP/FOS
Maize Starch	51.5	41.85	44.2
Fructooligosaccharides	0	0	2.5
Sugar Beet Pulp (10-15% sugar)	0	10	5
Fish meal (70.6% crude protein)	20	20	20
Dextrose	15	15	15
Soy Oil	0.5	1.4	0.9
Cellulose (Arbocel)	5	5	5
Premix (maize)	1	1	1
Soy isolate	4	3	4
$CaCO_3$	0.35	0.17	0.29
CaH_2PO_4	0.34	0.33	0.33
$KHCO_3$	1	0.85	0.85
L-Lysine HCl	0	0.04	0.02
DL –Methionine	0.17	0.18	0.18
L-Threonine	0.11	0.12	0.12
L-Tryptophan	0.06	0.06	0.06
Diamol[b]	1	1	1
Crude Protein	17.97	18.03	18
Total Energy (kcal/kg)	2527	2526	2527

[a] No added copper or antibiotics.

[b] Diamol - Diatomaceous shell powder – inert material used as a marker for nutritional studies.

DNA isolation. Three grams (wet weight) of thawed faecal samples were homogenised in 50 ml of ice-cold 0.05 M potassium phosphate buffer (pH 7.0), and aliquots of 1 ml stored at – 20 °C. Total genomic DNA was extracted from pig faeces by a bead-beating method as previously described (40). Agarose gel 1.2 % (wt/vol) containing ethidium bromide was used to check the amounts of DNA visually.

PCR amplification. Primers U0968f-GC (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and L1401r (5'-CGG TGT GTA CAA GAC CC-3') (23) were used to amplify V6-V8 regions of 16S rRNA. This primer pair is specific for bacterial 16S rRNA and yields amplicons of 470-bp length. PCR was performed using the Taq DNA polymerase kit from Life Technologies (Gaithesburg, Md.). PCR mixtures (50µl)

contained 0.5µl of Taq polymerase (1.25 U), 20mM Tris –HCl (pH 8.5), 50mM KCl, 3.0mM MgCl2, 50 mM each dNTP, 5 pmol of the primers U968-GC and L1401, 1µl of DNA diluted to approximately 1ng and UV sterile water. The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) by using the following program: 94 ^{o}C for 5 min, and 35 cycles of 94 ^{o}C for 30sec, 56 ^{o}C for 20 sec, 68 ^{o}C for 40 sec, and 68 ^{o}C for 7 min last extension. Aliquots of 5µl were analysed by electrophoresis on 1.2 % agarose gel (wt/vol) containing ethidium bromide to check the sizes and amounts of the amplicons.

TABLE 2. Details of piglets investigated, including diet, experiment number, environmental temperature (ºC) from day 6, and litter number.

Diets	SBP/FOS			SBP			Control		
Exp.[a]	Piglet	TºC[b]	Litter	Piglet	TºC[b]	Litter	Piglet	TºC[b]	Litter
1	A1	15	1	B1	15	2	C1	15	3
1	A2	15	1	B2	15	2	C2	15	3
1	A3	24	1	B3	24	2	C3	24	3
1	A4	24	1	B4	24	2	C4	24	3
2	A5	15	4	B5	15	5	C5	15	6
2	A6	15	4	B6	15	5	C6	15	6
2	A7	24	4	B7	24	5	C7	24	6
2	A8	24	4	B8	24	5	C8	24	6
3	A9	15	7	B9	15	8	C9	15	9
3	A10	15	7	B10	15	8	C10	15	9
3	A11	24	7	B11	24	8	C11	24	9
3	A12	24	7	B12	24	8	C12	24	9

[a] Exp.- experiment number; [b] T- environmental temperature from day 6 till day 13.

Denaturing gradient gel electrophoresis. The amplicons obtained from the faecal-extracted DNA were separated by DGGE according to the specifications of Muyzer *et al*., (21) using a Dcode TM system (Bio-Rad Laboratories, Hercules, Cal.). Electrophoresis was performed in 8% polyacrylamide gel 37.5:1 acrylamide-bisacrylamide (dimensions 200x200x1 mm) using a 38%-48% denaturing gradient. The gels were electrophoresed for 16 hrs at 85 V in 0.5 x TAE buffer (16) at a constant temperature of 60 ºC. The gels were stained with AgNO3 (26).

Analysis of the DGGE gels. DGGE analysis of all samples was repeated twice. All gels were scanned at 400 dpi and analysed using the software of Molecular Analyst/PC (version 1.12, BioRad, Hercules, Calif.). Firstly, a number of bands per lane was assessed by using bands searching algorithm within the program. A manual check was done and the DGGE fragments constituting less than 1% of the total area of all bands were omitted. When one and the same

DGGE band had a value lower than 1% of the total area of all bands in the first replicate and higher than 1% in second one we calculated an average value based on the two runs. Bands above 1% of the total area of all bands in two replicates of one sample were considered as dominant DGGE bands and included in the further analysis. The similarity between the DGGE profiles was determined by calculating a band similarity coefficient (*SD*) (Dice: *SD*=2nAB/(nA+nB), where A is the number of DGGE bands in a line 1, B represents the number of DGGE bands in lane 2, and nAB is the number of common DGGE bands (9, 20, 30, 31). Secondly, as a parameter for the structural diversity of the microbial community, the Shannon index of general diversity, *H'* (5, 18, 29), was calculated by using the following function: $H' = -\Sigma P_i \log P_i$, where Pi is the importance probability of the bands in a lane. *H'* was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak height in the densitometric curves. The importance probability, Pi, was calculated as: $Pi = n_i/H'$, where n_i is the height of a peak and *H'* is the sum of all peak heights in the densitometric curve.

Statistical analysis. For statistical analysis calculations were made to determine band similarity coefficient (*SD*), number of DGGE bands, and the Shannon index of general diversity. Differences between diets for these parameters, were tested for significance using Tukey's studentised range test of multiple comparisons (32) according to the following:

$Y = \mu + D_i + \varepsilon_{ij}$, where Y is the result, μ the mean, D effect of the diet, and ε_{ij} the error term. All statistical analyses were performed using the SAS GLM procedure (11).

Cloning of the PCR amplified products. PCR was performed with a *Taq* DNA polymerase kit from Life Technologies using primers 8f and 1510r (12), which amplify the bacterial 16S rRNA. Amplification was carried out as described previously (40). The PCR product was purified with the QIAquick PCR purification kit (Westburg, Leudsen, The Netherlands) according the manufacturer's instructions. Purified PCR product was cloned into a pGEM-T (Promega, Madison, Wis., USA). Ligation was done at 4 ºC overnight followed by transformation into competent *E.coli* JM109. The colonies of ampicillin-resistant transforms were transferred with a sterile toothpick to 15 µl TE buffer and boiled for 15 min at 95 ºC. Immediately, PCR was performed with pGEM-T specific primers T7 (5'-AAT ACG ACT CAC TAT AGG-3') and SP6 (5'-ATT TAG GTG ACA CTA TAG-3') (Promega) to check the size of the inserts by using the cell lysate as a template. Plasmids containing an insert of approximately 1.6 kb were used to amplify V6-V8 regions of 16S rRNA. The amplicons were compared with the bands of DGGE profiles that comprised more than 1% of the total area of all bands. Plasmids containing an insert corresponding to a dominant band

were grown in Luria broth liquid media (5 ml) with ampicillin (100 µg/ml). Plasmid DNA was isolated using the Wizard Plus purification system (Promega) and used for sequence analysis.

Preparation of the clone - specific probe. Probes for clones A9 (AF349429), A17 (AF349417), and A22 (AF349420) were generated by PCR amplification of the V6 region from position 971 to 1057 of the 16S rRNA as described (7). PCR products were purified with the QIAquick PCR purification kit. The DNA was resolved in 50 µl UV sterile water and then labeled using Prime-a-Gene Labeling System Kit (Promega, Madison, Wis., USA) and [α^{32}P] ATP as specified by the manufacturer and used as a probe.

Southern blot hybridisation. Electroblotting of denaturing gradient gels were carried out according to Muyzer *et al*., (22). PCR products were transferred from the polyacrylamide gels to Hybond-N^+ nucleic acid transfer membranes (Amersham International, Bucks, United Kingdom) by using a Bio-Rad model SD semi-dry electrophoretic transfer cell. Prehybridisation and hybridisation were done as previously described (7). A detection screen (Molecular Dynamics, Hercules, USA) was incubated with the hybridised membrane and the probe signals were detected with a Phosphor Imager SF (Molecular Dynamics, Hercules, USA).

Sequence analysis. Purified plasmid DNA (approximately 1 µg/ml) was used for sequence analysis of the cloned 16S rRNA by using a Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, United Kingdom) according to the manufacturer's specifications using the T7 and SP6 primer or 1100r primer (5'- GGG TTG CGC TCG TTG –3') 5' – end labeled with IRD-800. Sequences were automatically analysed on a LI – COR DNA Sequencer 4000L (LiCor, Lincoln, Nebr., USA) and corrected manually. These sequences were also compared to those available in public databases by using BLAST-analysis (10). The partial and complete 16S rRNA sequences were checked for chimerical constructs by RDP CHECK_CHIMERA program (15). None of the sequences were found to be PCR- generated chimers.

Nucleotide sequence accession numbers. The sequences of the pig faecal 16S rRNA clones were deposited in the GenBank. The new sequences, with their accession numbers in parentheses, are: A2 (AF349418), A4 (AF349425), A4-1 (AF349426), A8 (AF349428), A9 (AF349429), A11 (AF349430), A12 (AF349415), A13 (AF349416), A17 (AF349417), A20 (AF349419), A22 (AF349420), A29 (AF349421), A29-1 (AF349422), A36 (AF349423), A39 (AF349424), A47 (AF349427).

RESULTS

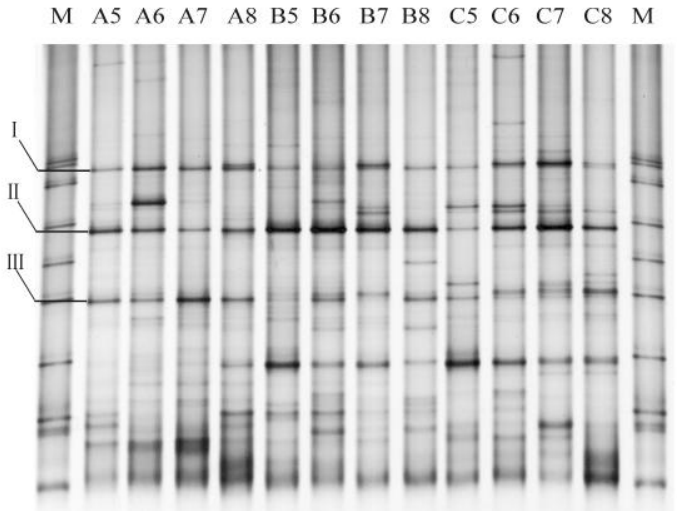
Outline of the feeding trials and analysis of the pig faecal bacterial community before weaning. A feeding trial was performed in triplicate in which a total of 36 piglets were fed 13 days one of three diets: a basic diet without fermentable carbohydrates (control), one containing 10% sugar beet pulp (SBP), and one containing 5% SBP and 2.5% fructooligosaccharides (FOS) (Table 2). On day 6 of each experiment, the ambient temperature was reduced from 24 to 15°C for half of the piglets, but no significant effect of this was found for any aspect of the microbial community structure, so it is not discussed further.

In order to assess the variability between the bacterial populations in the piglets before weaning, faecal samples were taken immediately prior to the feeding trial (day 1). A representative analysis of the PCR fragments generated with primers 968GC and 1401r and analysed by DGGE is shown in Fig. 1. This gel consists of 16S rRNA PCR amplicons derived from faecal samples of twelve piglets on day 1. The number of bands in the DGGE profiles varied from 20 to 28. Several bands were common in all samples before weaning (Fig. 1.) A comparative analysis of DGGE profiles of faecal amplicons from day 1 from all piglets of nine litters was performed. Dice coefficients for pairwise comparison between DGGE profiles of piglets within the same litter were calculated and found to vary between 47 to 83.7%.

The Shannon index of diversity, *H'*, from the DGGE banding pattern of a sample was calculated on the basis of the number and relative intensity of bands on a gel lane. Thus, *H'* was used as a parameter that reflects the diversity of the whole microbial community. *H'* index calculated for all piglets from all three replicate experiments at day 1 ranged between 1.06 to 1.46 (mean 1.38 ± 0.086). These values indicated differences in the DGGE band intensity and number of bands between the **Effect of dietary fermentable carbohydrates on the development of the faecal bacterial community of weaned piglets.** Faecal samples from 36 individual pigs that participated in the trial (Table 2) were analysed by DGGE. The samples were collected immediately post-weaning and were used to monitor the effect of non-digestible carbohydrates on the microbial community. The faecal samples collected from the piglets of the first feeding trial were used to validate the reproducibility of the DGGE fingerprints. The DNA extraction from each sample was repeated twice. After PCR amplification and separation on DGGE two aliquots of one faecal sample had band similarity coefficient (*SD*) higher than 95% (data not shown), therefore they were considered as identical. Further, amplicons of 16S rRNA DGGE from all faecal samples obtained at days 1, 2, 5, 6, 7, 8, 13 were separated by DGGE on eighteen gels. The DGGE profiles of faecal

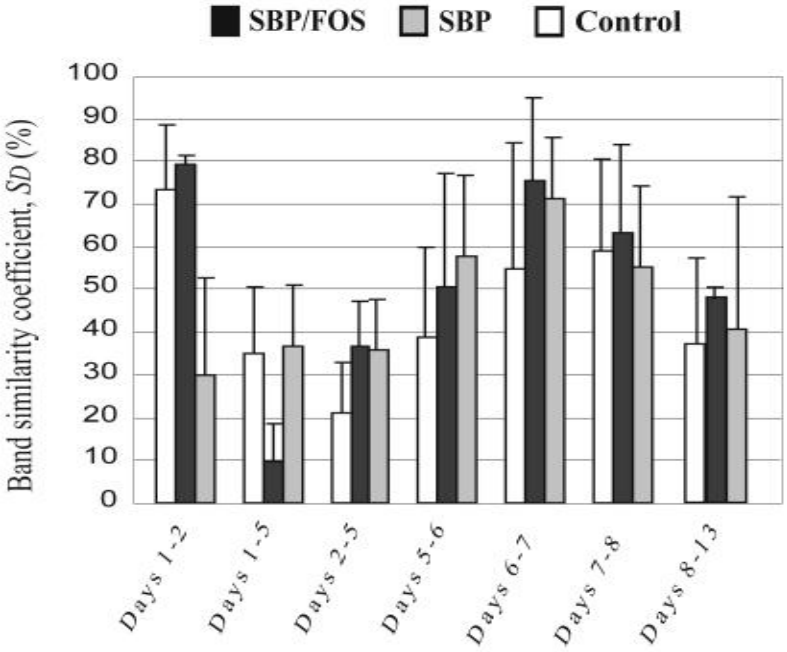
samples from pigs on one of three different diets were compared. The similarity indices of all possible pairs of gel tracks were calculated. In parallel, analyses were performed based on the Shannon index of diversity, based on the mean of the DGGE bands of twelve piglets on the same diet in time and the average number of common bands nAB within the individual piglet between days 5 and 13. individual piglets at the beginning of each experiment.

Figure 1. DGGE of PCR products of V6 to V8 regions of 16S rRNA of piglet faecal samples from day 1 of experiment 2. A5, A6, A7, A8 – piglets on a diet SBP/FOS, B5, B6, B7, B8 piglets on a diet SBP, C5, C6, C7, C8 - piglets on a control diet. M, marker. I, II, III- indicate bands common to most piglet faecal samples.



The mean values of the Dice coefficients for pairwise comparison of DGGE profiles for the piglets on the same diet examined during the three experiments are shown in Fig. 2. During the first two days after weaning no major changes in the *SD* values were observed for the twelve piglets fed with the control diet and that containing SBP and FOS. However, there were remarkable differences in banding patterns between day 1 and day 5 after weaning as well as between day 2 and day 5 for all faecal samples from pigs on the three diets. Differences were found both in the position of specific bands and in the number of bands (data not shown). Changes in the prominent bands were reflected in the lowest Dice coefficients for pairwise comparison between samples from day 1 and 5 and between day 2 and 5. These results suggest that during the week following introduction of the solid diet, dynamic changes were occurring in the bacterial community.

Figure 2. Dice coefficients for pairwise comparison between piglets on the same diet in time. Diet Control, mean values of *SD* from the twelve piglets fed with control diet, experiments 1, 2 and 3. Diet SBP/ FOS, mean values of SD from the twelve piglets, experiment 1, 2 and 3. Diet SBP, mean values of *SD* from the twelve piglets, experiment 1, 2 and 3. x, comparison between the DGGE profiles from different days. y, Dice coefficients for pairwise comparison in %. Bars indicated the standard deviation between the DGGE profiles from piglets on the same diet.

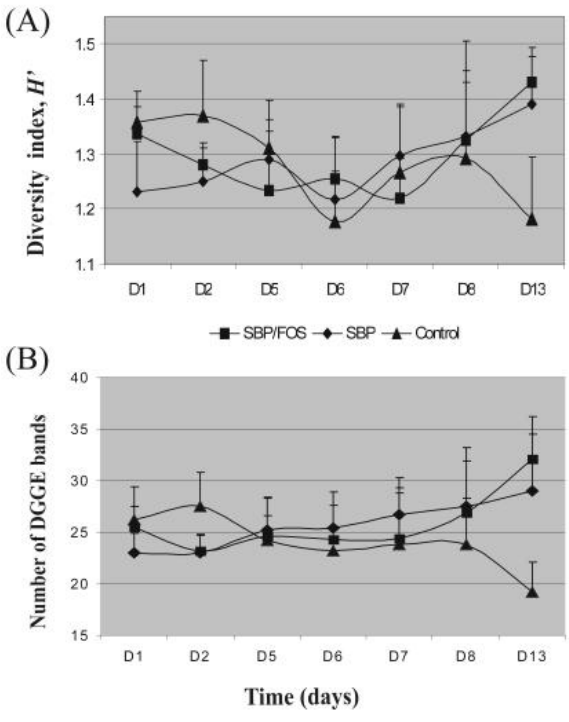


To examine the impact of fermentable carbohydrates in the diet on the stability of the dominant DGGE bands, Dice coefficient for pairwise comparisons were calculated between DGGE amplicons from days 5-6, 6-7, 7-8, 8-13 (Fig. 2). A comparison of the results from piglets fed either of the three diets showed that those piglets fed the diet containing SBP and that containing SBP and FOS had a higher similarity index during the period of almost 10 days compared to piglets fed with the control diet. This suggests that the stimulation of fermentation by the presence of fermentable carbohydrates (SBP or SBP/FOS) in the diet resulted in faster stabilisation of the bacterial community at this critical period after weaning. However, the mean values of the Dice coefficients for pairwise comparison between the groups were not significantly different ($P>0.05$), due to the large individual variation between piglets on the same diet.

In order to compare the diversity of the predominant bacterial population in piglets fed one of the three diets, the numbers of predominant fragments in the DGGE profiles and the Shannon's index of general diversity were calculated at day 1, 2, 5, 6, 7, 8, 13 for the three experiments. (Fig. 3)

Statistical analysis showed that at the beginning of each experiment up to day 8, neither the number of DGGE bands nor the Shannon's index were statistically different among the treatment groups. However, at the end of the experiments (day13) the bacterial community diversity of faecal populations from piglets fed with SBP/FOS diet (*H'*=1.43 ± 0.06) and SBP (*H'*=1.39 ± 0.08) were greater (P<0.05) than in piglets fed the control diet (*H'*=1.18 ± 0.11) (Fig. 3A). The total number of bands at day 13 was also significantly higher (P<0.05) in the groups fed with diets containing fermentable carbohydrates (for SBP/FOS diet = 32 ± 4, SBP diet = 29 ± 5) compared to the control diet (19± 2) (Fig. 3B). In addition, the relative mobility of each band within all 18 DGGE gels was calculated in order to find the number of common bands (nAB) between days 5 and 13. The mean of the number of DGGE bands per track from day 13 of all piglets fed the three diets, was compared with the mean of the number of common DGGE bands. The mean value of nAB in the samples from pigs fed with diet containing SBP and FOS was 14 ± 3 or 42 % of all bands per track, 12 ± 3 or 41% of all bands in samples from piglets fed with a diet containing only added SBP, were higher (P<0.05) than for pigs fed with the control diet 6 ± 2 or 30%. The mean of the common bands for the group fed with the diet containing SBP and FOS compared with that of the group fed SBP was not significantly different (p>0.05).

Figure 3. Analysis of DGGE banding patterns in time day 1, 5, 6, 7, 8, 13. (A) Diversity index. SBP- piglets fed with SBP, Both- piglets fed with SBP/FOS, con – control. (B). Number of DGGE ampilicons in time according to the three diets.



These differences in Shannon's index of general diversity, total number of DGGE amplicons and also the number of common bands indicate a shift in the bacterial populations present in the GI tract that occurred due to the presence of fermentable carbohydrates in the diet.

Identification of cloned 16S rRNA sequences in DGGE patterns. To study the effect of fermentable carbohydrates (SBP and FOS) on the phylogenetic diversity of the predominant bacteria, the 16S rRNA from a faecal sample of a single pig fed a diet SBP/FOS was amplified, cloned and sequenced. V6 and V8 regions of the 16S rRNA were amplified of the cell lysates of 73 transformants. The mobility of these amplicons after DGGE were compared to those obtained from rRNA of the piglets fed 13 days with the SBP/FOS diet. Thirty-six clones were assigned to one of the 16 dominant bands in the DGGE profiles, while 37 did not match any of the detectable bands. The majority (15 out of 16) of the dominant bands showed less than 97% similarity with known sequences in the database (Fig.5). This indicates that most of these sequences were derived from new, as yet undescribed bacterial species. The phylogenetic analysis based on 16S rRNA gene showed that, except three clones: A8 (AF349428), A4 (AF349425) and A4-1 (AF349426) (Fig.5), the rest 13 sequences were related to species of the *Clostridium coccoides* rRNA group of species (cluster XIVa) and *Clostridium leptum* subgroup (3).

Our investigations were intended to reveal whether the different diets led to the appearance of common PCR amplicons for all piglets fed that diet by two weeks after weaning. Sixteen clones assigned to the 16 prominent DGGE bands from a single pig were compared with the bands in the DGGE profiles from piglets fed all three diets. Based on identical mobility within the gel, three common DGGE bands were present only in pigs that were fed with the SBP/FOS diet. These bands indicated with 1, 2 and 3 in Fig. 4 and 5 matched with the clones A 22, A9 and A17 were related to *Ruminococcus* sp. (97%), *Ruminococcus obeum* (95%), and *Ruminococcus* sp. (str. BIE 41) (93%). In addition, an amplicon with a sequence resembling that of *Ruminococcus* sp. (str. BIE 41) was also common in all DGGE profiles from the pigs on the SBP diet. None of 16 clones could be matched to the patterns derived from the piglets fed with the control diet. The high resolution of DGGE does not exclude the possibility that two different 16S rRNA sequences might migrate to exactly the same position. Visual matches for the three *Ruminococcus*- like species A9 (AF349429), A17 (AF349417), and A22 (AF349420) (Fig. 4) were confirmed with clone-specific V6 probe Southern blot hybridisation. A highly specific hybridisation was obtained with a clone A9 (AF349429) related to *Ruminococcus obeum* (Fig. 4 B). Similar results were obtained with probes A17 and A22 (data not shown).

Figure 4. (A) DGGE of PCR products of V6 to V8 regions of 16S rRNA of faecal samples at 13 day after weaning, experiment 2. A5, A6, A7, A8 – piglets on a diet SBP/FOS, B5, B6, B7, B8 piglets on a diet SBP, C5, C6, C7, C8 - piglets on a control diet (Table 2). M, marker. Fragments that are indicated by numbers were identified by the clone library and V6 Southern hybridisation. The origin of the fragments and the corresponding clone are: 1, A 22 *Ruminococcus* sp. (AF349420); 2, A 9 *Ruminococcus obeum* (AF349429); and 3, A 17 *Ruminococcus* sp. (str. BIE 41) (AF349417). (B) Southern hybridisation of an electroblotted DGGE (Fig. 5 A) with probe derived from clone A9 *Ruminococcus obeum* (AF349429) indicated with number 2. The PCR products were loaded as described in the legend of Fig. 5 A. The number 2 indicates the place of hybridisation of the clone A9 with its self. A5, A6, A7, A8 the place of specific hybridisation with PCR amplicons derived piglets that were fed 13 days with a diet contained SBP and FOS; B5, B6, B7, B8 piglets on a diet SBP, C5, C6, C7, C8 -piglets on a control diet (Table 2).

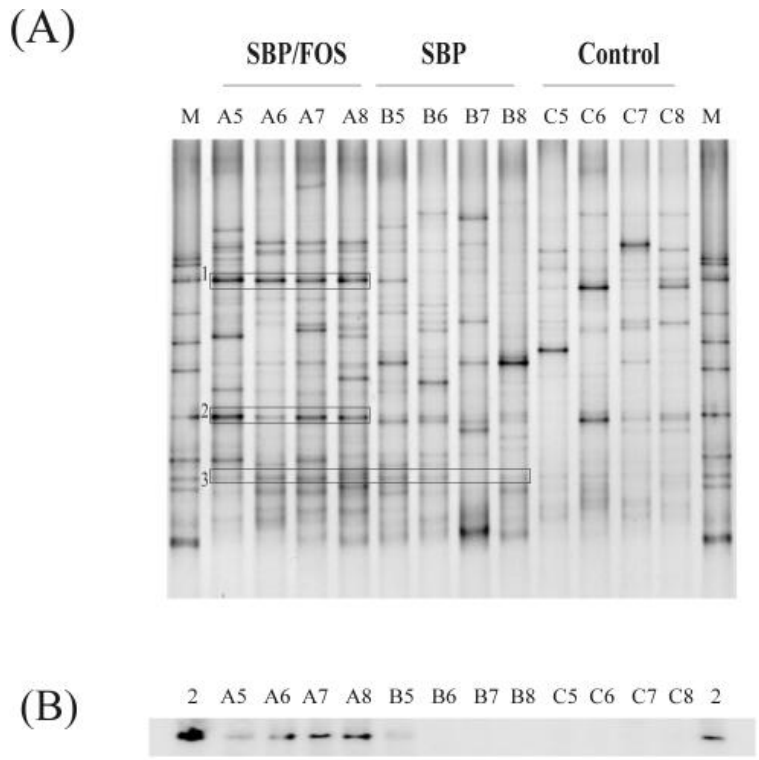
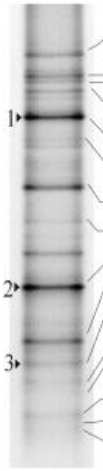


Figure 5. Clones with the percentage of similarity to known sequences in Genbank and the RDP database, and sequence lengths that were retrieved from piglet (A5) fed 13 days diet with FOS and SBP (see Table 2 and Fig. 4 A). Fragments that are indicated by numbers were used for V6 Southern hybridisation.



Clone	Closest relatives	Sequence similarity (%)	Sequence length (bp)
A39	*Ruminococcus obeum*	94	1038
A2	*Clostridium celerecrescens*	91	1503
A36	*Eubacterium formicigenerans*	96	1198
A11	*Eubacterium eligens*	95	379
A22	*Ruminococcus* sp.	97	1515
A47	*Ruminococcus torques*	93	1422
A20	*Clostridium indolis*	93	1469
A13	*Clostridium indolis*	93	1513
A9	*Ruminococcus obeum*	95	1481
A8	Uncultured rumen bacterium JM 19	94	1025
A17	*Ruminococcus* sp. (str. BIE 41)	93	1513
A29-1	*Ruminococcus callidus*	94	1493
A4	*Megasphaera elsdenii*	96	1525
A12	*Ruminococcus* sp.	93	1527
A29	*Ruminococcus obeum*	93	1498
A4-1	*Prevotella* sp.	92	1198

DISCUSSION

This study describes the application of DGGE, cloning, sequencing and specific V6 hybridisation of 16S rRNA to monitor the effect of diet containing non-digestible fermentable carbohydrates (SBP and FOS) on the development of the faecal bacterial community of weaning piglets. The results show that diet containing SBP with or without FOS lead to a higher bacterial diversity. A proposed mechanism is that non-digestible carbohydrates reach the large intestine and provide a source of energy for those members of the bacterial community, which can utilise the dietary fibers comprising predominantly pectin and cell walls.

Recently, it has been shown that both DGGE and TGGE are sensitive tools that can be used to demonstrate the differences between bacterial communities from different individuals (30, 40). Zoetendal e*t al*. (40) reported that TGGE profiles 16S rRNA amplicons from faeces obtained from two unrelated individual persons were host-specific and stable over a period of at least six months. Similar results have been described for pig faecal microbiota using an approach based on DGGE and unique amplicons specific for each pig were observed even when the piglets were fed with the same diet (40). This study also demonstrated a significant stability of the individual faecal bacterial population in pigs in the age period between 28 and 49 days and weaned at approximately three

weeks. In the present study, we found a significant change in the pig faecal bacterial community after weaning at approximately 28 days.

We found that the DGGE profiles of 16S rRNA gene PCR amplicons were not affected by reducing environmental temperature from 24ºC to 15ºC. To our knowledge that is the first attempt to monitor changes in the faecal bacterial community structure prior to environmental stress and further experiments with different temperatures or other forms of stress are needed to confirm these results.

The pig's intestinal microflora undergoes a rapid ecological succession during the period from birth to weaning (34). During and after birth, the young animal becomes contaminated with a variety of microbes from the birth canal and the immediate environment (33). The microflora remains fairly stable in terms of species after this initial colonisation, and for as long as the piglets receive sow's milk (17). However, the introduction of solid food causes major qualitative and quantitative alterations in the microflora. For example, strict anaerobes such as *Bacteroides,* become established in the large intestine, and this corresponds with a decline in the numbers of facultative anaerobe organisms (28).

It has also been shown that the type of diet can influence the bacterial community structure in the colon of pigs (4, 14, 19). Present results indicate that faecal microbiota from the piglets fed with a diet containing the fermentable carbohydrates SBP and FOS, had more stable DGGE profiles from about day 5 of weaning. The DGGE profiles on day 5 and day 13 from piglets fed with a diet containing either SBP and FOS or SBP contained approximately twice as many common 16S rRNA amplicons bands compared with that from pigs fed the control diet. The higher number of common DGGE bands in piglets fed with the SBP/FOS diet may reflect an increasing diversity of the pig microbiota. However, an alternative explanation may be the appearance of species with multiple copy number of slightly different 16S rRNA operons (23, 27).

Nearly half of all clones in our 16S rRNA library did not match any visible bands in the DGGE profile. DGGE visualised only the dominant bacterial fraction in pig faeces, while a cloning approach randomly selected 16S r DNA amplicons. It has previously been shown that all ribotypes matching clearly visible fingerprint bands only represent approximately one-half of all of the rRNA extracted from the soil (6). This suggests that a large number of less dominant bacteria do not form detectable DGGE bands, although some of them can be selected using a cloning approach (40).

The 13 sequences corresponding to dominant bands in the DGGE profile of pigs fed with the SBP/FOS diet were related to two phylogenetic groups- *Clostridium coccoides* group- eleven sequences and *Clostridium leptum* subgroup- two sequences. The sequences fell into cluster XIVa

(3) which included many *Clostridium* spp., *Butyrivibrio* spp., and *Ruminococcus* spp. Three clones related to *Ruminococcus obeum* – like amplicons were found on three different positions in DGGE profiles. Similar results were observed for *Ruminococcus*-like sequences isolated from human faeces (40) that were found in different positions on the TGGE fingerprint. The result suggests an abundance of species related to *Ruminococcus* in pig faeces. The genus *Ruminococcus* consists of anaerobic cocci and the members of this group have previously been isolated from both human (8) and pig faeces (19).

Four of the clones, which corresponded with four dominant bands, showed the highest similarity with *Clostridium* and *Eubacterium* spp. Since their partial sequences showed a similarity lower than 97%, it is likely that they represented a new, as yet uncultured *Clostridium* spp.

Beyond the individual variations between DGGE profiles from pigs on the same diet there were also some bands in common. V6 Southern hybridisation confirmed the presence of three *Ruminococcus*- like species in the predominant DGGE amplicons derived from pigs fed with diet SBP/FOS.

It can be concluded that the diet containing fermentable carbohydrates affects the bacterial diversity in piglets faeces approximately two weeks after the administration. It has been shown that the pig microbiota changes quite dramatically after weaning, and that the addition of non-digestible but fermentable carbohydrates can lead to a higher bacterial diversity and more rapid stabilisation of the microbial community.

ACKNOWLEDGEMENTS

The research was partly supported by the Product Board Animal Feed, The Netherlands". S. R. Konstantinov is grateful for fellowship from the Wageningen Institute of Animal Sciences, and Zhu thanks the National Natural Sciences Foundation in China for its support (grant no: 39870588 and 30025034). We also thank Wilma Akkermans - van Vliet and Hans Heilig for technical assistance and advice for all the analyses.

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CHAPTER 4

Specific Response of a Novel and Abundant *Lactobacillus amylovorus*-like Phylotype to Dietary Prebiotics in the Guts of Weaning Piglets

This chapter has been published in:

Applied and Environmental Microbiology (2004) **70**: 3821-3830

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ABSTRACT

Using 16S rRNA gene-based approaches, we analyzed the responses of ileal and colonic bacterial communities of weaning piglets to dietary addition of four fermentable carbohydrates (inulin, lactulose, wheat starch, and sugar beet pulp). An enriched diet and a control diet lacking these fermentable carbohydrates were fed to piglets for 4 days (n = 48), and 10 days (n = 48), and the lumen-associated microbiota were compared using denaturing gradient gel electrophoresis (DGGE) analysis of amplified 16S rRNA genes. Bacterial diversities in the ileal and colonic samples were measured by assessing the number of DGGE bands and the Shannon index of diversity. A higher number of DGGE bands in the colon (24.2 ± 5.5) than in the ileum (9.7 ± 4.2) was observed in all samples. In addition, significantly higher diversity, as measured by DGGE fingerprint analysis, was detected in the colonic microbial community of weaning piglets fed the fermentable-carbohydrate-enriched diet for 10 days than in the control. Selected samples from the ileal and colonic lumens were also investigated using fluorescent in situ hybridization (FISH) and cloning and sequencing of the 16S rRNA gene. This revealed a prevalence of *Lactobacillus reuteri* in the ileum and *Lactobacillus amylovorus*-like populations in the ileum and the colon in the piglets fed with fermentable carbohydrates. Newly developed oligonucleotide probes targeting these phylotypes allowed their rapid detection and quantification in the ileum and colon by FISH. The results indicate that addition of fermentable carbohydrates supports the growth of specific lactobacilli in the ilea and colons of weaning piglets.

INTRODUCTION

Diet, microbiota and gastrointestinal tract interactions of mammals are extremely complex, and are the result of millions of years of co-evolution between the higher vertebrates and their microbiota. As a consequence, any major changes in lifestyle and diet are likely to place stress on the stability of these interactions and affect the entire gastrointestinal (GI) tract ecophysiology. In contrast to the gradual weaning of human babies, piglets within a production environment are weaned at an early stage with solid feed and transported to production farms. This combination of stress factors can lead to diarrhoea, a reduced growth rate, and in some cases, even death (52). In order to enhance growth and suppress the activity of the gut microbiota, antimicrobial compounds have been fed to weaning pigs for more than four decades (5). Nowadays, the emergence of antibiotic resistance in the human commensal bacteria has raised concerns about the impact of anitimicrobial compounds for agricultural use (51), and accelerated the search for alternative nutritional strategies, such as the addition of probiotics and prebiotics (61). These approaches have become an increasingly important consideration in swine nutrition, because of accumulating evidence as to their potential benefits in animals and humans and the possibility that they replace in-feed antibiotics. The development of such dietary strategies requires a combination of *in vitro*, *in vivo*, and challenge studies, involving both expertise in animal nutrition, and an evaluation of the composition and activity of the indigenous microbiota throughout the GI tract.

In the past the microbial community in the GI tract of pigs has been studied intensively, but most attention was paid to easily cultivable commensal bacteria and a number of opportunistic pathogens (9, 54). Many of the strictly anaerobic GI tract bacteria are still difficult to cultivate and therefore remain undetectable using conventional techniques (56, 60). Recent phylogenetic analysis based on the *in-vitro* amplification of 16S rRNA gene and other phylogenetic markers by polymerase chain reaction (PCR) techniques have revealed a dramatically higher diversity than described previously by cultivation (18, 26, 42). A combination of PCR and fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal fragment length polymorphism (T-RFLP) has led to new insights in the pig intestinal microbial ecology, and on the effect of different dietary strategies and host factors on the bacterial community composition (23, 24, 27).

It has been recognized that a stable indigenous microbiota in the intestine can prevent colonization by pathogens (47, 59). This so-called colonization resistance may be of utmost importance for animals, especially at stressful times such as weaning. Promotion of

colonization resistance through the addition of prebiotics has been suggested as a comparatively easy way to improve enteric health (4, 6, 62). Prebiotics have been used to induce the colonization of bacteria such as lactobacilli and bifidobacteria, considered to be beneficial for the host (11, 12, 58). Stimulation of the *Lactobacillus* population within the gastrointestinal tract of piglet is of specific importance, not only due to their potential effect on gut function and health (54, 55), but also because of their possible antagonistic activities toward other bacteria (17, 50). Lactobacilli establish early in the piglet intestine, and although succession occurs throughout the pig's lifetime, they remain a predominant part of the intestinal bacterial community (3, 38, 54, 57). Numerous studies have suggested that some prebiotics may specifically stimulate intestinal lactobacilli. The application of lactobacilli as probiotics or therapeutic supplements has also been studied (45, 46). However, little is known of the response of the bacterial community to such dietary interventions.

This work describes changes in the predominant ileal and colonic bacterial populations in weaning piglets that were fed a diet containing four added fermentable carbohydrates, namely inulin, lactulose, wheat starch and sugar beet pulp. The data indicate that the incorporation of these four ingredients in the diet results in outgrowth of lactobacilli in the small intestine and higher diversity in the colon. Two particular phylotypes related to *L. amylovorus* and *L. reuteri* were the most prevalent throughout the gut of piglets fed with the prebiotics as demonstrated by DGGE of 16S rRNA gene amplicons in combination with sequence analysis. Newly developed DNA oligonucleotide probes targeting these key species allowed their rapid detection and quantification in the ileum and colon of piglets by FISH.

MATERIALS AND METHODS

Animals, diets and sampling. All the procedures involving animals were conducted in accordance with the Dutch law on experimental animals and had been approved by the Animal Experimental Committee of Wageningen University.

Three identical, but independent feeding experiments including a total of 108 piglets (crossbred Hypor×Pietrain) were started immediately at the time of weaning (25–28 days old). Each experiment had 36 piglets. At the start of the experiment (day 1), 4 piglets were sacrificed. The remaining 32 piglets were offered one of two diets (16 piglets per diet): HF diet containing four added fermentable carbohydrates namely: lactulose, inulin, sugar beet pulp and wheat starch, and the LF diet with a low concentration of fermentable carbohydrates

(Table 1). The diets were composed in such a way that total energy and protein content were comparable. On day 4 and 10 of each experiment, 8 piglets were sacrificed per treatment. Samples were divided into aliquots, one of which was used for genomic DNA extraction followed by 16S rRNA gene targeted PCR-DGGE analysis, cloning and sequencing. In parallel, aliquots from the same samples were fixed for fluorescent *in situ* hybridization (FISH) and for determination of the lactic acid concentration.

TABLE 1. Composition of the diets (g/kg), LF - a diet with a low concentration of fermentable carbohydrates, HF- a diet containing lactulose, inulin, sugar beet pulp and wheat starch.

Diets /Ingredients	LF	HF
Maize starch	504.8	368.1
Sugarbeet pulp	0	50.0
Inulin	0	7.5
Lactulose (~50%DM)	0	20.0
Wheat starch	0	50.0
Fishmeal	200.0	200.0
Soya isolate	50.0	45.0
Dextrose	150.0	150.0
Soya oil	15.0	30.0
Cellulose (Arbocel)	50.0	50.0
Premix	10.0	10.0
Chalk	2.5	1.5
Monocalcium phosphate	1.5	1.5
$KHCO_3$	12.0	12.0
L-lysine HCl	0.6	0.7
DL-methionine	2.0	2.0
L-threonine	1.0	1.1
L-tryptophan	0.6	0.6

DNA isolation. DNA isolation from lumen samples (0.2 grams) was done by using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA). Agarose gel 1.2% (w/v) electrophoresis in the presence of ethidium bromide was used to check visually for DNA quality and yield.

PCR amplification. All primers used in this study are listed in Table 2. Primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 were used to amplify the V6–V8 regions of 16S rRNA gene. PCR was performed using the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures (50 μl) contained 0.5 μl of *Taq* polymerase (1.25 U), 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 3.0 mM $MgCl_2$, 200 μM of each dNTP, 5 pmol of the

primers, 1 µl of DNA diluted to approximately 1 ng/µl and UV sterile water. The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and the cycling consisted of 94°C for 5 min, and 35 cycles of 94°C for 30 s, 56°C for 20 s, 68°C for 40 s, and 68°C for 7 min final extension. Aliquots of 5µl were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing ethidium bromide to check for product size and quantity.

TABLE 2. List of oligonucleotide primers (I) and probes (II) used in this study.

name	sequence (5'-3')	Reference
I		
[a]S-D-Bact-0011-a-A-17	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	(26)
[a]S-G-Lab-0159-a-S-2	GGAAACAG(A/G)TGCTAATACCG	(16)
[a]S-*-Univ-0515-a-A-24-GC	CGCCGGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGGATCGTATTACCGCGGCTGCTGGCA	(16)
[a]S-G-Lab-0677-a-A-17	CACCGCTACACATGGAG	(16)
[a]S-D-Bact-0968-a-S-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGC ACGGGGGGAA CGCGAAGAACCTTAC	(39)
[a]S-D-Bact-1401-a-A-17	CGGTGTGTACAAGACCC	(39)
[a]S-Univ-1100-a-A-15	GGGTTGCGCTCGTTG	(25)
[a]S-D-Bact-1492-a-A-19	GGTTACCTTGTTACGACTT	(26)
T7	TAATACGACTCACTATAGG	Promega
Sp6	GATTTAGGTGACACTATAG	Promega
II		
[a]S-D-Bact-0338-a-A-17	GCTGCCTCCCGTAGGAGT	(14)
[a]S-G-Lab-0158-a-A-20	GGTATTAGCA(C/T)CTGTTTCCA	(14)
[a]L-*-OTU171-0088-a-A-18	CGCTTTCCCAACGTCATT	this study
[a]L-*-OTU173-0085-a-A-18	CCATCGTCAATCAGGTGC	this study

[a]Nomenclature according to (1).

To investigate the *Lactobacillus*-specific GI-tract bacterial community by DGGE, a specific nested PCR approach was chosen. For the initial amplification, S-D-Bact-0011-a-A-17 and S-G-Lab-0677-a-A-17 primers were employed (16) using the following cycling conditions: pre-denaturation at 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 sec, 66^{o}C for 20 s, at 68^{o}C for 40 sec, and a final extension at 68^{o}C for 7 min. PCR products were then used as templates in nested PCR reactions, using: S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-24. The cycling program was identical with the one used for the amplification of the V6–V8 regions of 16S rRNA gene.

DGGE analysis. The amplicons obtained from the lumen -extracted DNA were separated by DGGE according to the specifications of Muyzer et al. (36) using a Dcode TM system (Bio-

Rad Laboratories, Hercules, CA). Electrophoresis was performed in an 8% polyacrylamide gel of 37.5:1 acrylamide–bisacrylamide (dimensions 200×200×1 mm) using a 38–48% denaturing gradient (35) for separation of PCR products obtained with primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17, whilst gradients of 30-60% were employed for the separation of the S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-24 generated amplicons. The gels were electrophoresed for 16 h at 85 V in 0.5×TAE buffer (31) at a constant temperature of 60°C and subsequently stained with $AgNO_3$ (49).

Analysis of the DGGE gels. Analysis of all DGGE samples was done as described previously (24). Briefly, all gels were scanned at 400 dpi and analysed using the software of Molecular Analyst/PC (version 1.12, Bio-Rad, Hercules, CA). First, a number of bands was assessed per lane using the bands searching algorithm within the program. A manual check was done and the DGGE fragments constituting less than 1% of the total area of all bands were omitted from further analysis. Second, as a parameter for the structural diversity of the microbial community, the Shannon index of general diversity, *H'* (8, 24, 32), was calculated using the following function:

$$H' = -\Sigma P_i \log P_i,$$

where P_i is the importance probability of the bands in a lane. *H'* was calculated on the basis of the bands on the gel tracks that were applied for the generation of the dendrograms by using the intensities of the bands as judged by peak height in the densitometric curves. P_i, was calculated as: $Pi = n_i/H'$, where n_i is the height of a peak and *H'* is the sum of all peak heights in the densitometric curve. The similarity between the DGGE profiles was determined by calculating a band similarity coefficient (S_D) (Dice: $S_D=2n_{AB}/(n_A+n_B)$, where n_A is the number of DGGE bands in lane 1, n_B represents the number of DGGE bands in lane 2, and n_{AB} is the number of common DGGE bands (24).

Statistical analysis. For statistical analysis, the number of DGGE bands, the Shannon index of general diversity and the band similarity coefficient (S_D) were calculated. Differences between diets for these parameters were tested for significance using Tukey's Studentised range test of multiple comparisons (53) according to the following equation: $Y=\mu+D_i+\epsilon_{ij}$, where *Y* is the result, μ the mean, *D* the effect of the diet, and ϵ_{ij} the error term. All statistical analyses were performed using the SAS GLM procedure (19).

Generation and screening of 16S rRNA gene clone libraries. PCR was performed with a *Taq* DNA polymerase kit from Life Technologies using primers S-D-Bact-0011-a-S-17 and S-D-Bact-1492- a-A-19. Amplification was carried out as described previously (24). The PCR product was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands) according to the manufacturer's instructions. Purified PCR product was cloned into pGEM-T (Promega, Madison, WI) using competent *E. coli* JM109 as a host. The colonies of ampicillin-resistant transformants were transferred with a sterile toothpick to 15 µl TE buffer and boiled for 15 min at 95 °C. Immediately, PCR was performed with vector specific primers T7 and SP6 to check the size of the inserts using the cell lysate as a template. Plasmids containing an insert of approximately 1.6 kb were used to amplify V6–V8 regions of 16S rRNA gene. The amplicons were compared with the bands of DGGE profiles that comprised more than 1% of the total area of all bands. Clones representing an insert corresponding to a dominant band were grown in Luria Broth liquid medium (5 ml) with ampicillin (100 µg ml^{-1}). Plasmid DNA was isolated using the Wizard Plus purification system (Promega), and used for sequence analysis of the cloned 16S rRNA gene by using a Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, UK) according to the manufacturer's specifications and using either the T7 and SP6 primers or S-Univ-1100-a-A-15 labelled with IRD-800. Sequences were automatically analysed on a LI-COR DNA Sequencer 4000L (LiCor, Lincoln, NE) and corrected manually. Sequences were also compared to those available in public databases by using BLAST analysis (2). The partial and complete 16S rRNA gene sequences were checked for chimeric constructs by the RDP CHECK_CHIMERA program (30). None of the sequences were found to be PCR-generated chimeras.

Cloning and sequencing of DGGE bands after *Lactobacillus* specific PCR amplification. Representative bands were excised from DGGE gels using QIAEXII Gel extraction kit (Westburg) according to the manual instructions. After reamplification using the original S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-24 primers set, cloning and a sequencing analysis was carried out as previously described.

Design and validation of oligonucleotide probes for FISH analysis. Nearly complete 16S rRNA sequences of *Lactobacillus amylovorus*- like and *L. reuteri*-like [this study] and closely related *L. amylovorus*- like (OTU 171) and *L. reuteri*-like (OTU 173) from pig intestine (26) were aligned, and probes targeting these sequences were designed using the ARB software package (29). Probes were designed taking into consideration: the type and the

position of nucleotide mismatches with sequences of related species, a G+C content of over 50%, and a length of approximately 18 nucleotides. Sequence comparison using the ARB, Check Probe and BLAST programs confirmed that the targeted regions were conserved among the 16S rRNA sequences of OTU 171 (*L. amylovorus*-like) isolated from pig intestine and *L. kitasatoi* isolated from chicken (34) (band B, Fig. 1Fig. 1), and to *Lactobacillus reuteri*- like (OTU 173) (band A, Fig 2). Probe OTU171-0088-a-A-18 was found to match also the partial sequence of *L. galinarum* [NCBI accession No: X97898]. However, a comparison between the *L. galinarum*, OTU171 and *L. kitasatoi* sequences showed 100-99% homology among them based on 600 bp (*E. coli* position 20-620).

Reference strains *L. amylovorus* DSMZ 20531 and *L. reuteri* DSMZ 20015 were used as positive control (Table 3). Nine reference *Lactobacillus* strains and *Enterococcus fecalis* DSMZ 20478 frequently found in the GI tract were used as negative control to evaluate the specificity of the newly designed probes. The temperature of hybridization was 50^{o}C and, if needed, formaldehyde was added to increase the specificity (15).

TABLE 3. Bacterial strains, their sources, media used for their cultivation and FISH results for the probes L-S-OTU171-a-A-0088 (I) and L-S-OTU173-a-A-0085 (II); +, hybridization signal; -, no hybridization signal.

Strains	Source[a]	Medium[b]	I	II
Lactobacillus amylovorus	DSMZ 20531	MRS	+	-
Lactobacillus crispatus	DSMZ 20584	MRS	-	-
Lactobacillus reuteri	DSMZ 20015	MRS	-	+
Lactobacillus reuteri	VTT E92142	MRS	-	+
Lactobacillus acidophilus	VTT E96276	MRS	-	-
Lactobacillus acidophilus	ATCC4356	MRS	-	-
Lactobacillus brevis	VTT E91458	MRS	-	-
Lactobacillus buchneri	VTT E93445	MRS	-	-
Lactobacillus plantarum	VTT E79098	MRS	-	-
Lactobacillus rhamnosus LGG	ATCC 53103	MRS	-	-
Enterococcus faecalis	DSMZ 20478	WW	-	-

[a]DSMZ-German Collection for Microorganisms and Cell Culture, 38124 Braunschweig, Germany. VTT- VTT culture collection, P.O. Box 1504, FIN-02044, Finland. ATCC-American Type Culture Collection, 12301, Rockville, MD 20852, MRS, Lactobacilli MRS broth, Difco, Sparks, MD 21152.

Reference strains used in this study were obtained from sources as indicated in Table 3. The strains were cultivated as recommended by the culture collections in the respective catalogues. Exponentially grown cells were harvested at 5,000 x g for 10 min, washed with 0.2 μm pore size filtered phosphate–buffered saline (PBS; per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4; pH 7.2) and diluted 1:3 with 4% paraformaldehyde in PBS.

After fixation at 4°C for 16h, cells were stored in 50% ethanol-PBS at –80°C for subsequent FISH analysis.

Collection and preparation of ileum and colon lumen samples for FISH. Ileal and colonic lumen samples from 108 experimental piglets were processed as described previously (10). In short, 0.5 g of lumen samples was resuspended in 4.5 ml of PBS and vortexed with 5-6 glass beads (diameter = 3 mm) for at least 3 min to homogenize the sample. After centrifugation at 700 x g for 1 min, 1 ml of the supernatant was added to 3 ml of 4% paraformaldehyde in PBS and stored for 16 hours at 4°C. After washing twice with PBS the fixed cells were stored in 50% ethanol-PBS at -80°C until further use.

Enumeration of bacteria by FISH. For microscopic analysis, fixed cells were spotted on gelatin-coated glass slides and dried for 20 min at 50°C. The optimal cell concentration for counting using the different probes was determined using dilution series of the lumen samples. After drying of the slides, the cells were dehydrated for 3 min in 50%, 70% and finally 96% ethanol/H_2O. Ten microliters of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% (wt/vol) SDS) containing 10 ng/µl Cy3-labelled lactobacilli probes or 5 ng/µl FITC –labeled S-D-Bact-0338-a-A-17 (Table 2) was added to each well, followed by incubation at 50°C for 16 hours. After hybridization the slides were washed in 50 ml hybridization buffer for 10 min. For total cell counts 4', 6-diamino-2-phenylindole (DAPI) at a final concentration of 100 ng/ml was added to the washing buffer. After rinsing the slides in double distilled water they were immediately air-dried and mounted in Vectashield (Vector Labs, Burlingame, CA). Digital images of the slide were analyzed and fluorescence positive cells were counted using Qwin image analysis software (Leica Microsystems, The Netherlands). For each analysis 25 microscopic fields were counted.

Lactic acid analysis. The lactic acid concentration in ileal lumen was analyzed by High Performance Liquid Chromatography (Jasco instruments) using column (Supelcogel, C-610H, 30cm*7.8mm ID) and precolumn (Supelcoguard, C-610H, 5cm*4.6mm ID) with 1% H_2SO_4 as mobile phase. The concentrations were determined by UV detection at 210 nm.

Nucleotide sequence accession numbers. The sequences reported in this study were deposed under the following accession numbers AY493201- AY493245 in the GenBank.

RESULTS

Animal observations. Animals remain healthy throughout the experimental period. The average weaning weight of piglets was 7.5 kg. At the end of the experiment, no significant difference in body weight gain was observed between the dietary treatments.

Effect of fermentable carbohydrates on the bacterial diversity in the ileum and colon. A comparative 16S rRNA gene- targeted DGGE fingerprinting analysis of bacterial communities was performed for ileal and colonic lumen samples of piglets that were fed two different diets. Samples were analyzed from piglets that were sacrificed at the day of weaning (n=12), 4 days (n=48) and 10 days after weaning (n=48), respectively. The number of DGGE bands and Shannon index of general diversity were assessed for each sample and subjected to statistical analysis according to GI tract location across all time periods. For all samples, a comparison between the two locations along the GI tract revealed a statistically higher number of DGGE bands (24. 2 ± 5. 5, $p<0.05$) in the colonic compared to the ileal lumen (9.7 ± 4. 2). The Shannon index of diversity in the colon (1.43 ± 0.3, $p<0.05$) was also higher compared with the ileum (0. 86 ± 0. 26). No significant differences in the number of DGGE bands or diversity were detected at Day 4 in the ileum, nor in the colon of piglets fed the different diets. However, by day 10 after weaning, the diversity was significantly higher in the colonic samples of HF piglets, as evidenced by the number of bands (27. 5 ± 5.6, $p<0.05$) compared with the LF group (20.5 ± 6.3). There was no statistical difference between the number of DGGE bands and the diversity index in the ileal samples by day 10.

The influence of the diet on the bacterial community structure in the ileal and colonic lumen of HF and LF group piglets at days 4 and 10 was further elucidated. By day 4, there were no DGGE bands detected in one dietary group, which were completely absent in the other (data not shown). In contrast, a simple visual comparison of the DGGE banding patterns by day 10 of the experiment revealed a marked difference between the ileal samples of the two dietary groups. A representative DGGE analysis of the PCR fragments generated with primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 is shown in Fig. 1 A. Two particularly strong bands (A and B) were present in the ileal samples from piglets fed with HF diet at day 10. Band B was also detected in 18 out of 24 of the colon samples of the HF diet at day 10 and was absent in pigs fed with LF diet (data not shown). The ileal samples of the LF group, on the other hand, were dominated by another band located at the upper most part of the DGGE gels (band C, Fig 1A). Band C was not present in the samples of the HF diet. To obtain an

objective interpretation of the electrophoretic patterns of the HF and LF ileum, the samples were subjected to a numerical analysis based on the Dice similarity coefficient followed by cluster analysis. The similarity was visualized using the UPGMA algorithm (Fig.2). Cluster analysis revealed that all 24 samples of HF diet fed piglets formed a coherent cluster with similarity indices above 60 %. Within this cluster, 20 out of 24 samples grouped together with similarity indices higher than 75 %. The low similarities between HF and LF samples confirmed the visual differences in their DGGE fingerprints (Fig. 1). The average similarity index of the HF colon samples was 45% (data not shown). Taken together, the results obtained after DGGE analysis demonstrate that bacterial composition in the ileum and colon of piglets was modulated by the HF diet by day 10 of the experiment, which suggests a uniform response of the bacterial community to this dietary intervention.

Identification of cloned 16S rRNA generRNA gene sequences in DGGE patterns. In order to identify changes in the bacterial diversity detected by DGGE analysis, the 16S rRNA generRNA gene from the ileal lumen samples of four HF-fed piglets (10 days) and LF-fed piglets was amplified and cloned into *E. coli*, and 15 clones per sample were partially sequenced (Table 4). To identify the dominant bands A and B that appeared in 90% of samples 10 days after starting the HF diet, together with a third distinct band (C) in the LH diet (Fig. 1A), V6 to V8 regions of the 16S rRNA gene were amplified from the cell lysates of a total of 120 transformants. The mobility of these amplicons during DGGE was compared to those obtained from rRNA gene of the piglets fed 10 days with the HF and LF diets. Sixty-one percent of the clones were assigned to one of the dominant bands in the DGGE profiles, while 39% did not match any of the detectable bands. The clones from the four different clone libraries corresponding to bands A, B and C were completely sequenced. The 16S rRNA gene sequences of the clones representing band A were identified as *Lactobacillus reuteri*-like or OTU173, while band B showed similarity to OTU171 or *Lactobacillus amylovorus*-like (26). Clones matching the position of band C were 98% similar to *Sarcinia ventriculi* (Fig. 1A).

Although the amplification of the V6 to V8 regions using general bacterial primers allowed for the visualization of the major differences between the HF and LF samples, and to screen the 16S rRNA gene clone libraries, it yielded a poor resolution of *Lactobacillus* populations. Therefore, specific amplification of the *Lactobacillus* GI-tract bacterial community was used to screen the clones matching to one of the dominant bands after V6-V8 16S region DGGE analysis. *Lactobacillus*- specific amplification in combination with DGGE analysis confirmed the predominance of one particular phylotype related to *L. amylovorus*, while a

band related to *L. reuteri* was not consistently found in the samples of the piglets fed with the HF diet (Fig. 1 B). *L. acidophilus* was also present in samples of HF and LF irrespective of diet.

Figure 1. Effect of the fermentable carbohydrates containing diet on the ileum bacterial community by day 10 of the experiment. (A) DGGE of PCR products of V6 to V8 regions of 16S rRNA gene of lumen samples at 10 day after weaning. 1 to 6, piglets on HF diet. 7-12, piglets on LF diet. M, marker. Fragments that are indicated by A, B, C were identified from 16S rRNA gene clone libraries. (B) Monitoring of the *Lactobacillus*-like community of piglets. DGGE analysis of amplicons generated by nested PCR with primers S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-24, originating from: 1 to 6 – piglets on HF diet, 7-12 piglets on LF diet. The dominant fragments in *Lactobacillus*-like patterns were identified by the clones corresponding to *L. acodophilus*, *L. reuteri* and *L. amylovorus*.

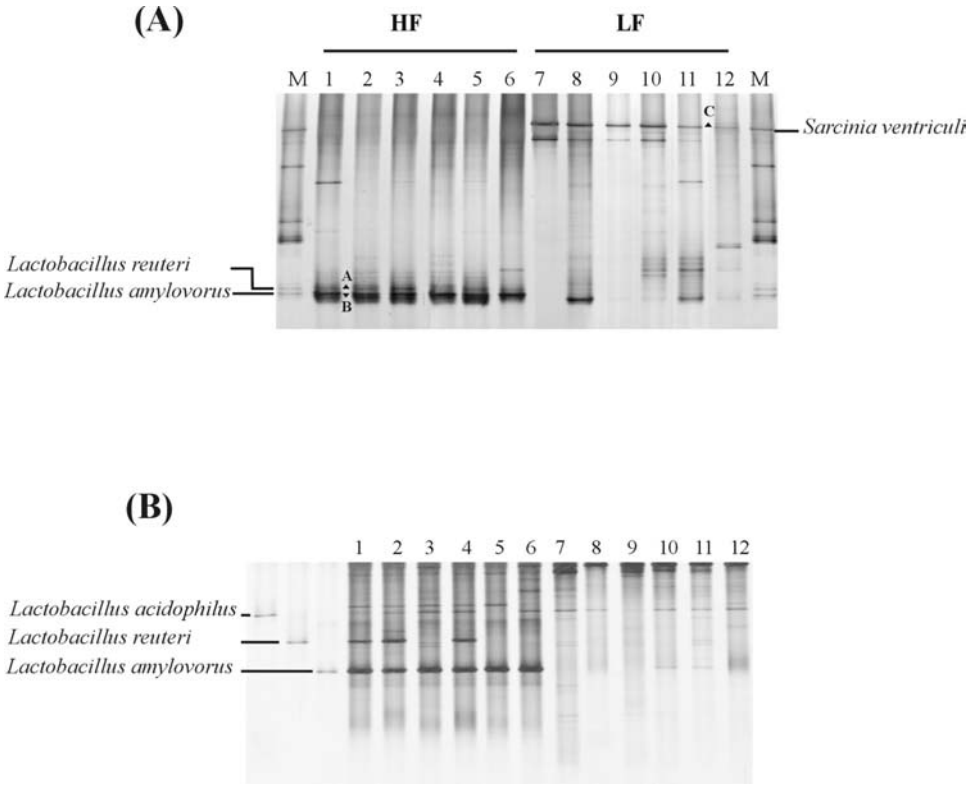
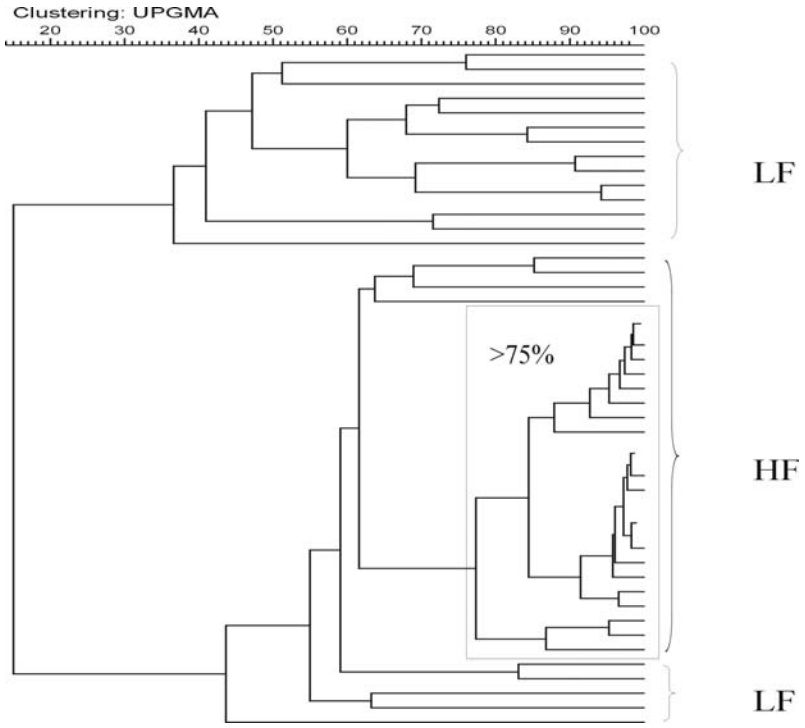


Figure 2. Similarity index of DGGE profiles obtained from ileum microbiota of 48 piglets fed either HF or LF diet for 10 days. The normalization of the DGGE gels was done with respect to the reference standards included in three gels containing the ileal lumen samples of HF and LF from the three replicate experiments. The Dice coefficient of similarity between banding patterns of different gels was calculated. This allowed the generation of a dendrogram and the samples were grouped according to the similarity of their community profiles.



To confirm the visual match between the 16S rRNA gene clones and the DGGE bands, the HF diet-specific bands were excised from DGGE gels after *Lactobacillus*- specific PCR, reamplified and sequenced. Sequencing analysis of the bands (A and B) confirmed their identities as *L. reuteri* and *L. amylovorus*- like (26). Interestingly, of the four HF diet samples, an average of 4 other phylotypes related to *L. mucosae* (97%), *L. gallinarum* (97%), and two different *L. species* clones: one oral (98%, accession no. AY005048) and one isolated from swine production facilities (99%, accession no. AY017059) were found (Table 4). However, their sequences did not match to any visible DGGE bands. In comparison, the *Lactobacillus* diversity in LF was predominated by *L. acidophilus*-like related sequences. The results of the clone libraries showed increased lactobacilli diversity in the HF samples,

while DGGE analysis suggested a specific outgrowth of *L. amylovorus*-like phylotypes in the terminal ileum of weaning piglets.

Development and evaluation of FISH probes specific for *Lactobacillus amylovorus* and *Lactobacillus reuteri*- like. Potential probes were identified based on the alignment of the complete 16S rRNA sequences of the clones matching DGGE bands A and B (Fig. 1) and related *Lactobacillus* spp. (Table 3). The probes were experimentally validated by performing FISH analysis on a range of *Lactobacillus* species and other bacteria that are commonly found in large numbers in the pig GI tract (26, 54). A constant temperature of 50^{o}C for 16 h. and 0 % (vol:vol) of formamide in the hybridization buffer were used, resulting specific hybridization only with the respective target strains (Table 3).

TABLE 4. Clones with the percentage of identity to known sequences in Genbank, that were retrieved from ileal lumen samples of four HF-fed piglets and four LF-fed piglets at 5 weeks of age. The clones are listed according to their abundance in the eight different 16S rRNA gene cloning libraries. The total number of sequenced clones was 60 for both HF- and LF-groups.

No	Closest relative in Genbank	GenBank accesion no.	Number of Clones HF	Number of Clones LF	Average similarity (%)
1.	*Lactobacillus amylovorus*-like	AF371469	22	0	98
2.	*Lactobacillus reuteri* (DSM 20016^{T})	X76328	11	2	98
3.	*Lactobacillus acidophilus johnsonii*	M99704	1	19	99
4.	*Lactobacillus* sp. oral clone CX036	AY005048	7	0	98
5.	*Lactobacillus* sp. CLE-4	AY017059	4	0	99
6.	*Lactobacillus mucosae*	AY445125	2	1	97
7.	*Lactobacillus gallinarum*	AJ242968	3	1	97
8.	*Sarcina ventriculi* (DSM 286)	X76649	0	12	98
9.	*Salmonella* sp.	AF130955	1	5	95
10.	*Pasteurella aerogenes*	M75048	1	4	93
11.	*Clostridium lituseburense*	M59107	1	3	97
12.	*Haemophilus* sp.	M75077	1	3	96
13.	*Moraxella lacunata*	AF005161	1	2	97
14.	*Veillonella atypical*	X84007	1	2	98
15.	*Leptotrichia* sp. (oral clone)	AY349385	1	2	96
16.	*Ruminococcus albus*	AF104843	1	1	91
17.	*Ruminococus callidus*	X85100	0	1	95
18.	*Holdemania filiformis*	Y11466	0	1	91
19.	*Calonectris diomedea*	Y16359	1	1	96
20.	*Veillonella parvula*	AF439640	1	0	90

Subsequently, the validated probes were used to enumerate target bacteria in individual ileal and colonic samples of piglets fed the different diets for 10 days. To evaluate whether the microbiota was affected by the diet, the total cell counts, and the total bacterial and lactobacilli/enterococci counts were compared between the HF and LF diet (Table 5). Within the HF, the lactobacilli/enterococci were significantly higher ($p<0.05$) than in the LF. The hybridization with L-*-OTU171-0088-a-A-18 probe detected the OTU171 phylotype in 83 % of the ileal samples and 75 % of the piglet colonic samples of the HF diet, while no hybridization positive cells were obtained for the LF diet (Table 5). In comparison, *L. reuteri*-like related population was detected in 75 % of the ileal lumen samples and 41 % for the colonic lumen from the HF diet using the L-*-OTU173-0085-a-A-18 probe.

TABLE 5. FISH results for the total cell counts (DAPI staining), total bacterial (S-D-Bact-0338-a-A-17) and lactobacilli/enterococci counts (S-G-Lab-0158-a-A-20) (I). Specific counts for the probes L-S-OTU171-a-A-0088 (*L. amylovorus*-like) and L-S-OTU173-a-A-0085 (*L. reuteri*-like) for ileal and colonic lumen samples of piglets fed 10 days HF or HS diet (II). Counts are expressed as mean ± SD cells. g^{-1}.

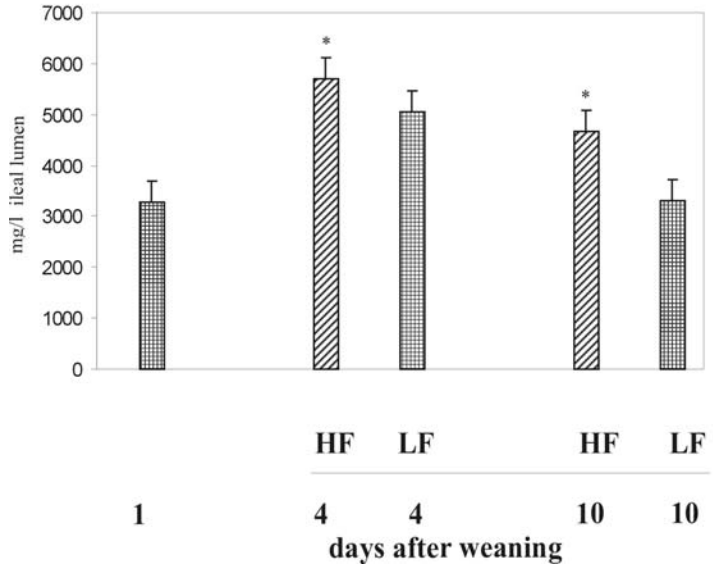
	HF		LF	
	Ileum (n=24)	Colon (n=24)	Ileum (n=24)	Colon (n=24)
I				
Total cell count	2.1±1.1 X 10^8	3.1±2.4 X 10^{10}	1.2±1.2 X 10^8	2.3±1.4 X 10^{10}
Total bacterial count	1.89±1.4 X 10^8	2.7± 1.5 X 10^{10}	1.17±1.1 X 10^8	1.94±0.9 X 10^{10}
Lactobacilli/enterococci	1.5±0.6 X 10^8*	3.8± 2.1 X 10^8	0. 4±0.3 X 10^7	6.3±3.1 X 10^8
II				
L. amylovorus-like				
Probe: L-S-OTU171-a-A-0088				
Piglets colonized *N* (%)	20 (83)	18(75)	n.d	n.d
Median count	1.3x10^8	3.7x10^9	n.d	n.d
Range	0.64-2.4x10^8	1.3-5.6x10^9	n.d	n.d
L. reuteri- like				
Probe: L-S-OTU173-a-A-0085				
Piglets colonized *N* (%)	18 (75)	10 (41.2)	n.d	9 (37.5)
Median count	7.7x10^7	5.8x 10^7	n.d.	5.5x 10^7
Range	4.4 –12x10^7	5.3-6.2x10^7	n.d	5.1-6.3x10^7

n.d.= no bacteria detected; * significant differences from the respective values compared (in bold) at $p<0.05$.

Lactic acid concentration in ileum lumen. Lactic acid was measured in the lumen samples from the terminal ileum of all piglets from days 1, 4 and 10 after the introduction of the diet (Fig. 3). By days 4 and 10, a significantly higher lactic acid concentration was recovered in the samples of HF diet compared with LF. As lactic acid is a common end-product of fermentation of lactobacilli, these results were in agreement with the outgrowth of

lactobacilli in the terminal ileum as demonstrated by 16S rRNA gene based DGGE and FISH analysis, and suggests that the lactobacilli were not only present, but also metabolically active.

Figure 3. Luminal lactic acid concentration (mg/l) in the terminal ileum of piglets. The data is expressed as a mean value plus standard error mean for all samples, * significantly different concentration of lactic acid ($p<0.05$).



DISCUSSION

The microbiological results reported here, indicate that the addition of specific fermentable carbohydrates to the diet can lead to a shift in both the composition and activity of the microbial community of the small and large intestine of weaning piglets. Two particular phylotypes related to *Lactobacillus amylovorus* and *L. reuteri* were the most prevalent populations in the ileum of piglets fed the HF diet for ten days, as demonstrated by DGGE analysis and a phylotype-specific 16S rRNA targeted FISH analysis. In addition, bacterial diversity was increased by day 10 in the colon of the HF group, as evidenced by the higher number of DGGE bands and the Shannon index of diversity in the corresponding samples. Given current concerns of European farmers about the forthcoming ban on antibiotics as

growth promoters, and the idea that the addition of prebiotics may act to enhance colonization resistance by stimulation of appropriate commensal bacteria, these results show that careful design of the diet, can indeed stimulate supposedly beneficial bacteria. They also show that other species can be suppressed. These findings therefore, are not only interesting for piglet microbiology and nutrition at the time of weaning, but also provide new insights into the effect of prebiotics on the indigenous *Lactobacillus* communities of piglets.

The availability of fast sequencing techniques offers an unprecedented opportunity to conduct comprehensive surveys of pig microbial communities (18, 26). Results based on comparative sequence analysis of the 16S rRNA gene and on the chaperonin-60 gene documented the complexity of the intestinal microbial community and suggested that the majority of the bacterial species colonizing the GI tract in pigs have not yet been characterized. However, cloning and sequencing is time-consuming and may limit the number of samples that can be processed. Thus, the high sample throughput required to determine community responses to experimental treatments such as introduction of prebiotics or probiotics needs yet to be achieved by the analysis of multiple clone libraries. Alternatively, denaturing gradient gel electrophoresis (DGGE) has been introduced into microbial ecology (35-37) as one attempt to obtain an overview of the structural diversity of microbial communities. Individual DGGE bands can be assigned to cultured organisms or retrieved ribosomal sequences (23, 24). This is usually not possible in activated sludge, sediments, soil, and other highly diverse microbial systems because the banding patterns are too complex (8). However, the number, precise position, and intensity of the bands reflect the number and relative abundance of dominant rRNA gene types in the sample and thus allow a comparison to be made of microbial communities with each other. By applying this approach to piglet GI-tract lumen samples, a distinct diversity value for each sample was obtained and changes in community diversity over time in different experiments was observed. In agreement with previous analyses of 16S rRNA gene libraries obtained from pig ileum and colon samples (26), the results reported in this study showed a significantly lower diversity in the ileum compared to the colon. Further elucidation of the diet effect by using 16S rRNA gene PCR-DGGE analysis unveiled the impact of the ileum microbiota in the utilization of the fermentable carbohydrates. Marked differences in the bacterial communities composition were demonstrated by Day 10 of the experiment (Fig. 1 A, B) between ileal samples from piglets fed with HF or LF diets. While this has not been previously demonstrated by culture-independent approaches, there are numerous studies showing that the ileum of pigs harbors a diverse and active bacterial population (21) and reviewed by (61). Furthermore, the increased

diversity in the colon of piglets fed the HF diet as demonstrated by DGGE is in agreement with our earlier data (24). Such a strong effect of the diet on the porcine colon and faecal bacterial populations has also been demonstrated when the animals were fed different diets (27) containing fermentable carbohydrates and after introduction of an exogenous *Lactobacillus* strain (50).

The combination of 16S rRNA gene directed DGGE, cloning and sequencing in this study has identified the phylogenetic changes in the piglets microbiota and highlighted the outgrowth of *L. amylovorus*- like populations in the ileum of HF fed group piglets. However, because these approaches are all based on PCR amplification methods, the results cannot be converted to actual bacterial numbers. FISH, in combination with microscopic analysis has provided a powerful tool in detecting and quantifying various bacterial genera including *Lactobacillus* in human faeces (14, 15). Sequences related to *L. amylovorus* or phylotype OTU 171 were recovered from the colonic wall and lumen of a pig (42) and found to be the most abundant *Lactobacillus* phylotype in the GI tract of Danish pigs of different ages and feeding regimes (26). The same phylotype was detected independently to predominate in the small intestinal microbiota of weaning piglets based on 16S rRNA gene-sequence analyses and DGGE (22). Since the current results suggest a significant stimulation of this bacterium in the presence of the HF diet, a DNA oligonucleotide probe targeting this phylotype was developed and validated for FISH analysis. After validation, the probe was used to quantify the number of hybridized cells in the ileal and colonic lumen of piglets fed 10 days with HF or LF diet. The FISH results showed that the size of OTU 171- related populations varied from 0.64×10^8 to 2.4×10^8 /g of ileal lumen of 20 out of 24 piglets (Table 5). The results were in agreement with the DGGE analysis where the phylotype was detected as a dominant DGGE band in 80% of the analyzed piglets fed 10 days with HF diet.

Various studies on the effect of prebiotic oligosaccarides on the colonic microbiota in humans have reported a stimulation of lactobacilli by inulin and lactoluse (48) and reviewed by Rastall (43). However, in many of the *in vitro* and *in vivo* experiments further characterization beyond the genus level has not been achieved (43). Populations of lactobacilli related to *L. amylovorus* and *L. reuteri* have been identified as a common inhabitant of the human and animal intestine. The properties of the type strains are also well established. They are known for their ability to degrade starch. In addition, both strains produce bacteriocins, potentially suppressing other populations within the intestinal microbiota (13). However, the extrapolation of functional properties from well-characterized cultured strains to the related phylotypes *L. amylovorus*-like and *L. reuteri*-like may not be

justified. In particular, a high level of 16S rRNA gene relatedness (>97.5%) was found between the type strains of *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. kitastonis* (34) and the most abundant *L. amylovorus*-like phylotype detected in the study. Therefore, a study on the physiological and genomic properties of a large collection of *L. amylovorus*-like populations isolated from the pig intestine is underway in our laboratory. The results will be described in a separate paper.

In the current study sugar beet pulp (SBP) with a significant fermentable carbohydrate (including cell walls) content was also included. The cell wall component of the diet has previously been found to affect not only the microbial fermentation in the GI tract of pigs (21), but also to play a role in stimulation or inhibition of certain pathogens in the intestine (20). The addition of the SBP to the diet of pigs was reported to reduce the population of coliforms (44), while others suggested an increased proliferation of the pathogenic *Escherichia coli* if the piglets were fed with fiber-enriched diet (33). The effect of dietary fiber on the development of swine dysentery is currently also under discussion. As shown by some reports, diets with low fiber and resistant starches prevent the pigs from infection with *Brachyspira hyodysenteriae* (7, 40, 41), while others were not able to confirm these findings (27, 28). Our results suggest that the combination of fermentable dietary fiber and oligosaccharides may specifically stimulate the *L. amylovorus*-like population along the gut of weaning piglets.

ACKNOWLEDGEMENTS

This research was financially supported by the European Communities project HEALTHYPIGUT (QLK5-LT2000-00522). We thank Wilma Akkermans-van Vliet, Dick Bongers, Cornelia Malin, and Yanka Georgieva for their technical assistance. We are grateful to Maria Saarela for providing the *Lactobacillus* VTT strains.

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CHAPTER 5

Lactobacillus sobrius sp. nov., a Novel Fructooligosaccharides-Utilizing Isolate Abundant in the Intestine of Weaning Piglets

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ABSTRACT

To obtain porcine isolates related to *Lactobacillus amylovorus*, we screened strains from piglet intestine grown on *Lactobacillus*-specific MRS agar for hybridization to a fluorescent 16S rRNA-targeted DNA probe. Six isolates were acquired and further characterized by phenotypic and molecular taxonomic methods. The isolates were Gram-positive, catalase-negative, facultatively anaerobic rods. They had similar phenotypic characteristics and displayed genomic DNA relatedness values of >78%, indicating that they belong to a single species. Comparative 16S rRNA gene sequence analysis demonstrated that the novel isolates were members of *Lactobacillus* rRNA group I, which includes *L. delbrueckii*, the type species of the genus. Based on 16S rRNA sequence identity, *L. kitasatonis* (99%), *L. crispatus* (98%), and *L. amylovorus* (97%) were the nearest relatives of the new isolates, but their DNA relatedness was found to be lower than 49%. One of the isolates, strain OTU171_001^T was further characterized using physiological and biochemical tests. The strain utilized fructooligosaccharides and sugar beet pulp homofermentatively. Together, the results allowed genotypic and phenotypic differentiation of strain OTU171_001^T from those related species that showed 16S rRNA gene sequence similarity values greater than 97%. Strain OTU171_001^T merits species status, and the name *Lactobacillus sobrius* sp. nov. is proposed with the type strain OTU171_001^T (DSMZ 16698).

A modified version of this chapter has been submitted for publication

INTRODUCTION

Lactobacilli are characterized as Gram-positive, non-spore forming rods, and are catalase-negative, non-motile, do not usually reduce nitrate, and utilize glucose fermentatively (5). Members of the *Lactobacillus* are not only found on plants and in plant-derived materials such as silage, grains and foods but also in the gastrointestinal (GI) tract of humans and animals (17). Recently, the predominance of a particular *Lactobacillus* phylotype, defined as *L. amylovorus*-like or operative taxonomic unit (OTU) 171, has been detected in the intestine of healthy pigs (11). Further studies showed, a specific response of this novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary oligosaccharides in the guts of weaning piglets (9). Subsequent phylogenetic analysis revealed a high level of 16S rRNA gene relatedness (>97.5%) between the *L. amylovorus*-like phylotype and the type strains of *L. amylovorus*, *L. crispatus*, *L. gallinarum*, and *L. kitasatonis* (9). A recent study has highlighted the physiological and genomic differences between the phylogenetically closely related species *L. kitastonis, L. crispatus*, *L. amylovorus* and *L. gallinarum* (16). Hence, culturing and further analysis was needed in order to gain insight into the systematic position of *L. amylovorus*-like strains from the intestine of weaning piglets. Here, we report on the isolation of *L. amylovorus*-like strains from pig intestinal sources, and their characterization using a polyphasic approach. The results of this polyphasic approach indicate that these strains represent a novel species for which the name *Lactobacillus sobrius* sp. nov. is proposed.

MATERIALS AND METHODS

A DNA oligonucleotide probe L-*-OTU171-0088-a-A-18 (5'-CGC TTT CCC AAC GTC ATT-3') (9) targeting the 16S rRNA of *L. amylovorus*-like phylotype OTU171 was used for screening of a range of *Lactobacillus* isolates from piglets (21 days of age) housed at different locations. In total 192 isolates grown on *Lactobacillus* selective agar MRS (Difco, Le Point de Claix, France) were screened by fluorescence *in situ* hybridization (FISH) using the CY3-labelled phylotype specific probe in combination with image analysis as described (9). Two *Lactobacillus amylovorus*- like strains were identified in the faeces of piglets housed on a farm near Wageningen, the Netherlands and indicated further as OTU171_001^T

and OTU171_002 in this study. Three strains (OTU171_003, OTU171_004, OTU171_005) were isolated from the faeces of piglets kept on a farm near Bologna, Italy, and one isolate was found in the ileal lumen sample of a piglet reared on a farm near Bristol, UK (OTU171_006). The six isolates were selected for further characterization by phenotypic and molecular taxonomic methods. *L. acidophilus* DSMZ20079^T, *L. amylovorus* DSMZ 20531^T, *L. crispatus* DSMZ 20584^T, *L. gallinarum* DSMZ 10532^T, *L. helveticus* DSMZ 20075^T, and *L. kitasatonis* JCM1039^T were used as reference strains. Further cultivation of *Lactobacillus* isolates and reference strains was anaerobically on MRS agar or in MRS broth at 37 °C.

Strains were tested for carbohydrate fermentation abilities using the API 50 CHL system (bioMérieux). In addition, the degradation of fructooligosaccharides (FOS) and sugar beet pulp (SBP) by *L. sobrius*_001^T was tested using MRS as basal media (without carbohydrates) as described by (1). The bacterial culture was propagated at 37°C, aerobically in MRS basal media consisted of: 1% bactopeptone (wt/vol), 0.5% yeast extract (wt/vol), 0.2% dipotassium phosphate (wt/vol), 0.5% sodium acetate (wt/vol), 0.2% ammonium citrate (wt/vol), 0.02% magnesium sulfate (wt/vol), 0.005% manganese sulfate (wt/vol) , 0.1% Tween 80 (vol/vol), 0.003% bromocresol purple (vol/vol). The MRS basal medium was autoclaved and after filter sterilization, and either D-glucose (dextrose), D-fructose, or FOS (Raftilose P95, Orafti) were added to a concentration 1% sugar (wt/vol). In the case of SBP degradation, the basal media was supplied with 1% SBP (wt/vol) and then boiled for 20 min in a water bath (100^{o}C). Without carbohydrate supplementation, the bacterial growth in the semisynthetic medium was unable to sustain above OD600 nm= 0.25.

Cell shape, size and arrangement, Gram-stain and colonial appearance were determined by using cells grown on MRS agar plates for 2 days at 37 °C. Production of gas from glucose was also examined. Catalase formation, and growth at 15 and 45 °C were done using MRS broth as the basal medium.

16S rRNA gene sequences (approximately 1.5 kb) were determined for the six representative strains. The 16S rRNA gene (corresponding to *Escherichia coli* positions 8–1492) was amplified by PCR, using primers S-D-Bact-0011-a-A-17 (AGA GTT TGA T(C/T)(A/C) TGG CTC AG), and S-D-Bact-1492-a-A-19 (GGT TAC CTT GTT ACG ACT T) (11) and the product was further cloned and sequenced as described (9). The sequence of OTU_001^T was deposed under the accession number AY700063 in the GenBank. Phylogenetic analysis was performed using the ARB software package (12). In order to estimate the approximate genome size of the isolates and to analyze their genomic diversity, pulsed-field gel electrophoresis (PFGE) of *Apa* I digested chromosomal DNA was performed as described

previously (15). The genome size of the isolates was calculated according to the λ PFG standard ladder using the program Quantity One (Bio-Rad). G+C content was calculated based on the determination of genomic DNA (14). DNA-DNA relatedness was analyzed by filter hybridization according to (8), except that for the nick translation [α-^{32}P] dCTP was used, and the hybridization temperature was 59°C (±25°C below Tm). Extraction of whole-cell proteins and their separation by SDS-PAGE was done using standard protocols (13), and SDS-PAGE protein fingerprints were compared using the Bionumerics software package version 3.0. (Applied Maths).

RESULTS

The *L. amylovorus*-like isolates hybridizing to the OTU171 probe were Gram-positive, non-spore-forming and non-motile rods and colonies of these strains were white with circular to irregular shapes. All strains displayed similar fermentation characteristics, as measured by API 50 CHL. Phenotypic characteristics that differentiate the strains from other reference strains are summarized in Table 1. In contrast to *L. kitasatonis*, all new isolates were able to ferment D-raffinose. Strain OTU171_001^{T} was also tested for ability to ferment FOS and SBP (Fig. 1). The strain grew in MRS-FOS to a final cell density OD600=1.81. This MRS-FOS growth was slower compared to MRS-D-glucose, but similar to the cell density after strain inoculation on MRS-D-fructose (Fig 1). A positive growth, with the approximately the same rate as the growth on MRS-D-glucose, was obtained on MRS-SBP broth as well (OD600=2.7). The strains produced DL-lactic acid, did not grow at 15 °C, but did grow at 45 °C, were catalase-negative and did not produce gas from glucose. The DNA G+C content of the six strains ranged from 35-36 mol% (Table 1).

Almost-complete 16S rRNA gene sequences of six representative isolates showed high sequence similarities (>99 %). Subsequent phylogenetic analysis confirmed the association between the newly isolated strains and species that belong to the *Lactobacillus delbrueckii* group of the genus *Lactobacillus* (2, 16) (Fig. 2). High levels of sequence relatedness were found with *L. kitasatonis* (99%), *L. crispatus* (98%), *L. amylovorus* (97%) and *L. gallinarum* (97%).

Figure 1. Growth of OTU171_001^T strain in MRS broth containing either 1% FOS, 1% D-glucose, 1% D-fructose or 1% SBP.

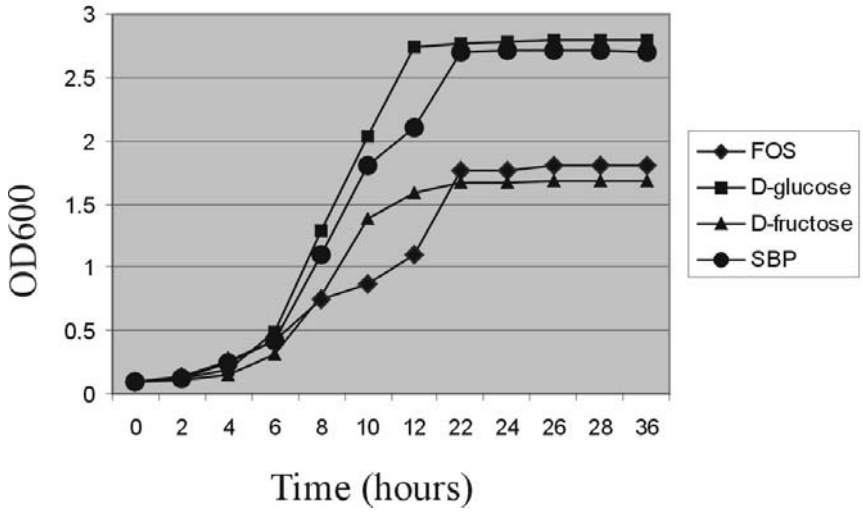
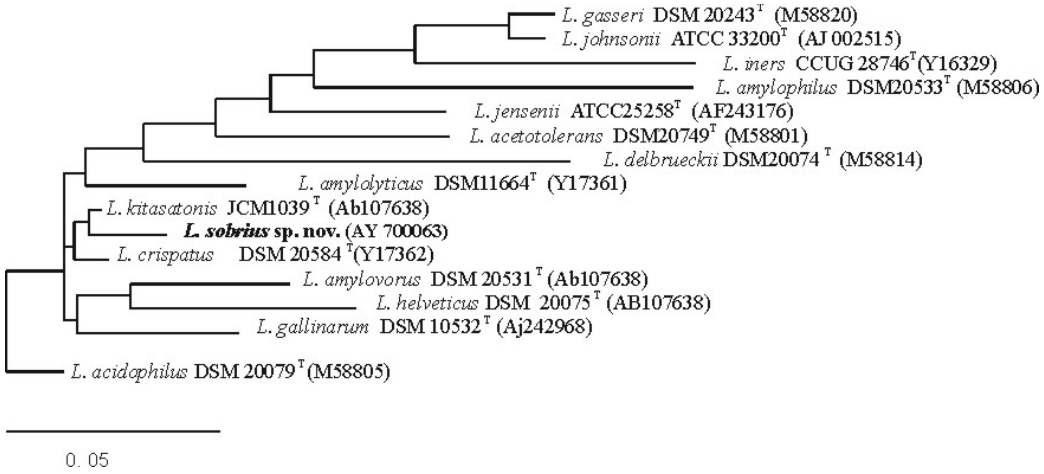


TABLE 1. DNA G+C content and phenotypic characteristics of *Lactobacillus amylovorus*-like strains and closely related lactobacilli. Taxa: 1, *L. amylovorus*-like (OTU171) strains; 2, *L. kitasatonis*; 3, *L. amylovorus*; 4, *L. crispatus*; 5. *L. gallinarum*; 6, *L. gasseri*; 7, *L. acidophilus*; 8, *L. johnsonii*. Data about the taxa 2 to 8, belonging the *L. acidophilus* group, is from (16); +, Positive; D, strain-dependent; D-, usually negative; -, negative; W, weak reaction. All strains produce acid from D-glucose, galactose, D-fructose, D-mannose and maltose and produce DL-lactic acid. No strains produce acid from arabinose, xylose, rhamnose, melezitose or sorbitol.

Characteristics	1	2	3	4	5	6	7	8
DNA G+C content (mol%)	35-36	37-40	36-38	33-36	36-37	33-34	32-40	33-35
Growth at 15°C	-	-	-	-	+	-	-	+
Fermentation of:								
D-Raffinose	+	-	D	+	+	-	+	D
Sucrose	+	+	+	+	+	+	+	+
Lactose	+	DW	+	+	D	+	+	+
Mannitol	DW	DW	-	D	-	-	-	-
Cellobiose	D	D	D	+	+	+	+	+
Salicin	DW	D	+	+	+	+	+	+
Ribose	DW	-	-	D	-	-	-	-
Trehalose	D	D-	-	D	-	+	+	D
Melibiose	D	-	D	+	+	W	-	D
Amygdalin	DW	-	D	+	+	+	+	+

The analysis of the isolates' whole-cell proteins by SDS-PAGE displayed marked differences in comparison with those of the reference strains (Fig. 3). An abundant 50kD protein was detected in the SDS fingerprints of all new isolates. After cluster analysis of the SDS-PAGE protein profiles, the fingerprints of the new isolates formed a coherent cluster with a similarity higher than 85%, while they were only distinctly related to the examined type strains with a similarity index below 70%.

Figure 2. Phylogenetic analysis of strain OTU171_001^T (*L. sobrius*) and selected members of *L. delbrueckii* 16S rRNA gene cluster of the genus *Lactobacillus*. The tree was calculated using the neighbor-joining method with partial 16S rRNA gene sequences (*E. coli* positions 107-1433), using the ARB software package (12). The bar represents 5% sequence divergence.



Furthermore, the new isolates were compared to the closest type strains by DNA-DNA hybridization. Labelled DNA of OTU171_001^T, OTU171_002 and OTU171_003 reassociated at high level (78-100%) with unlabelled DNA from the six OTU171 strains, while only low levels of reassociation (2-49%) were observed with the examined closely related *Lactobacillus* species (Table 2). The results indicated that the isolates, analyzed during the course of the study, belong to a single species that differ from the closest type strains.

Figure 3. Relationships between the whole-cell proteins of the examined *Lactobacillus* type strains and OTU171 isolates after SDS-PAGE analysis.

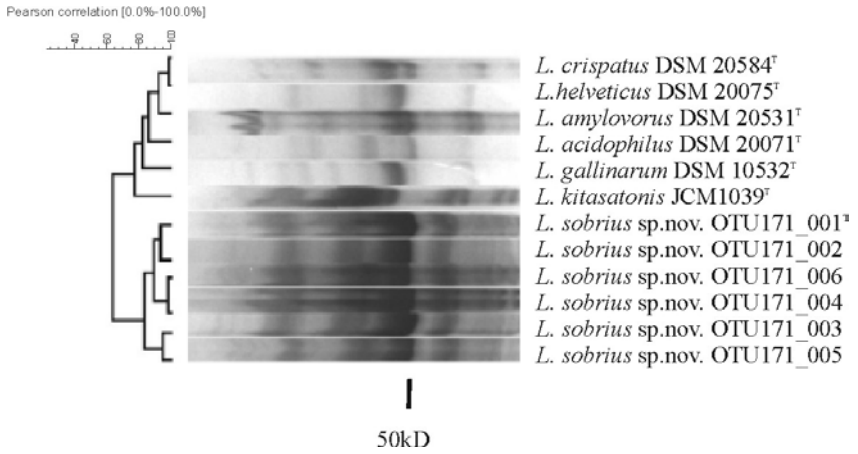


TABLE 2. DNA relatedness among *L. sobrius* sp. nov. and phylogenetically closely related *Lactobacillus* species. All values are mean values of two analyses with a standard deviation below 5%.

Strain	Reassociation (%) with DNA from strain:			
	OTU171_001^T	OTU171_002	OTU171_003	JCM1039
1. *L. sobrius*				
OTU171_001^T	100	98	90	45
OTU171_002	99	100	84	46
OTU171_003	95	90	100	35
OTU171_004	96	92	93	36
OTU171_005	99	92	100	25
OTU171_006	78	81	93	29
2. *L. kitasatonis* JCM1039^T	41	40	38	100
3. *L. amylovorus* DSMZ 20531^T	49	44	47	25
4. *L. crispatus* DSMZ 20584^T	2	3	2	18
5. *L. gallinarum* DSMZ 10532^T	22	24	31	-
6. *L. acidophilus* DSMZ20079^T	13	12	13	-
7. *L. helveticus* DSMZ 20075^T	11	13	14	-

-, not tested.

To further assess genomic diversity of the six isolates, the PFGE profiles of *Apa* I digested chromosomal DNA were visually compared. All isolates displayed distinct PFGE profiles from each other (data not shown). The average genome size was 1.2 Mb.

Based on the reported data, we propose the new species *L. sobrius* with the type strain OTU171_001^T.

Lactobacillus sobrius **(so' bri.us. L.masc. adj. sobrius, sober/moderate; in Calvinism: hard working, moderate person.)**

Cells are Gram-positive, non-motile, non-spore-forming rods that are 0·6–1·0 μm in width, and 2·0–20·0 μm in length, and occur singly, in pairs or often in long chains. Colonies are 1.4-2 mm in diameter, circular to slightly irregular to rough in form and with white colour, when the organism is grown on MRS agar at 37 °C for 2 days. There is no growth at 15 °C, but the bacterium grows at 45 °C. The organism is facultatively anaerobic and produces D- and L-lactic acid homofermentatively. Catalase is not produced. Acid is produced without gas formation from D-glucose, D-mannose, maltose, galactose, D-fructose, lactose, esculin, sucrose, amidon, mannitol (2 of 6 strains, including strain OTU171_001^T), cellobiose (3 out of 6 strains, including strain OTU171_001^T), salicin (2 out of 6 strains, including strain OTU171_001^T), trehalose (2 out of 6 strains, including strain OTU171_001^T), amygdalin (2 out of 6 strains, including strain OTU171_001^T, weak reaction), *N*-acetylglucosamine (2 out of 6 strains, including strain OTU171_001^T), arbutin (2 out of 6, including strain OTU171_001^T), ribose (2 out out of 6 strains, including strain OTU171_001^T, weak reaction), glycogene (3 out of 6 strains, fermented by strain OTU171_001^T), 5 ceto-gluconate (2 strains out of 6, fermented by strain OTU171_001^T, weak reation). FOS and unidentified compounds of SBP are also fermented by OTU171_001^T. There is no acid formation from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, melezitose, rhamnose, adonitol, β methyl–xyloside, sorbitol, L-sorbose, dulcitol, inositol, α methyl-D-mannoside, α methyl-D-glucoside, arbutin, inulin, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 ceto-gluconate. G+C content is 35-36 mol%. The habitat of *L. sobrius* is the porcine intestine. The type strain is OTU171_001^T (NCCB 100067; DSM 16698).

DISCUSSION

Here we report on the isolation and characterization of a *Lactobacillus sobrius* sp. nov. The bacterium is homofermentative organism abundant in the intestine of healthy piglets before weaning. Its ability to degrade FOS, and a mixture of substrates such as SBP, may potentially lead to its selective enrichment in the intestine of weaning piglets after their dietary addition. Such stimulation, if true, may potentially reduce the factors that predispose the piglets to morbidities after weaning.

Weaning is a serious stress factor in the development of pigs. Within the intensifying agricultural environment, piglets are weaned at an early age (3-4 weeks) with solid feed and transported to production farms. This combination of stress factors can lead to diarrhoea, a reduced growth rate, and in some cases, even death (9). In order to enhance growth and suppress the growth of Gram-negative bacterial pathogens antimicrobial compounds have been fed to weaning piglet a long time (4). The emergence of antibiotic resistance in the human commensal bacteria has raised concerns about the impact of anitimicrobial compounds for agricultural use. In this respect, prebiotics, probiotics, or synbiotics (combination of pre, and probiotics) are nutritional strategies, alternative to antibiotics, aiming to keep the weaning piglets healthy (9).

Prebiotics, such as FOS, and partially SBP, are defined as food ingredients that are neither hydrolyzed not adsorbed in the stomach or gastrointestinal tract, but rather confer beneficial effects on the host by selectively stimulating the growth of selected *Lactobacillus* or *Bifidobacterium* strains. In contrast, it has been demonstrated that eight strains *of Escherichia coli, Salmonella* spp. and other enteric bacteria were unable to use FOS as a sole carbon source (7). Furthermore, the dietary addition of FOS and SBP was found to improve the porcine microbial balance after weaning (10).

Probiotics are living microorganisms whose oral administration is aiming to promote health and growth, and has a potential to re-establish the natural balance and return the host to normal health and nutrition (3). Such bacterial therapy is partly based upon the hypothesis that specific strains from the indigenous microbiota may exert anti-pathogenic activities (6). In the current study, *L. sobrius* sp. nov. was found as a common commensal in the intestine of piglets before weaning. Hence, we hypothesise that a further dietary supplementation of a *L. sobrius* sp. nov. culture may enhance the pathogenic exclusions and improve the piglets' health and performance during the critical weaning period.

ACKNOWLEDGEMENTS

This research was financially supported by the European Communities EC project HEALTHYPIGUT (QLK5-LT2000-00522). We are grateful to Wilma Akkermans – van Vliet for excellent assistance with the sequencing analysis. Professor Dr. Hans G. Trüper, Department of Microbiology, Universität Bonn, Germany is specially acknowledged for his help with the etymology of the *Lactobacillus sobrius* name.

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CHAPTER 6

Lactobacillus sobrius sp. nov. strain 001^T rom the Resident Porcine Gastrointestinal Microbiota Protects Pig Enterocytes from Enterotoxigenic *Escherichia coli* K88 Induced Damages

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ABSTRACT

Bacterial therapy for man and farmhouse animals is largely based on the concept that specific strains selected from the commensal gut microbiota are involved in resistance to intestinal diseases. The aim of this study was to analyze the possible protective effects of two phylogenetically closely related *Lactobacillus* species, against intestinal damages promoted by an intestinal pathogen. Differentiated swine intestinal epithelial cell line (IPEC-1) was infected with enterotoxigenic *Escherichia coli* K88 (ETEC) and treated with a novel porcine isolate *Lactobacillus sobrius* sp. nov. strain 001^T (LS1), and with *L. amylovorus* (DSMZ 20531^T). The protective effects of the two lactobacilli were assessed by measuring pathogen adhesion and invasion, cell membrane permeability, alterations in tight junctional and cytoskeletal proteins and cytokine expression. ETEC was able to adhere and invade the IPEC-1 cells, and caused disruption of membrane permeability and integrity. Treatment of infected cells with LS1 strongly reduced the number of adhered and internalized ETEC. Moreover, upon LS1 treatment the localization and distribution of tight junction and cytoskeleton proteins were preserved. LS1 was also able to prevent the ETEC-induced up regulation of IL-1β and IL-8 and down-regulation of IL-10. These protective effects were not found with *L. amylovorus* (DSMZ 20531^T) treatment. Together, the data indicate that LS1 exerts a species-specific protection against the harmful effects of ETEC by different mechanisms.

A modified version of this chapter has been submitted for publication

INTRODUCTION

The intestinal epithelium constitutes the major barrier that separates the external from the internal environment and represents the first line of defence against pathogens and dangerous environmental agents. Tight junctions play a major role in regulating epithelial permeability and avoiding the entry of external molecules (2). They encircle the cells at the apical end of the lateral membrane and are composed of an array of proteins, including the integral membrane protein, occludin, and cytosolic peripheral membrane proteins, the zonula occludens proteins (ZO-1, ZO-2, ZO-3), in close apposition to the actin and myosin ring (32). Enteric pathogens may cause several sorts of damages to intestinal cells. This include disruption of mucosal barrier that allows indiscriminate passage of luminal antigens, and interference in the epithelial cell signalling, which controls both the transcellular and paracellular secretion pathways. Such an impairment of the mucosal immune system may lead to the development of inflammatory reactions (9, 30, 32).

Infection by enterotoxigenic *Escherichia coli* K88 (ETEC) is one of the principal causes of diarrheal disease in pigs during the pre- and post-weaning period. Among the different ETEC strains, those expressing K88 fimbrial adhesins are the most prevalent (13). The pathogenic action of ETEC is exerted through binding of K88 fimbriae to specific intestinal receptors, allowing release of enterotoxins, which induce massive fluid and electrolyte secretion, resulting in diarrhea (17). Probiotic lactobacilli have potential to overcome intestinal disorders, due to their ability to maintain or restore normal microflora, inhibit pathogen adhesion to intestinal wall, secrete antimicrobial peptides and prevent a number of intestinal disorders including inflammatory diseases (3, 16, 31, 33). It has been suggested that the beneficial effect of various lactobacilli is related to the species-specific properties (19). Moreover, protective effects of several *Lactobacillus* species have been demonstrated for man and farmhouse animals (10).

Previous studies have shown that strains closely related to *Lactobacillus amylovorus* are widely distributed as common porcine intestinal commensals (18). Their phenotypic and molecular taxonomic characterization, however, revealed significant differences with the type strain of *L. amylovorus* (DSMZ 20531^T), and the name *Lactobacillus sobrius* was proposed (Chapter 5). Given the fact that this particular *Lactobacillus* species is an abundant isolate cultured from the intestine of a healthy piglet, the aim of the present study was to examine a possible protective role of *L. sobrius* strain 001^T (LS1) and phylogenetically closely related *L. amylovorus* (DSMZ 20531^T) against ETEC-induced cellular damages, by

investigating pathogen adhesion and invasion, cell membrane permeability, alterations in tight junctional and cytoskeletal proteins and cytokine expression. As a cellular model, we have used the IPEC-1 cell line derived from the small intestine of a newborn unsuckled piglet (12) that can differentiate and exhibit enterocytic features, including microvilli and tight junctions (28).

MATERIALS AND METHODS

Epithelial cell culture. The IPEC-1 cells (obtained from Drs Berschneider H.M. and Black D.D.), were grown in complete DMEM/F-12 medium (Eurobio, Milan, Italy) supplemented with 5% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 15 mM Hepes, 5 µg/L epidermal growth factor (Becton Dickinson, Milan, Italy), ITS (10 µg/ml insulin, 5.5 µg/ml transferrin; 5 ng/ml selenium; Sigma, Milan, Italy), 10^5 U/l penicillin and 100 mg/l streptomycin. According to the different experiments, IPEC-1 cells were grown on Transwell filters, tissue culture plates or glass coverslips as described below, at 37°C in an atmosphere of 5% CO_2 and 95% air at 90% relative humidity. After confluency, cells were cultured for 8-10 days without FCS to allow differentiation. Medium was changed three times a week.

Bacterial growth. ETEC strain K88 (provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Reggio Emilia, Italy) was grown in Luria-Bertani (LB) broth containing 1% tryptone and 0.5% yeast extract (both from OXOID, Basingstoke, UK), plus 1% NaCl, pH 7.0. After overnight incubation at 37°C with vigorous shaking, bacteria were diluted in fresh LB and grown until mid-log phase for all experiments. Bacterial cells were harvested by centrifugation at 3,000 x g for 10 min at 4°C, and then resuspended in antibiotic-free DMEM/F-12 medium for all experiments.

Two different lactobacilli were used: *L. amylovorus* (DSMZ 20531^T) type culture 20531 (commercial strain isolated from cattle waste) (23), and *L. sobrius* strain 001^T (LS1), isolated from pig intestine (Chapter 5). The lactobacilli were grown in DeMan Rogosa Sharp (MRS) medium (DIFCO, Milan, Italy) at 37°C under anaerobic conditions without shaking. After overnight incubation, they were diluted in fresh MRS, grown until mid-log phase and processed as for ETEC. The viability of ETEC, *L. amylovorus* (DSMZ 20531^T) and LS1 grown on DMEM/F-12 medium did not differ from that of bacteria grown on LB or MRS media, as tested in preliminary experiments.

Bacterial concentrations of both ETEC and lactobacilli were determined in preliminary experiments by densitometry and confirmed by serial dilutions followed by viable plate counts (colony forming units, CFU) on LB agar after 16 h incubation for ETEC, and on MRS agar after 48 h incubation for the two lactobacilli.

Bacterial adhesion. Bacterial adhesion was tested by two different methods, the agar plating and fluorescent *in situ* hybridization (FISH) assays. IPEC-1 cells (1 x 10^6 cells/well) were grown and differentiated on 24-well plates for agar plating assay or on glass cover slips in 24-well plates for the FISH assay. They were treated with ETEC (1 x 10^8 bacteria/ml) and with LS1 or *L. amylovorus* DSMZ (1 x 10^9 or 1 x 10^{10} bacteria/ml). After incubation at 37°C for 1.5 h, non-adherent ETEC cells were removed by several washes with Hank's balanced salt solution (HBSS; 137 mmol/L NaCl, 5.36 mmol/L KCl, 1.67 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1.03 mmol/L $MgSO_4$, 0.44 mmol/L KH_2PO_4, 0.34 mmol/L Na_2HPO_4, 5.6 mmol/L glucose).

For agar plating assay, IPEC-1 cells were lysed with 1% Triton-X-100 and adhered ETEC was quantified by plating appropriate serial dilutions of lysates on LB agar. Preliminary experiments confirmed that the two lactobacilli were not able to form colonies after overnight incubation on LB agar at 37°C in aerobic conditions.

The FISH assay was performed as described (18). Briefly, IPEC-1 cells were fixed with ice-cold 96% ethanol and PBS (1:1) for 2h at 4°C, placed onto gelatin-coated glass slides and dried for 20 min at 50°C. The cells were then gradually dehydrated in 50%, 70% and 96% ethanol/H_2O for 3 min. The hybridization was carried out using a *L. amylovorus* specific probe, L-*-OTU171-0088-a-A-18 (CGC TTT CCC AAC GTC ATT), and 5 ng/µl FITC-labelled universal bacterial specific probe, S-D-Bact-0338-a-A-17 (GCT GCC TCC CGT AGG AGT) in the respective buffers at 50°C for 16 hours. After the washing step, the digital images of the slide were analyzed and fluorescence positive cells were recorded using Qwin image analysis software (Leica Microsystems, The Netherlands).

Membrane permeability. IPEC-1 cells (5 x 10^5 cells/filter) were differentiated on Transwell filters (polyethylene terephtalate filter inserts for cell culture of 6.5 mm diameter, 0.45 µm pore diameter; Becton Dickinson). The membrane integrity was determined by transepithelial electrical resistance (TEER), as previously reported (29). The TEER was monitored with a Millicell Electrical Resistance System (Millipore, Milan, Italy) and expressed as Ohm x cm^2. The TEER was checked before each experiment and only cell monolayers with TEER > 1000 Ohm x cm^2 were used. To test the effect of ETEC on membrane integrity, cells were infected

with 1 x 10^8 bacteria/ml for 5 h. To assay the effect of lactobacilli on cell permeability, several concentrations of LS1 and *L. amylovorus* (DSMZ 20531^T) (from 1 x 10^8 to 1 x 10^{10} bacteria/ml) were added to the apical compartment of Transwell filters for 5 h. To verify the probiotic protective effects, LS1 or *L. amylovorus* (DSMZ 20531^T) (1 x 10^9 or 1 x 10^{10} bacteria/ml) were apically added to the cells together with ETEC for 2 h. In other experiments, IPEC-1 cells were pretreated with LS1 or *L. amylovorus* (DSMZ 20531^T) for 8 h before ETEC infection. The TEER was measured every 30 min.

Immunolocalization of tight junction and cytoskeleton proteins. IPEC-1 cells (1x10^6 cells/well) were differentiated on glass coverslips in 24-well plates. They were treated with ETEC (1x10^8/ml) and LS1 or *L. amylovorus* (DSMZ 20531^T) (1x10^9 bacteria/ml) for 1.5 h. Nonadherent bacteria were removed by several washes with Ca-Mg containing PBS (PBS$^+$, Sigma). Localization of ZO-1, occludin and F-actin was analyzed according to Ferruzza *et al.*, (8). Briefly, for ZO-1 and occludin analysis, the cells were fixed with ice cold absolute methanol and treated with rabbit anti-ZO-1 polyclonal antibody and mouse anti-occludin monoclonal antibody (Zymed Laboratories, Milan, Italy). For secondary detection, the cells were incubated with rodopsin-conjugated mouse anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Milan, Italy) for ZO-1 and occludin respectively. For F-actin analysis, the cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and treated with 1% bovine serum albumin (BSA) containing 0.4 μg/ml fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma). After several washes with 0.1 % BSA and treatment with 50 μg/ml RNAase (Roche Diagnostics Milan, Italy), the cells were incubated with 0.001 % propidium iodide in PBS$^+$. Stained monolayers were mounted on glass slides in vectashields (Vector Laboratories, Milan, Italy) and analyzed under a ZEISS fluorescence microscope.

ETEC invasion. Internalization of viable bacteria was assayed by gentamicin protection assay, as previously described (29). IPEC-1 cells (1 x 10^6 cells/well) were differentiated on 24-well plates and infected with ETEC (1 x 10^8 CFU/ml). To test the protection of the two *Lactobacillus* spp. against ETEC invasion, cells were treated with ETEC (1 x 10^8 CFU/ml) and LS1 or *L. amylovorus* (DSMZ 20531^T) (1 x 10^9 and 1 x 10^{10} CFU/ml) for 1.5 h. After extensive washes with HBSS, cells were incubated with culture medium containing 50 mg/L gentamicin sulphate (Sigma), for 2.5 h to kill residual viable extracellular bacteria. As control for bacterial killing, gentamicin was added to some wells at the beginning of bacterial

treatment. Cells were then washed with HBSS and lysed with 1% Triton-X-100. Viable intracellular bacteria were quantified by agar plating.

Cytokine mRNA. IPEC-1 cells were differentiated in 6-well plates at 4 x 10^6 cells/well. ETEC (4 x 10^8 CFU/well) and lactobacilli (4 x 10^9 CFU/well) were added either separately or together for 2 h. This time was chosen because preliminary experiments indicated that alterations in cytokine mRNA levels by ETEC infection were most evident after 2 h of treatment. Total RNA was extracted with TRIZOL reagent (Life Technologies, GIBCO BRL, Milan, Italy) and 1 μg of RNA was subjected to reverse transcription-polymerase chain reaction to analyze the abundance of mRNAs encoding: interleukin (IL)-1β, IL-8 and IL-10. Each cytokine was co-amplified with glyceraldheyde-3-phosphate-dehydrogenase (GAPDH) as internal control. The sequences of primers are shown in a previous study (7). The primers were provided by MWG Biotech (Florence, Italy). The PCR products were analyzed on agarose gel and the intensity of the bands was measured by Scion image software (Scion Corporation, Frederick, MD). The ratio of cytokine/GAPDH mRNA intensities was used to evaluate the level of each cytokine mRNA.

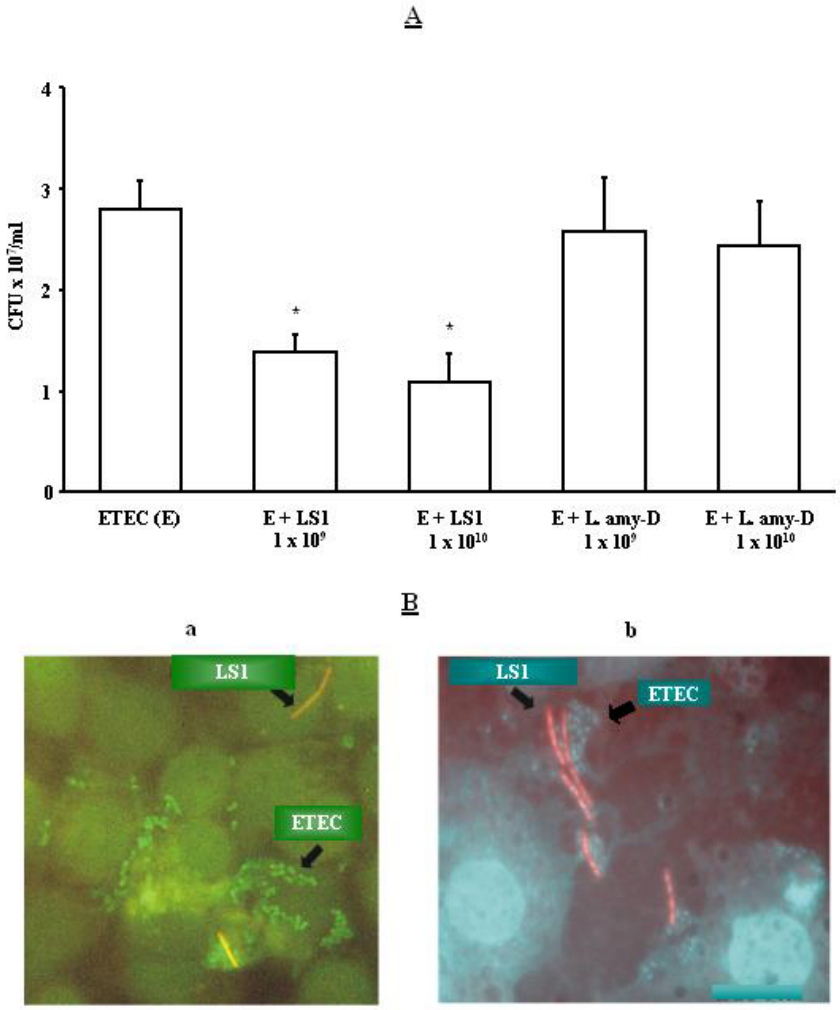
Statistical analysis. The significance of the differences was evaluated by one-way ANOVA followed by Fisher's test for all experiments except for TEER analysis. Repeated measures analysis (mixed model) was used to analyze time and treatment effect on TEER. Huynh-Feldt correction for F-values was applied due to the assumption that circularity of the variance covariance matrix was violated. Significance was set at p values < 0.05. For multiple comparison, Bonferroni correction was used. All statistical analyses were performed with SPSS software program (version 8.0; SPSS Inc, Chicago,IL, USA).

RESULTS

Reduction of ETEC adhesion. We have previously shown that ETEC was able to adhere to human Caco-2 cells(29). Here, we report that this strain of ETEC can also adhere to IPEC-1 cells. Indeed, when adhesion was measured by agar plating assay, an elevated number of viable bacteria was found attached to the cells (Fig. 1A). Treatment of the IPEC-1 cells with LS1 was able to strongly reduce the number of adhered ETEC, and this effect was achieved already with the lower concentration of the probiotic (1 x 10^9 CFU/ml). Opposite to this result, *L. amylovorus* (DSMZ 20531^T) did not protect the cell against ETEC adhesion, even at

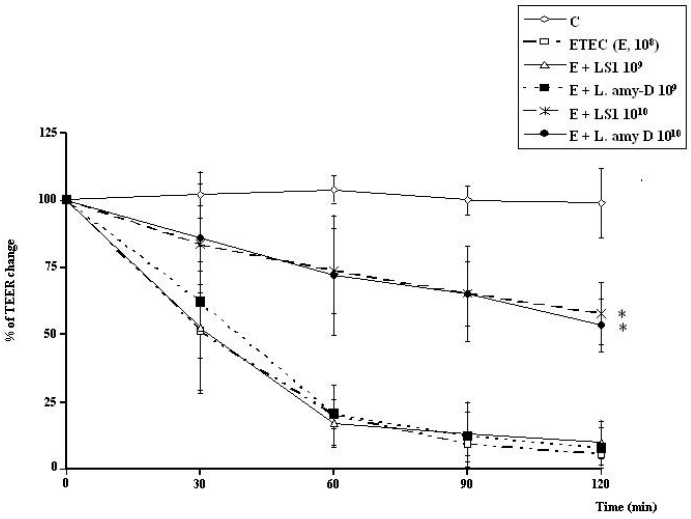
higher concentration (1 x 10^{10} CFU/ml), suggesting a species-specific effect. Complementary to the plate counting assay, ETEC and LS1 attachment was also detected by FISH method (fig 1B). The different fluorescent probes revealed that both ETEC and LS1 were detectable on IPEC-1 cells (Fig. 1B, a). Interestingly, co-aggregation of LS1 and ETEC were evidenced after microscopic examination (Fig. 1B, b). In addition, *L. amylovorus* (DSMZ20531^T) attachment was also observed by FISH (data not shown).

Figure 1. Reduction of enterotoxigenic *Escherichia coli* K88 (ETEC) adhesion to IPEC-1 cells by *L. sobrius* strain 001^T (LS1) and *L. amylovorus* (DSMZ 20531^T) (*L. amy*-D). Cells differentiated on 24-well plates were treated with ETEC (1 x 10^8 CFU/ml) and different concentrations of probiotics (1 x 10^9 or 1 x 10^{10} CFU/ml) for 1.5 h. A: number of adhering ETEC determined by agar plating and reported as colony forming units (CFU). Data means (SD), n=5 (in triplicate). *p<0.05 compared with E. B: FISH assay. a: adhesion of ETEC and LS1; b: co-aggregation of ETEC and LS1.



Effect of bacteria on epithelial permeability. Membrane damage by ETEC and the potential protection by lactobacilli were investigated by measuring membrane permeability by TEER. First, we have assayed the effect of the two *Lactobacillus* species when added alone to the IPEC-1 cells. Both lactobacilli did not alter membrane permeability when added to the cells at the concentrations of 1 x 10^9CFU/ml or 1 x 10^{10}CFU/ml (data not shown). A drastic fall of TEER was induced by ETEC infection (1 x 10^8CFU/ml), already after 30 minutes of infection (Fig. 2). When the two lactobacilli were added together with ETEC to the cells, a protection from the membrane damage was observed with the higher concentration of probiotics (1 x 10^{10} CFU/ml), since the decrease of TEER was strongly reduced (Fig. 2).

Figure 2. Effects of *L. sobrius* strain 001^T (LS1) and *L. amylovorus* (DSMZ 20531^T) (*L. amy*-D) on tight junction permeability of IPEC-1 cells infected with enterotoxigenic *Escherichia coli* K88 (ETEC). Cells differentiated on Transwell filters (5 x 10^5 cells/filter) were untreated (C) or apically treated with ETEC (1 x 10^8 CFU/ml) and different concentrations of the two strains of lactobacilli (1 x 10^9 or 1 x 10^{10} CFU/ml). Transepithelial electrical resistance (TEER) was measured every 30 min, from 0 to 2 h. Values are means (SD), n=≥ 5 (in triplicate). *p < 0.05 compared with C and with E, E + LS1 10^9 and E + L. amy D 10^9.



Protection of membrane barrier. To investigate whether the membrane barrier disruption caused by ETEC was associated with a perturbation of cytoskeleton and tight junctions, and whether lactobacilli could inhibit such alterations, immunolocalization of some of the most

important tight junction and cytoskeleton proteins was analyzed. Uninfected IPEC-1 cells showed uniform distribution of ZO-1 and occludin around the circumferences of the cells, an even distribution of peri-junctional F-actin (Fig. 3Aa, Ba, Ca,). Treatment of IPEC-1 cells with LS1 did not modify the localization of ZO-1, occludin and F-actin (Fig. 3Ab, Bb, Cb), whereas treatment with *L. amylovorus* (DSMZ 20531^T) induced a partial disappearance of ZO-1, occludin and F-actin (Fig. 3Ac, Bc, Cc). Immunofluorescence of ZO-1 in ETEC infected cells showed an opening of tight junction as indicated by loss of continuity of cell-cell contact (Fig. 3Ad). Infection with ETEC caused a reduction of fluorescence intensity of occludin indicating a reduction of the protein around the periphery of the cell, as well a scattered distribution of the protein inside the cell (Fig. 3Bd). A partial disorganization associated with uneven distribution of F-actin (Fig. 3Cd) was also induced by ETEC. When the cells were treated with ETEC together with LS1, a correct distribution of tight junction and cytoskeleton proteins was found (Fig. 3Ae, Be, Ce). In contrast, *L. amylovorus* (DSMZ 20531^T) treatment was not able to protect the ETEC-induced cytoskeleton and tight junction alterations (Fig. 3Af, Bf, Cf). These data suggest again a species-specific effect of LS1.

Reduction of ETEC invasivity. In a previous study we have found that a certain number of ETEC was able to invade Caco-2 cells (29). Thus, in this study we have investigated whether the same strain of pathogen invaded IPEC-1 cells and whether the two lactobacilli strains could inhibit this invasion (Fig. 4). Infection of IPEC-1 with ETEC resulted in internalization of certain number of pathogen into the cells. An almost complete inhibition of ETEC invasivity was observed after coincubation of ETEC with LS1 at a concentration of 1 x 10^{10} CFU/ml, and a lower inhibition with 1 x 10^9 CFU/ml. The highest concentration *L. amylovorus* (DSMZ 20531^T) (1 x 10^{10} CFU/ml) was able to reduce the ETEC invasivity, but at lower extent than LS1.

Regulation of cytokine gene expressions. To investigate whether the damage caused by ETEC was associated with development of inflammation and whether this was counteracted by lactobacilli, the expression of pro- and anti-inflammatory cytokines was analyzed (Fig. 5). While treatment of the cells with LS1 did not induce any alteration on the expression of these cytokines as compared to untreated cells, *L. amylovorus* (DSMZ 20531^T) induced an increase of pro-inflammatory cytokines IL-1β and IL-8. Infection of IPEC-1 cells with ETEC caused an up-regulation of IL-1β and IL-8, and a down-regulation of the anti-inflammatory cytokine IL-10, as compared to untreated cells. The increase of IL-1β and IL-8 was not induced when the cells were treated with LS1 together with ETEC. In contrast, *L. amylovorus* (DSMZ

20531^T) did not counteract the ETEC-induced IL-1β and IL-8 expression. The two strains of lactobacilli were both able to neutralize the ETEC induced decrease of IL-10.

Figure 3. Immunofluorescence localization of ZO-1, occludin and F-actin. Differentiated IPEC-1 cells ($1x10^6$ cells) on glass coverslips in 24-well plates were treated with enterotoxigenic ETEC ($1x10^8$CFU/ml) and *L. sobrius* strain 001 (LS1) or *L. amylovorus* (DSMZ 20531^T) ($1x10^9$ CFU/ml), for 1.5 h. For ZO-1 and occludin, cells were fixed with methanol and labelled with rabbit anti-ZO-1 or mouse anti-occludin polyclonal antibody. For F-actin, cells were fixed with paraformaldehyde, permeabilized and labelled with fluorescein isothiocyanate (FITC)-conjugated phalloidin. Immunofluorescences were analyzed under fluorescence microscope. a: control; b: LS1; c: *L. amylovorus* (DSMZ 20531^T); d: ETEC; e: ETEC + LS1; f: ETEC + *L. amylovorus* (DSMZ 20531^T). Arrow indicates loss of cell-cell contact.

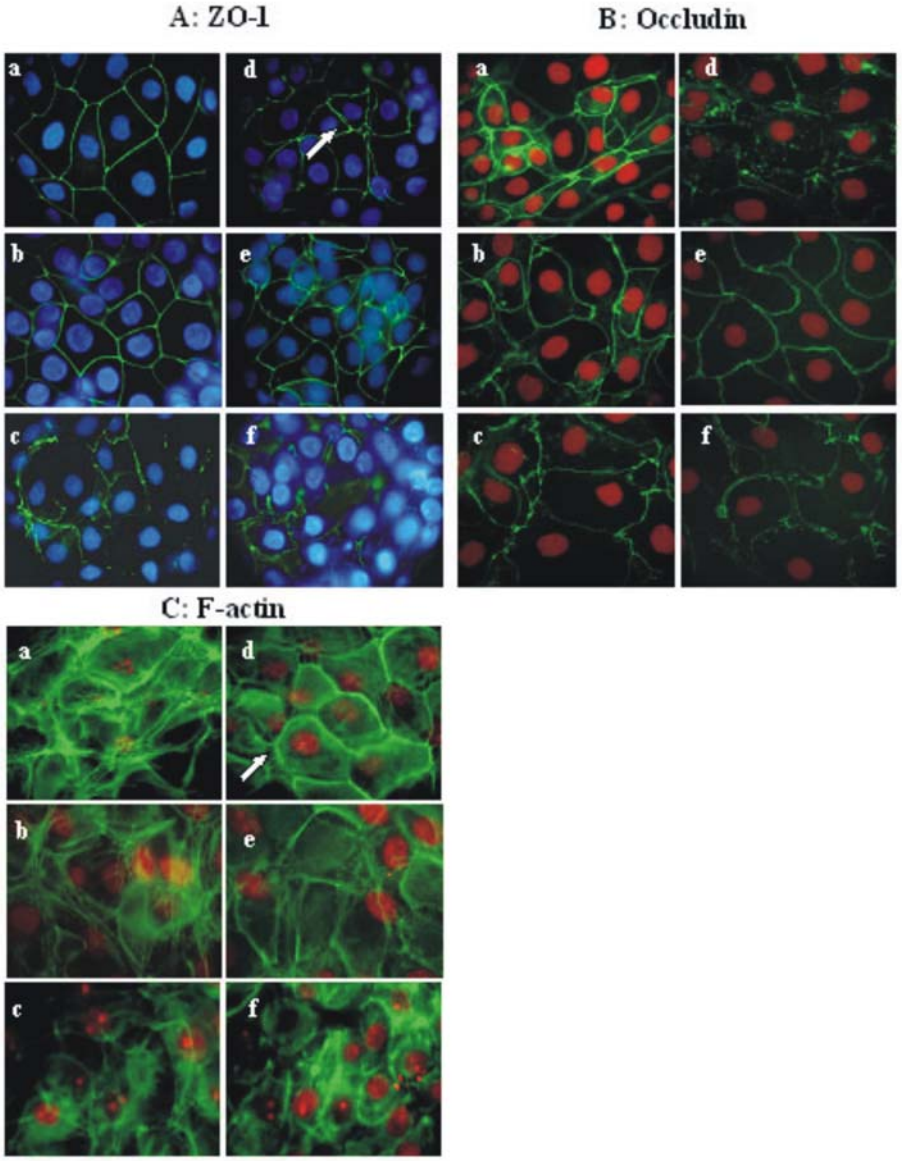
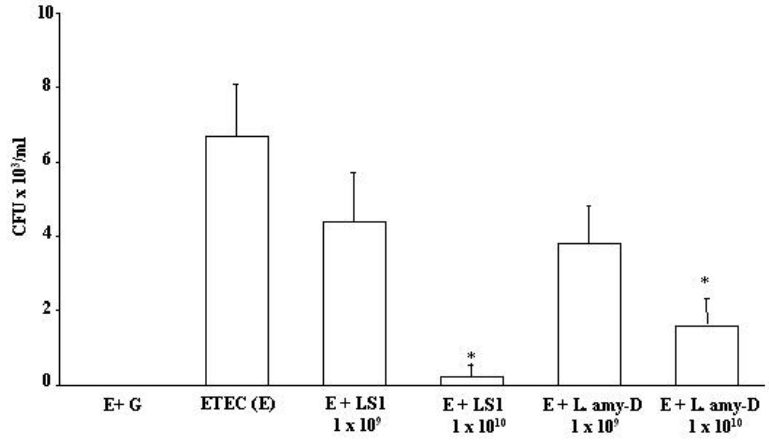


Figure 4. Reduction of enterotoxigenic *Escherichia coli* K88 (ETEC) invasivity by *L. sobrius* strain 001^T (LS1) and *L. amylovorus* (DSMZ 20531^T) (*L. amy*-D). Differentiated IPEC-1 cells on 24-well plates ($1x10^6$ cells/well) were treated with ETEC (1×10^8 bacteria/ml) and different concentrations of lactobacilli (1×10^9 or 1×10^{10} CFU/ml), for 1.5 h. Gentamicin sulphate (50 mg/l) was added for additional 2.5 h. As control for bacterial killing, gentamicin was added to some cells together with ETEC at 0 h (E+G). Viable internalized ETEC cells were quantified by agar plating and reported as colony forming units (CFU). Data are means (SD), n=8. *p<0.05 compared with E.

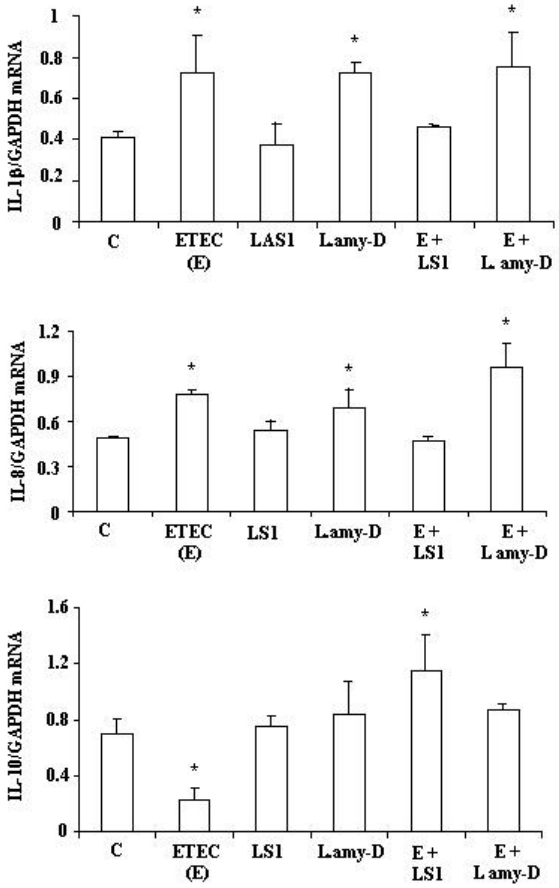


DISCUSSION

In the present study, strong protective effects of the novel porcine isolate *L. sobrius* (LS1), but not of *L. amylovorus* (DSMZ 20531^T) against ETEC induced epithelial damages to pig intestinal cells was demonstrated. The data indicate that LS1 determinants could be responsible for the unique resistance of the epithelial cells to ETEC proinflammatory stimuli. ETEC must first adhere to intestinal epithelial cells to release enterotoxins and initiate intestinal damages. Limiting access of pathogens to intestinal epithelial cells is one of the activities exerted by several probiotics (15, 31) Here, we show that LS1 was able to strongly inhibit ETEC adhesion, whereas *L. amylovorus* (DSMZ 20531^T) was ineffective. This inhibition was likely achieved by competition with the pathogen for binding sites to the epithelium. Moreover, the co-aggregation of LS1 with ETEC was highlighted by the FISH assay indicating a direct LS1-ETEC interaction. At what extent the LS1-ETEC contacts interfered with the pathogenic attachment, is still unknown. Although the *Lactobacillus* species used here are closely related, a recent study has demonstrated their significant

phenotypic and molecular taxonomic differences (Chapter 5). Several *L. sobrius* isolates have also been identified as common porcine intestinal commensals. Specifically, the LS1 strain has been isolated from the faeces of a healthy neonatal piglet (Chapter 5). In contrast, the type strain of *L. amylovorus* (DSMZ 20531^T) has been cultured from cattle waste-corn. Both *Lactobacillus* species attached to IPEC-1 cells, however, only LS1 reduced the pathogenic adhesion, indicating a species-specific effect.

Figure 5. Gene expressions of pro- and anti-inflammatory cytokines. Differentiated IPEC-1 cells on 6-well plates (4 x 10^6 cells/well) were untreated (C) or treated with enterotoxigenic *Escherichia coli* K88 (ETEC) (4 x 10^8 cells/well) and *L. sobrius* strain 001^T (LS1) or *L. amylovorus* (DSMZ 20531^T) (*L. amy*-D) at a concentration of 4 x 10^9 cells/well, for 2 h. The expression of interleukin IL-1β, IL-8, and IL-10 was analyzed by RT-PCR and are reported as densitometric values of each cytokine mRNA normalized to GAPDH mRNA. Data are means (SD); n=≥ 6. *p<0.05 compared with C.



Although reduced, the ETEC adhesion was not completely prevented by LS1 treatment. A certain amount of the pathogen did attach to the cells and thus ETEC was potentially able to target certain membrane barrier function of the epithelium, and to trigger further inflammatory cascade.

Tight junctions form the barrier to the diffusion of macromolecules through the paracellular space (2). Zonula occludens are the major proteins of tight junctions, and bind to the transmembrane occludin proteins and to the apical perijunctional actomyosin ring. The interaction between tight junctions with the cytoskeleton is fundamental to maintain barrier function (32). Disruption of epithelial barrier in the gut by bacterial infections has been hypothesized as the main pathogenic factor involved in several inflammatory intestinal diseases (20, 30). In this study we show that the monolayers of IPEC-1 cells, as other intestinal cell lines, have well defined tight junctions as indicated by the immunofluorescence localization of ZO-1, occludin and F-actin. In agreement with previous findings that enteric pathogens may cause loss of membrane barrier (2), we report that infection of IPEC-1 cells with ETEC led to disruption of tight junctions with loss of cell-cell contact, and disorganization of cytoskeleton. Whether this was achieved by release of bacterial proteases or through binding of bacterial toxins to the tight junctions proteins, as demonstrated for some pathogen toxins (2), remains to be investigated. Various *Lactobacillus* strains have been shown to protect the intestinal epithelial barrier function. (20, 27) The two *Lactobacillus* species used in this study partially inhibited the increased membrane permeability induced by ETEC in a dose dependent manner. However, the analysis of tight junctional and cytoskeleton proteins clearly show that only the new porcine isolate LS1 is able to counteract the membrane barrier disruption caused by ETEC, by maintaining the correct localization of ZO-1 and F-actin. Our results indicate again a species-specific effect of LS1, since *L. amylovorus* (DSMZ 20531^T) was unable to prevent the membrane damages. The disruption of membrane barrier by ETEC likely allowed the invasion of of ETEC, although in a restricted amount. Also in this case, the main protective effect was seen by LS1 that blocked the internalization of the pathogen, whereas *L. amylovorus* (DSMZ 20531^T) only partially inhibited the ETEC invasion.

Many cytokines have been shown to regulate tight junction structure and function (35). For instance, IL-1β may lead to loss of occludin and ZO-1 (4). While IFNγ may disrupt barrier integrity (37), IL-10 can prevent IFNγ-mediated decrease of barrier function (21). Furthermore, IL-8 release has been associated with pathogen-induced alterations of tight

junctions (25). In agreement with these findings, we have found that disruption of membrane barrier by ETEC was associated with an increase of IL-1β and IL-8, and a decrease of IL-10. Treatment with LS1 prevented the dysregulation of these cytokines, suggesting that the protective effect on membrane integrity is mediated by cytokine regulation. Interestingly though, *L. amylovorus* (DSMZ 20531^T) was able to neutralize the decrease of IL-10 upon ETEC attachment, but did not counteract the pathogen induced up-regulation of the IL-1β and IL-8 expression pattern. Hence, further studies are needed on the specific LS1 cues modulating the inflammatory cytokines regulation during ETEC infection.

It is still unclear whether the breakdown of barrier integrity is the primary cause of development of inflammation or vice versa the inflammation leads to loss of the barrier. The cytokines studied, other than mediators of tight junctions structure and function, play a key role in the regulation of inflammatory processes (1, 14) Thus, LS1 could prevent the ETEC K88 induced inflammatory disease through regulation of cytokines, which are crucial for membrane barrier homeostasis and development of inflammatory reactions.

Although the unique LS1 determinants mediating the protective effect remain to be identified, various cell wall components could have played a key role. The cell wall of Gram-positive bacteria, such as lactobacilli, is mainly composed of peptidoglycan and essential teichoic acid polymers (22), which include lipoteichoic acids (LTAs) (24). LTAs from two *Lactobacillus* strains appear to be involved in the downregulation of cytokine expression after bacterial stimulation (34). LTAs from gram-positive organisms, and thus present in LS1, have been identified as ligands for toll-like receptor (TLR)-2 (36). Several TLRs are constitutively expressed by intestinal epithelial cells (5) and appear to be responsible for immune cell activation by bacterial products (6, 26). Among them, TLR-2 has been also shown to directly enhance tight junctions stabilization through activation of protein kinase C. (6). Based on these consideration, we can hypothesize that LS1 may induce TLR signaling that lead to immunological reaction against the pathogen and/or directly to restoration or stabilization of membrane barrier integrity. However, other pattern-recognition receptors such as C-type lectin receptors (CLRs) may also be involved in the observed immunological reaction (11). At what extend the immune responses reported in our study involved a signalling through TLRs and/or CLRs, remains to be determined.

In conclusion**,** the results reported here indicate that the new porcine isolate LS1 exerts a species-specific protection against the harmful effects of ETEC, by competing with pathogen adhesion and preventing the membrane barrier disruption likely through regulation of cytokine expression.

ACKNOWLEDGEMENTS

We thank Dr. V. Panetta for valuable help in the statistical analysis, Drs Helene M Berschneider and Denis D. Black for their generous gift of IPEC-1 cell line and Prof. P. Bosi for kindly providing the ETEC strain. This work was supported by European Community grant, project Healthypigut, contract N: QLK5-CT-2000–00522. The authors are solely responsible for this publication, and the manuscript does not represent the opinion of the European Community, which is not responsible for the information delivered.

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CHAPTER 7

Representative Difference Analysis and Real-Time PCR for Strain-Specific Quantification of *Lactobacillus sobrius* sp. nov.

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ABSTRACT

Previous studies have shown that strains closely related to *Lactobacillus amylovorus* are widely distributed as common porcine intestinal commensals. Their phenotypic and molecular taxonomic characterization, however, revealed significant differences with the type strain of *L. amylovorus* (DSMZ 20531^T) and the name *Lactobacillus sobrius* sp. nov. was proposed. Current molecular ecological techniques, including ribosomal RNA-targeted approaches, are only of limited value for the assessment of such microdiversity that can have significant functional impact. Here we report on the development of a novel strain detection system based on isolation of specific genomic fragments by representational difference analysis (RDA) and their further detection by real-time PCR. RDA was firstly adapted to study the microdiversity between highly similar genomes of four *Lactobacillus sobrius* sp. nov. isolates. Unique genomic sequences for one of the isolates, *L. sobrius* sp. nov. strain 001^T, were revealed after subtractive hybridization. Furthermore, real-time PCR amplification of the strain-specific genomic fragments using Biorad iCycler equipment was evaluated. Real-time PCR detection of serially diluted DNA extracted from *L. sobrius* sp. nov. strain 001^T was linear for cell counts ranging from 10^7 to 10 cells per PCR assay. The detection specificity was validated by using genomic DNA of additional *L. sobrius* sp. nov. isolates and other phylogenetically closely related *Lactobacillus* spp. Applicability of the developed approach with intestinal samples was confirmed with porcine ileal digesta samples spiked with known numbers of *L. sobrius* sp. nov. strain 001^T cells. Together, a single-strain detection and quantification approach has been developed based on the combination of RDA and real-time PCR that may have several applications.

submitted for publication

INTRODUCTION

Molecular monitoring of the microbial content of foods and the mammalian host's gastrointestinal (GI) tract requires sensitive and reliable methods to distinguish between more or less closely related microorganisms at different levels of resolution. Current techniques, including genetic fingerprinting, gene sequencing, oligonucleotide probe hybridization and PCR with specific primers, discriminate strongly related bacteria with varying degrees of success (6, 11). Recently, a combination of cultivation-based methods with a molecular biological approach has showed that certain bacteria with identical 16S rRNA gene sequences represent distinct eco- and genotypes (4). Furthermore, porcine isolates characterized as *Lactobacillus sobrius* sp. nov. hybridized strongly to a fluorescent 16S rRNA-targeted DNA oligonucleotide probe specific for *Lactobacillus amylovorus* (Chapter 5). Moreover, a high level of 16S rRNA gene identity (>97 %) was found between the type strain of *L. sobrius* sp. nov. and the related species *L. amylovorus*, *L. crispatus* and *L. kitasatonis* (Chapter 5). Although phylogenetically closely related, their genomic relatedness was below 49% suggesting a significant microdiversity at both bacterial species and strain level undetected previously by 16S rRNA-targeted analyses. Hence, the understanding the functional impact of such microdiversity, naturally occurring in the intestinal microbial communities, requires the development of strain-specific detection assays.

Representational difference analysis (RDA) is a technique to reveal different nucleic acid stretches among two otherwise highly similar genomes or transcriptomes (8). The most prominent applications of RDA were on gene loss in tumor and healthy cells (7) and an analysis of virulence factors by comparing pathogens to harmless bacteria (13). The method has also been reported as quite difficult and tedious and the successful comparison of only two genomes has been considered as a technical achievement. Recently, RDA was significantly simplified and adapted to study the microdiversity of a novel lineage of predominant soil *Bacillus* sp. (3). The potential of the RDA to distinguish between closely related strains of lactobacilli, however, has not been examined.

Lactobacilli are members of the lactic acid bacteria, a broadly defined group characterized by the formation of lactic acid as sole or main end product of carbohydrate metabolism (17). They are encountered in a variety of environmental niches such as plant-derived raw materials and the GI tract of mammals (18). The applications of lactobacilli in the production of foods and feeds that require lactic acid fermentation, notably dairy products (yoghurt and cheese), have also been documented. While the lactic acid bacteria in the industrial setting

have been well studied, the lactobacilli inhabiting the GI tract of animals are much less known (17). A significant microdiversity has been reported for many intestinal lactobacilli, making them difficult to distinguish solely on the basis of the phenotypic characteristics or 16S rRNA gene-targeted phlylogenetic analysis (14, 15).

Here we used *L. sobrius* sp. nov. strain 001^T, a newly isolated abundant member of the porcine GI tract community, for a novel strain detection system development. *L. sobrius* 001^T strain-specific genomic fragments were firstly identified by RDA, and further detected by real-time PCR.

MATERIALS AND METHODS

Bacterial strains and media used in this study. Four *L. sobrius* sp. nov. isolates used in this study were: *L. sobrius* strain 001^T, identified in the faeces of piglets housed on a farm near Wageningen, the Netherlands, and *L. sobrius* strains 003, 004 and 005, isolated from faeces of piglets kept on a farm near Bologna, Italy (Chapter 5). *L. acidophilus* DSMZ20079^T, *L. amylovorus* DSMZ 20531^T, *L. crispatus* DSMZ 20584^T, *L. gallinarum* DSMZ 10532 ^{T} and *L. helveticus* DSMZ 20075 ^{T} were used as reference strains. The strains were propagated at 37°C, anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Difco, Le Point de Claix, France).

DNA isolation. After 24h of growth the cells were harvested at 5,000 x g for 10 min and washed with 0.2 μm pore size filtered phosphate–buffered saline (PBS; per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4; pH 7.2). The isolation of genomic DNA was done using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA). Agarose gel 1.2% (w/v) electrophoresis in the presence of ethidium bromide was used to check visually for DNA quality and yield.

Representative difference analysis (RDA) of *L. sobrius* strain 001^T genome. The RDA protocol was originally described by Felske (3) and applied to our samples with the following modifications.

Restriction digestion of *L. sobrius* strains genomic DNA. *L. sobrius* strain 001^T was used as TESTER, and *L. sobrius* strains 003, 004, and 005 as DRIVERS, respectively. Approximately 1 μg genomic DNA of the *L. sobrius* strains was digested with 10 U *Cfo*I (Promega, Madison, WI, USA) for 1.5 h at 37 °C in the respective buffer provided by the

manufacturer and in the presence of BSA (5ng/µl). The total digestion reaction had a volume of 20 µl.

Adapter ligation for DRIVER. The ligation of DRIVER adapters was carried out in the following reaction: 8 µl of the digested DNA was mixed with 8 µl DRIVER adapter [50 µM; 1:1 mixture of T7-long (THEBEN) with Short (KORINTH)], (Table 1), 1 µl (400 U) of T4 DNA ligase, 2 µl 10x ligase buffer and 1µl (25 µg ml^{-1}) BSA to a final volume of 20µl. The ligation mixture was incubated at 4 °C for 16 hours. Adapter residues were removed from the ligation reaction using DNA clean & concentrator-5 kit (Zimo Research, Orange, CA, USA). The DNA was eluted from the filter with 20µl mQ water.

TABLE 1. DNA oligonucleotides used in this study.

Oligonucleotides	Sequence (5'-3')	Target	Reference
T7-long (THEBEN)	TTTCTAATACGACTCACTATAGGCCGCCAGCG	RDA	(1)
SP6-long (SPARTA)	TTTATTTAGGTGACATAGATTAGGCCGCCA	RDA	(1)
Short (KORINT)	CTGGCGGCCTACCA	RDA	(1)
T7-RDA primers	AATACGACTCACTATAG	RDA	(3)
SP6-RDA primers	ATTTAGGTGACACTATAGA	RDA	(3)
S-G-Lab-0159-a-A-2	CGGTATTAGCACCTGTTTC	16Sr RNA	this study
L-*-OTU171-0077-a-S-2	ACTTCGGTAATGACGTTG	16Sr RNA	this study
OTU171_RDA_F	TTCTGCCTTTTTGGGATCAA	RDA(A)	this study
OTU171_RDA_R	CCTTGTTTATTCAAGTGGGTGA	RDA(A	this study
T7	TAATACGACTCACTATAGG		Promega
Sp6	GATTTAGGTGACACTATAG		Promega

DRIVER amplification. The DRIVER amplification was performed with *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD). The PCR reactions (50 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 300 µM each of dNTP, 0.8 µM of primer T7, 2.5 units of recombinant Taq DNA polymerase, and 5 µl template (ligation reaction). The samples were amplified in a thermocycler T1 Whatman Biometra starting with a predenaturation of 94 °C for 60 s and then using 40 cycles of 94 °C for 15 s, 44 °C for 30 s and 68 °C for 90 s and a final elongation of 4.5 min at 68 °C. This PCR product was purified with the QIAquick PCR purification kit (Westburg, Leusden, The Netherlands), eluted with 50 µl TE buffer and further used as the DRIVER.

TESTER production. For TESTER production, 1 µl DRIVER was digested with 10 U *Cfo*I for 1.5 h at 37 °C in the respective buffer plus BSA (5ng/µl) in 20 µl volume. The digested

DNA was purified using DNA clean & concentrator-5 kit (Zimo Research), and eluted with mQ in a final volume of 20 µl. Ten µl of this was amended with 7.5 µl TESTER adapter Sp6-long (SPARTA) and Short (KORINTH) (1:1 mixture of 50 µM each) and the ligation was achieved as described for the DRIVER above. Further, using a DNA clean & concentrator-5 kit (Zimo Research) the adapter residues were removed. The DNA was eluted from the filter with 50 µl MQ water, yielding the TESTER preparation. Considering the latter steps, using DRIVER and TESTER preparations at same volume in the RDA reaction will result in 100-fold DRIVER excess.

Subtractive hybridization and RDA-PCR. The subtractive hybridization was performed by mixing 5 µl each of DRIVER and TESTER, and 10 µl of 5 x EE buffer [50mM EPPS (SIGMA), 10 mM N-(2-hydroxyethyl) piperazine-N'-(3-propanesulfonic acid)(16), 5 mM EDTA, pH 8.0]. The reaction was covered by a drop of mineral oil and denatured for 5 min at 99 °C. Without cooling down, 5 µl of a 5 M NaCl solution was added and then the sample was hybridized at 67 °C for 3 h.

The PCR reactions contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 300 µM each of dNTP, 2.5 units of recombinant *Taq* DNA polymerase, and were prewarmed to 68 °C in a thermocycler T1 Whatman Biometra. Then 5 µl of the hybridization reaction were directly transferred from the 67 °C heating block into the 40 µl prewarmed PCR mix. The RDA amplification was performed starting with a preincubation of 1 min at 68 °C followed by the addition of 5 µl (1 µM) primer SP6 (during a pause 3 min at 4 °C) and then 45 cycles of 94 °C for 15 s, 44 °C for 30 s and 68 °C for 90 s, finishing with a final incubation for 4.5 min at 68 °C.

Electrophoresis and preparation of bands. The RDA reaction (50 µl) was mixed with 10 µl 6 x loading buffer and loaded on a 2 % agarose gel. Electrophoresis was done in TAE buffer until the loading buffer dye front reached approximately ¾ of the maximal running distance. The gel was amended with 5 µl of 1 % ethidium bromide solution per 100 ml gel for isolating single bands. Gel slices containing the RDA fragments were cut under UV light and forwarded to DNA purification using QIAEXII Gel extraction kit (Westburg) according to the manufacturer's recommendations.

Generation and screening of RDA clone libraries. A reamplification of the purified RDA fragments was done with a *Taq* DNA polymerase kit from Life Technologies using the original SP6 primer (Table 1). The PCR product was purified with the QIAquick PCR purification kit (Westburg) according to the manufacturer's instructions. Purified PCR

product was cloned into *E. coli* JM109 using the pGEM-T vector system (Promega). The colonies of ampicillin-resistant transformants were transferred with a sterile toothpick to 15 µl TE buffer and boiled for 15 min at 95 °C. Immediately, PCR was performed with vector specific primers T7 and SP6 (Table 1) to check the size of the inserts using the cell lysate as a template. Clones representing an RDA insert were grown in Luria Broth liquid medium (5 ml) with ampicillin (100 µg ml^{-1}). Plasmid DNA was isolated using the Wizard Plus purification system (Promega), and used for sequence analysis of the cloned RDA fragments by using a Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, UK) according to the manufacturer's specifications and using either the T7 or SP6 primer labelled with IRD-800. Sequences were automatically analysed on a LI-COR DNA Sequencer 4000L (LiCor, Lincoln, NE) and compared to those sequences available in public databases by using BLAST analysis (12)

Real-time PCR assay for quantification of *L. sobrius* strain 001^T. Strain-specific PCR primers targeting RDA fragments were designed using the primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Subsequently, the ARB software package (9) was employed for the design of a 16S rRNA-targeted species-specific primer set for the detection of *L. sobrius* strain 001^T. A BLAST search was performed to check the specificity of the DNA sequences of the primers. Furthermore, all primer sets were first tested with conventional PCR using the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures (25 µl) contained 1.25U µl of *Taq* polymerase (1.25 U), 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 3.0 mM $MgCl_2$, 200µM of each dNTP, 100 nM of each primer set and 1 µl of *L. sobrius* strain 001^T genomic DNA diluted to approximately 1 ng/µl and UV sterilized water (mili Q). The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and the cycling consisted of pre-denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 20 s, and 68°C for 40 s, and a final extension at 68°C for 7 min. Furthermore, the performance and optimal annealing temperatures of selected PCR primers, targeting RDA fragment A and the 16S rRNA gene of *L. sobrius* strain 001^T, were tested with gradient PCR using genomic DNA of *L. sobrius* stain 001^T and non-target test bacteria. Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, The Netherlands). A reaction mixture (25µl) consisted of 12.5 µl of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set, and 5 µl of the template DNA. The PCR conditions were: an initial DNA denaturation step at 95 °C for 3 min,

followed by 40 cycles of denaturation at 95 °C for 15 s, primer annealing and extension at 53.9–63.9 °C for 45 s. For further real-time PCR quantification analysis, the same program was used with a constant temperature of annealing and extension specific for the primer sets (Table 2). The fluorescent product was detected at the last step of each cycle. Following the amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by heating at 0.5°C/10s increments from 65 to 95°C, with continuous fluorescence collection. For determination of the number of *L. sobrius* strain 001^T cells in pure culture, *L. sobrius* strain 001^T was propagated at 37°, anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Difco, Le Point de Claix, France). The culture (2ml) was harvested after 24h by centrifugation at 5,000 x g for 10 min and washed with 0.2 μm pore size filtered PBS. The bacterial pellet was finally resuspended in 1 ml PBS. Ten μl of each culture was used for total cell counts determination based on 4', 6-diamino-2-phenylindole (DAPI) staining coupled to microscopy analysis as described (5). Isolation of genomic DNA from the remaining 990 μl bacterial culture was done using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA). Finally, *L. sobrius* genomic DNA was diluted to concentrations ranging from 10^7 to 10 cells per real-time PCR reaction and used for generation of real-time PCR standard curves. Furthermore, four porcine ileal lumen samples, obtained from piglets after a large feeding trial (Chapter 8), were spiked with different concentrations of *L. sobrius* strain 001^T (10^7 to 10^2 cells/ml). Isolation of genomic DNA was done using the Fast DNA Spin Kit (Qbiogene).

RESULTS

RDA analysis of *L. sobrius* strain 001^T and other related strains. RDA was firstly adapted to study the microdiversity between highly similar genomes of four *Lactobacillus sobrius* sp. nov. strains. To isolate specific genomic fragments of *L. sobrius* strain 001^T, high-quality genomic DNA was isolated from all four *L. sobrius* isolates (001^T, 003, 004, 005) and digested with *Cfo*I. The restriction enzyme recognizes a 4-bp sequence motif of high GC content (GCGC), which is present at a relatively low frequency in the DNA of the low GC GI tract bacteria. The restriction reaction yielded highest frequency of DNA fragments in the range of 0.7-3 kb (data not shown) that were further used to produce DRIVER and TESTER via different adaptors introducing a primer sequence. The half single strand–half double

strand adaptors set up only the primer sequence but not its complement. Following the TESTER and DRIVERs propagation, the DNA of *L. sobrius* strain 001^T (TESTER) was separately mixed with a 100-fold excess DNA from each of the related strains used as the DRIVERs (003, 004, 005). The resulting substractive hybridization reactions between the TESTER and the DRIVERs aimed to eliminate DNA fragments shared between the strains. The SP6 primer complements required for PCR are only present on TESTER homoduplexes after subtractive hybridization, allowing for the amplification of fragments unique to the TESTER.

Figure 1. RDA analysis of *L. sobrius* strain 001^T and other *L. sobrius* strains. Subtractive hybridization between: Lanes 1 and 2, *L. sobrius* strain 001^T (TESTER) and *L. sobrius* strain 003 (DRIVER); lane 3, *L. sobrius* strain 001^T (TESTER) and *L. sobrius* strain 004 (DRIVER); lane 4, *L. sobrius* strain 001^T (TESTER) and *L. sobrius* strain 005 (DRIVER). RDA fragments identified after sequencing analysis and BLASTx: **A**, hypothetical protein LJ1398 (36% on AA level) and **B**, hypothetical protein LJ1284 (52% on AA level) of *Lactobacillus acidophilus johnsonii* NCC 53 (gi/42519268); **C**, *Escherichia coli* putative cytoplasmic protein (41 % on AA level, gi/32470041); **D**, *Antrodia camphorata* manganese superoxide dismutase (95% on AA level, gi/33186704). M-standard 1kb DNA marker.

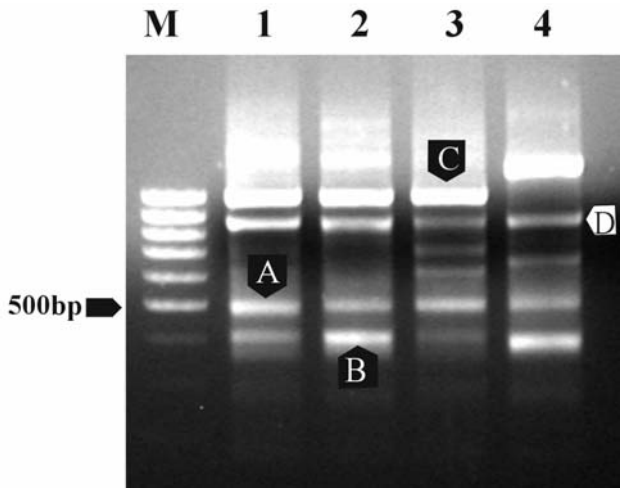

Figure 1 displays the clear RDA fragments obtained after PCR with SP6 primer (0.4-3 kb) while comparing *L. sobrius* strain 001^T with three highly related strains. Identical DRIVERs were found to yield the same RDA patterns (lane 1 and 2), suggesting minor tube-to-tube variation when the current protocol is carefully applied. Subsequently, the PCR fragments were isolated from gel, re-amplified, cloned and four of the fragments were sequenced. After BLASTx and BLASTn search, two of the sequences (Fig. 1; A, B) were found to show

homology with hypothetical protein LJ1398 (36% at AA level), and hypothetical protein LJ1284-encoding genes (52% at AA level) of *Lactobacillus acidophilus johnsonii* NCC 53 (gi/42519268). The other two RDA fragments (Figure 1; C, D) were most closely related to *Escherichia coli* putative cytoplasmic protein (41 % at AA level, gi/32470041), and *Antrodia camphorata* manganese superoxide dismutase-encoding genes (95% at AA level, gi/33186704), respectively.

TABLE 2. *L. sobrius* sp. nov. strains and phylogenetically closely related *Lactobacillus* species used in the validation of the primers targeting RDA fragment A (OTU171_RDA_F and OTU171_RDA_R) and 16S rRNA gene (L-*-OTU171-0077-a-S-2 and [a]S-G-Lab-0159-a-A-2) of *L. sobriu*s strain 001^T using real-time PCR; +, positive PCR result; -, no PCR signal. Numbers in brackets indicate optimal annealing temperatures.

Strain	RDA fragment A (60.3°C)	16S rRNA gene (62.5 °C)
1. *L. sobrius*_001^T	+	+
2. *L. sobrius*_003	-	+
3. *L. sobriu*_004	-	+
4. *L. sobrius*_005	-	+
5. *L. amylovorus* DSMZ 20531^T	-	+
6. *L. crispatus* DSMZ 20584^T	-	-
7. *L. gallinarum* DSMZ 10532^T	-	-
8. *L. acidophilus* DSMZ20079^T	-	-
9. *L. helveticus* DSMZ 20075^T	-	-

Development and validation of strain- and species-specific *L. sobrius* strain 001^T primers and their application for real-time PCR analysis. PCR primers were developed for each RDA fragment and tested on *L. sobrius* strain 001^T genomic DNA. Clear amplicons, without cross-reaction to other genomic fragments of *L. sobrius* strain 001^T were only found for the primer set targeting RDA fragment A (data not shown). Therefore, the latter primer set was further used for the strain-specific *L. sobrius* 001^T PCR amplification. Moreover, species-specific primers targeting the 16S rRNA gene of *L. sobrius* sp. nov. were also developed. The real-time PCR conditions for the two primer sets were first optimized, using amplification efficiency, linearity and specificity as criteria. The efficiencies of amplification, calculated from the formula Eff(n)= [$10^{(-1/slope)}$ -1], were between 95 and 99 % for the 16S rRNA gene targeting primers, and between 96% and 103% for the primers amplifying the RDA fragment. The linear range of amplification for both primer sets comprised DNA

dilutions equivalent to 10^7 to 10 cells per PCR (Fig. 2). Using temperature gradient PCR, the specificity of the primer sets was optimized against phylogenetically closely related *Lactobacillus* species, and, in the case of RDA fragment A targeting primers, also against other *L. sobrius* strains (Table 2).

Melting-curve analysis was used to detect the formation of the correct amplification product. For the *L. sobrius* 16S rRNA targeting primer set, primer dimers with lower Tm values were observed when small amounts of template DNA (up to 10 cells) were used in PCRs. Furthermore, there were not significant differences between the real-time PCR results obtained by quantifying *L. sobrius* strain 001^T in pure culture (data not shown), or when ileal samples were spiked with various amounts (10^7–10^2 cells) of *L. sobrius* strain 001^T (Table 3).

Figure 2. Real-time PCR quantification of dilution series (10^7 to 10 cells/per PCR) of *L. sobrius* strain 001^T genomic DNA using the strain-specific primer set.

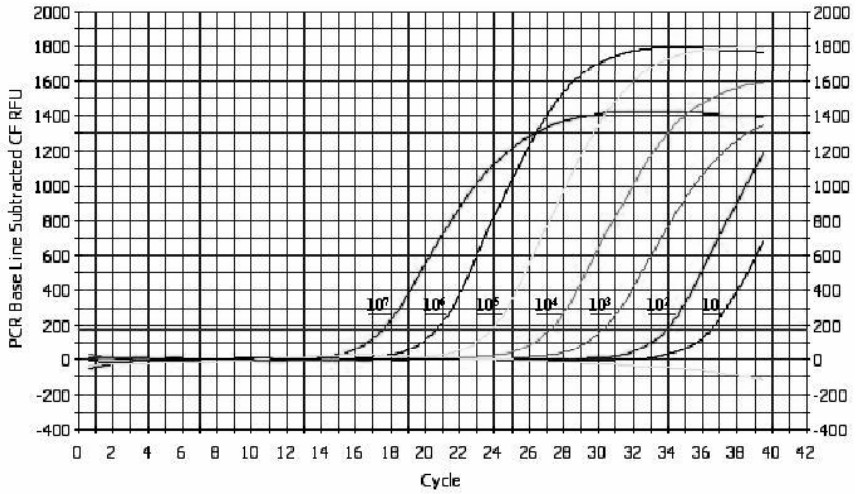

TABLE 3. Strain-specific real-time PCR detection in four porcine ileal samples spiked with various amounts of *L. sobrius* strain 001^T. Counts are expressed as mean of four ileal samples (cells. g^{-1}).

L. sobrius strain 001^T spiked in concentrations	1×10^3	1×10^5	1×10^7
Real-time PCR detection	1.1×10^3	1.2×10^5	1.1×10^7

DISCUSSION

Previous studies have shown that *L. sobrius* strain 001^T is a novel and abundant member of the porcine microbiota (5). Although closely related to *Lactobacillus amylovorus*, it has distinct phenotypic and molecular taxonomic characteristics (Chapter 5). In the present study, the specific quantification of *L. sobrius* strain 001^T was achieved by a combination of RDA and real-time PCR. The improved RDA method applied here, has previously been used to unveil variable genes among *Bacillus* isolates of a novel and abundant lineage in soil (3). Although it has also been suggested that the comprehensive collections of such variable genes might be applied to microarrays, the quantification of the microdiversity among closely related isolates by RDA has not been reported. Here, the RDA identified several strain-specific genomic fragments of *L. sobrius* strain 001^T. Selected RDA fragments were further sequenced. As expected, two sequences were found to show low homology with two hypothetical proteins of *L. acidophilus johnsonii* (Fig, 1; RDA fragments A and B). However, the other two sequences were not found in other lactobacilli, but were most closely related to *E. coli* putative cytoplasmic protein (41 % at AA level), and *A. camphorata* manganese superoxide dismutase-encoding genes (95% at AA level) (Fig. 1; C, D). Interestingly, we obtained clear amplicons only for the PCR primer set targeting RDA fragment A (Fig. 1), suggesting the limited applicability of the other RDA fragments for further PCR detection. Moreover, the specificity was confirmed by real-time PCR using genomic DNA of *L. sobrius* strain 001^T and other non-target *L. sobrius* strains and phylogenetically closely related *Lactobacillus* spp. In addition to the strain-specific primers, a *L. sobrius* species-specific 16S rRNA-targeted primer set was also designed and validated (Table 2). The species-specific primers showed a cross-reaction only with *L. amylovorus* (DSMZ 20531^T) genomic DNA, a fact explained by the identical primer binding sites. In addition, the further design of differential *L. sobrius* sp. nov. and *L. amylovorus* 16S rRNA-targeted primer pairs for real-time PCR was obstacled by the overall gene similarity above 97%. This particular problem of species detection was overcome when RDA fragment A was amplified by real-time PCR (Table 2). Although previous reports have successfully quantified the bifidobacterial species diversity in the human intestinal tract by 16S rRNA-targeted real-time PCR (10), this approach may be of a limited value when applied to some *Lactobacillius* spp. Furthermore, identical 16S rRNA gene sequences of planktonic bacteria have been shown that represent distinct eco- and genotypes (4). Thus, the results of our study demonstrate the potential of RDA combined with real-time PCR for selective detection,

enumeration and identification of closely related isolates colonizing different ecological niches with respect to their species delineation and strain differentiation.

Thus far, many phenotypic and molecular microbial ecological techniques for strain identification and quantification have been described. Although some classical strain identification methods have successfully been applied for isolating and enumerating specific strains from a mixed environment, there is a general awareness for the pitfalls of phenotypic characterization (2, 11). The observation of a similar phenotype does not always equate a similar, or closely-related, genotype. There has therefore been a shift towards cultivation-independent molecular approaches in order to provide more robust classification and differentiation. While these techniques might be biased as well (11), the RDA-real-time PCR method reported here can be combined in polyphasic strategies with additional classical strain identification methods, such as differential plating methodologies that have successfully been applied for isolating and enumerating specific strains (2). Together with other molecular approaches, including various genetic fingerprinting techniques, genetic marking, monoclonal antibodies assay or antibiotic resistance markers, the RDA may also be employed for species identification or subspecies discrimination. Alternatively, the method might be used in future as a cultivation-independent method aiming to quantify the microdiversity *in vivo*. Specifically, we anticipate that the RDA-real-time PCR approach would provide a reliable strain identification and further insight in the occurrence or/and activity of functional bacterial genes in the GI tract.

ACKNOWLEDGEMENTS

This work has been carried out with financial support of the European Community specific RTD programme "Quality of Life and Management of Living Resources" research project: HEALTHYPIGUT (QLK5-LT2000-00522). Dr. Andreas D. Felske is acknowledged for providing the improved RDA protocol.

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CHAPTER 8

Protective Effects of *Lactobacillus sobrius* sp. nov. in Piglets Challenged with Enterotoxigenic *Escherichia coli* K88

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ABSTRACT

Enterotoxigenic *Escherichia coli* K88 (ETEC) is one of the most common etiologic agents of swine post-weaning morbidity and mortality worldwide. The aim of the present study was to determine whether ETEC infection can be prevented by dietary supplementation with *Lactobacillus sobrius* sp. nov. strain 001^T, a newly cultured abundant member of the resident porcine gut microbiota. Therefore, piglets (n=48) were subjected to one of two dietary treatments, a control diet and a diet containing *L. sobrius* strain 001^T, and experimentally infected with ETEC. As revealed by a strain-specific real-time PCR assay, the presence of *L. sobrius* was accompanied by a significant reduction of ETEC prevalence in the porcine ileum at the end of the experiment. To determine to what extent the piglet immune system was stimulated by the administered *L. sobrius* strain 001^T, the concentration of total secretory IgA (sIgA) was determined in saliva, blood serum and intestinal jejunal secrete. At the end of the experiment, total sIgA contents in jejunum secrete and blood serum were not significantly affected by the *L. sobrius* supplementation. In contrast, total amount of sIgA in saliva was comparable to the level before the ETEC challenge in the *L. sobrius* fed group, while the sIgA amount decreased significantly in the control piglets ($P<0.05$). Moreover, an improved daily weight gain (+ 74%, $P<0.05$) was also observed in the piglets that received *L. sobrius* strain 001^T. Together, the data indicate that *L. sobrius* strain 001^T exerts a protective effect against the ETEC infection while improving the weight gain and immunity of weaning piglets.

submitted for publication

INTRODUCTION

Monogastric animals including pigs coexist with a diverse and dense commensal microbiota in their gastrointestinal (GI) tract that salvage energy from otherwise indigestible carbohydrates and protect the host from pathogens by forming a front line of mucosal defense. In contrast to the adult swine, the neonatal and weaning piglet is highly susceptible to enteric diseases (21). In the immediate post-weaning period, the balance between the development of so called healthy commensal microbiota or the establishment of a bacterial intestinal disease can be easily tipped toward disease expression (21). Bacteria that were associated with diarrhoeal disease after weaning include enterotoxigenic *Escherichia coli* K88 (ETEC) and other *E. coli* strains (post-weaning colibacillosis), and *Salmonella* spp. (1, 2, 21, 39). The attachment of ETEC to the enterocytes lining the small intestinal villi is mediated by adhesins known as K88 (or F4) (12). The degree of microbial adhesion is variable due to individual differences in the presence of intestinal receptors for the fimbriae of ETEC (52). After attachment to and colonization of the small intestinal enterocytes, ETEC provokes hypersecretory diarrhoea through the release of specific enterotoxins (21). Thus far, no effective vaccines are available to control post-weaning colibacillosis, and many pathogenic *E. coli* strains show resistance to multiple antibiotics (2). Although the pathogenic prevalence has been identified as primary infectious cause in post-weaning colibacillosis, there is abundant evidence to suggest that other factors are necessary for the manifestation of the disease (21, 30). The changes in the composition and activity of the small intestinal microbial community after weaning may be among the factors that predispose the animals to pathogenic infections. A significant decrease in the number of lactobacilli and an increase of coliforms has consistently been observed in the GI tract of piglets during the first weeks after weaning (13, 21, 24).

Addition of probiotics to the diet, defined as live microbial feed supplements (16), is an approach, alternative to antibiotics, for the prevention and treatment of some infectious intestinal diseases in humans and animals. Such dietary intervention is partly based on the concept that specific strains selected from the healthy gut microbiota may have powerful anti-pathogenic and anti-inflammatory properties, and therefore may provide resistance to intestinal diseases (23). Furthermore, commensal microbiota contributes to intestinal protection against pathogens by competition for nutrients and pathogen binding sites, and/or regulation of immune response. The host resistance to infection might also be improved by

the activities of the commensal bacterial such as the production of antimicrobial substances or the generation of restrictive physiological conditions, including acidification, caused by lactic acid and other fermentations (49).

As early as 1960 claims of improved weight gain and decreased incidence of infections are amongst several that have been made in association with probiotic supplementation for farmhouse animals (37). Solid scientific evidence supporting these and similar claims, however, is limited (15, 44, 48). At present, the United States Food and Drug Administration has approved only two probiotics formulations for use with poultry that were documented to decrease the needs of antibiotics use and pathogen levels (3, 48). With the forthcoming ban of antibiotics in the EU, several microbial cultures have been authorized as feed additives for pigs (44). These include several lactobacilli, notably due to their natural presence in the pig intestine, their positive effect on gut function and health, and their antagonistic activities toward other bacteria (48, 53). A critical phase in the development of farm animals is the transition to solid feed, also known as weaning, when a rapid proliferation of pathogens may occur. Hence, exogenous supplementation of lactobacilli has been reported among the possible alternatives to antibiotics for the reduction of post-weaning diarrhoea in piglets (54). The application of culture-independent approaches, based mainly on 16S ribosomal RNA (rRNA) gene analysis, has highlighted the specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to the weaning process and the addition of dietary prebiotics to early weaning piglets (25). Recently, several strains belonging to this phylotype have been isolated from the porcine intestine, and their detailed genotypic and phenotypic characterization has revealed that they belong to a new *Lactobacillus* species, for which the name *Lactobacillus sobrius* sp. nov. was proposed (Chapter 5). Given the fact that this particular *L. sobrius* is a member of the resident porcine gut microbiota before weaning and exerts anti-enteropathogenic ETEC activities *in vitro* (42), we hypothesized that its dietary supplementation would ensure animal growth and improve host immunity at weaning.

The present work describes the effects of a *L. sobrius* strain 001^T on pathogenic prevalence, growth performance and immunity of piglets orally challenged with ETEC. The results indicate that administration of the *L. sobrius* strain 001^T significantly reduced the prevalence of ETEC in the intestine and improved the growth and immunity of weaning piglets.

MATERIALS AND METHODS

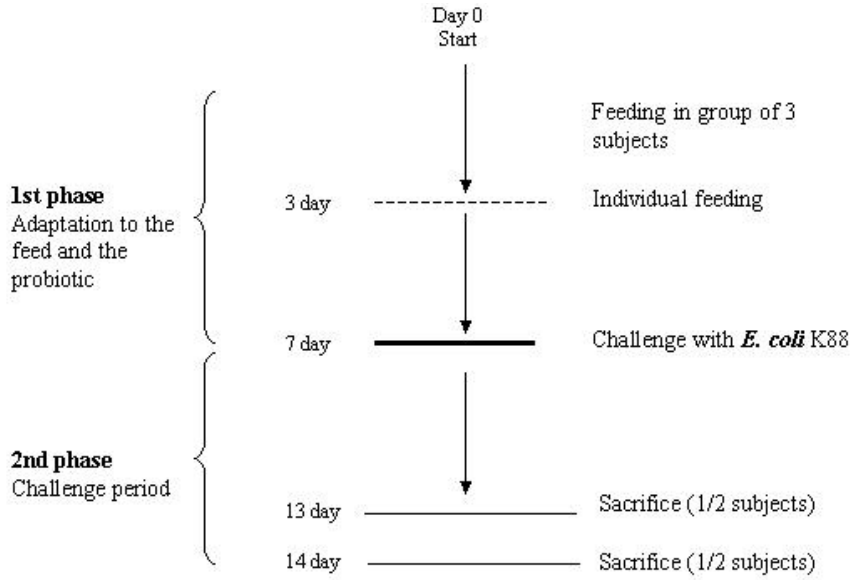
Experiment design. The animal experiment consisted of 3 replications. Each replication was done using 16 weaned piglets (landrace x large white) (21 days of age) assigned to one of two dietary treatments, a basic diet (control diet) and a basic diet containing *L. sobrius* strain 001^T (LAB diet). The piglets were balanced for litter and weight, and sex was registered. Piglets were fed a standard diet, containing 4% dried sugar beet pulp (Table 1).

Table 1. Composition of the basic diet.

Ingredients	g/kg
Corn extruded	299.2
Barley extruded	71.0
Rice flakes	133.0
Dried beet pulp	40.0
Soybean meal 44%CP	50.0
Soybean debitterized	77.0
Spray dried milk whey	135.0
Spray dried skimmed milk	135.0
Lard	48.0
HCl-Lysine	0.5
DL-Methionine	0.3
Monocalcium phosphate	10.0
Flavour	1.0

The LAB diet contained in addition to the basal diet 1 ml/day of a skimmed milk solution supplemented with 10^{10} CFU of *L. sobrius* strain 001^T, while 1 ml milk only was added to the control diet. The experimental period was divided in two phases (Fig. 1): an adaptation phase, when subjects were adjusted to the experimental diets (day 0 till day 7), and a challenge period (day 7 till day 14). The skimmed milk with and without *L. sobrius* strain 001^T was supplied orally by a sterile syringe on day 0 and day 1, and then till the end of the experiment in the trough, carefully mixed with the feed every morning. Pigs were penned on a mesh floor, in groups of four on day 0 and day 1, and then individually. On day 7, the piglets were challenged with 1.5 ml of a suspension containing 10^{10} CFU/ml enterotoxigenic *E. coli* K88 (ETEC).

Figure 1. Scheme of the trial.



Diarrhoea score and ETEC quantification. Diarrhoea scores were recorded by visual appraisal of each subject using a 5-point scoring system (1 to 5), the excrements being hard (score 1) and watery faecal excrements (score 5). Two and three days after the challenge, individual faecal samples were collected, and the quantification of ETEC and of total *E. coli* excretion, was done as reported previously (7). Four random samples per group per replication (in total 12 samples per diet) were used for further isolation of *L. sobrius* strains and their taxonomic characterization as described (Chapter 5).

Total secretory IgA(sIgA), and anti-*L. sobrius* strain 001^T and ETEC specific sIgA titres. At the day of challenge, saliva and blood samples were collected from all subjects. Saliva samples were collected again one day before the sacrifice, while blood samples were collected immediately before sacrifice. All sIgA determinations were done by ELISA. For total sIgA detection, 96-well microtiter plates were coated with Goat anti-pig sIgA, affinity purified (BETHYL Laboratories, Montgomery, U.S.A.) and diluted in carbonate-bicarbonate buffer, 50 mM, pH 9.4. Subsequently, phosphate–buffered saline (PBS) supplemented with 0.2% (v/v) Tween 20 was added to the wells to block the remaining binding sites. Pig Immunoglobulin Reference Serum (BETHYL, Laboratories, Montgomery, TX) was used as specific antibody for standard curve, Goat anti-Pig sIgA-HRP conjugate (BETHYL Laboratories, Montgomery, U.S.A) as secondary antibody and ABTS (ROCHE Diagnostics)

as enzyme substrate. Absorption was read at 405 nm by a microplate reader (Sunrise Microplate Reader, TECAN ITALIA). The concentration values was expressed as µg/ml. *L. sobrius* strain 001^T and ETEC-specific sIgA titres were determined according to (22) and (52), respectively.

Collection of samples at sacrifice. On days 13 or 14, all the piglets, equally distributed by treatment and selected on a random basis within each treatment, were anaesthetized with sodium thiopenthal (10 mg/kg body weight). Euthanasia was performed by intracardiac injection of Tanax® (0.5 ml/kg body weight; Intervet Italia, Peschiera Borromeo, Italy).

The small intestine was sampled at ¼ and ¾ of its total length for morphometric analysis of villi and crypts, and measured as reported (8). For the determination of sIgA, a 50 cm segment obtained from the final jejunum was immediately processed as described (11). Samples of gut content were also collected from stomach, duodenum, terminal jejunum, cecum and colon for pH measure. Lumen samples collected from the terminal ileum were divided into aliquots that were used for genomic DNA extraction by using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, U.S.A.) and fixed for fluorescent *in situ* hybridization (FISH), resprectively. A DNA oligonucleotide probe L-*-OTU171-0088-a-A-18 (Table 2) targeting the 16S rRNA of *L. sobrius* sp. nov. was used for FISH analysis of ileal lumen samples as reported (25).

Statistical analysis. Data were analyzed by analysis of variance using the GLM procedure (SAS version 8.1, SAS Institute, Cary, NC) with a 3-factor design, including diet, batch, sensitivity of intestinal villous to ETEC adhesion, and 1^{st} level interactions.

***Lactobacillus*-specific PCR amplification.** The PCR approach was applied as previously described (25) and the primers used in this study are listed in Table 2. PCR was performed using the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, MD). PCR mixtures (50 µl) contained 1.25U µl of *Taq* polymerase (1.25 U), 20 mM Tris–HCl (pH 8.5), 50 mM KCl, 3.0 mM $MgCl_2$, 200µM of each dNTP, 100 nM of the primers S-D-Bact-0011-a-A-17 and S-G-Lab-0677-a-A-17 and 1 µl of DNA diluted to approximately 1 ng/µl and UV sterilized water (mili Q). The samples were amplified in a thermocycler T1 Whatman Biometra (Göttingen, Germany) and the cycling consisted of pre-denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 66°C for 20 sec, and 68°C for 40 sec, and a final extension at 68°C for 7 min. PCR products were then used as templates in nested PCR reactions, using: S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-GC and following the cycling programme of 94°C for 5 min, and 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 68°C for 40 sec, and 68°C

for 7 min final extension. Aliquots of 5 µl were analyzed by electrophoresis on 1.2% agarose gel (w/v) containing ethidium bromide to check for product size and yield.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis. The amplicons obtained from the lumen-extracted DNA were separated by DGGE (Dcode TM system; Bio-Rad Laboratories, Hercules, U.S.A.) using a previously described protocol (25). In brief, electrophoresis was performed in an 8% polyacrylamide gel of 37.5:1 acrylamide–bisacrylamide (dimensions 200×200×1 mm) with a gradient of 30-60% for the separation of PCR products obtained with primers S-G-Lab-0159-a-S-20 and S-*-Univ-0515-a-A-GC. After a short run at 200 V for 5 min, the gels were electrophoresed for 16 h at 85 V in 0.5×TAE buffer at a constant temperature of 60°C and subsequently stained with $AgNO_3$.

TABLE 2. List of DNA oligonucleotides used in this study.

Oligonucleotides	Sequence (5'-3')	Target	Reference
D-Bact-0011-a-A-17	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	16S rRNA	(27)
S-G-Lab-0159-a-S-2	GGAAACAG(A/G)TGCTAATACCG	16S rRNA	(19)
S-*-Univ-0515-a-A-24-GC	CGCCGGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGGATCGTATTA CCGCGGCTGCTGGCA	16S rRNA	(19)
S-G-Lab-0677-a-A-17	CACCGCTACACATGGAG	16SrRNA	(19)
R 1401	CGGTGTGTACAAGACCC	16S rRNA	(36)
F 968	AACGCGAAGAACCTTAC	16S rRNA	(36)
K88AD_F	GGCACTAAAGTTGGTTCA	ETEC	(1)
K88AD_R	CACCCTTGAGTTCAGAATT	ETEC	(1)
S-G-Lab-0159-a-A-2	CGGTATTAGCACCTGTTTCC	16S rRNA	Chapter 7
L-*-OTU171-0077-a-S-2	ACTTCGGTAATGACGTTG	16S rRNA	Chapter 7
OTU171_RDA_F	TTCTGCCTTTTTGGGATCAA	RDA (A)	Chapter 7
OTU171_RDA_R	CCTTGTTTATTCAAGTGGGTGA	RDA (A)	Chapter 7

Reference strains used for real-time PCR analysis. *L. sobrius* strain 001^T was propagated at 37°C, anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Difco, Le Point de Claix, France), while ETEC was grown in Luria-Bertani broth containing 1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0. Both cultures (2ml) were harvested after 24h by centrifugation at 5,000 x g for 10 min and washed with 0.2 µm pore size filtered PBS (per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4; pH 7.2). The bacterial pellet was finally resuspended in 1 ml PBS. Ten µl of each culture was used for total cell counts determination based on 4', 6-diamino-2-phenylindole (DAPI) staining coupled to microscopy analysis as

described (25). Isolation of genomic DNA from the remaining 990 µl bacterial culture was done using the Fast DNA Spin Kit (Qbiogene). Finally, *L. sobrius* and ETEC genomic DNA were diluted to concentrations ranging from 10^8 to 10 cells per real-time PCR reaction and used for generation of real-time PCR standard curves.

Real-time PCR assay for quantification of *L. sobrius* strain 001^T and ETEC. Strain- and species-specific PCR primers targeting *L. sobrius* strain 001^T were used as described previously (Chapter 7) (Table 2). For ETEC quantification, primers K88AD_F and K88AD_R were employed (Table 2). In addition, 16S rRNA gene-targeted primers, 968F and L1401 (Table 2) were applied for total bacterial quantification. Real-time PCR was performed on an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, The Netherlands). A reaction mixture (25µl) consisted of 12.5 µl of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer set, and 5 µl of the template DNA. The PCR conditions for the specific *L. sobrius* strain 001^T quantification were: an initial DNA denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, primer annealing and extension at 60.3°C for 45 sec. The same conditions were used for the species-specific quantification of *L. sobrius* except that the primer annealing and extension was 62.5°C. For total bacterial quantification, conditions were 94 ^{o}C for 5 min, and 35 cycles of 94 ^{o}C for 30sec, 56 ^{o}C for 20 sec, 68 ^{o}C for 40 sec (37). For the determination of the number of *L. sobrius* strain 001^T and ETEC present in each sample, fluorescent signals detected from two serial dilutions were compared to a standard curve generated with the respective bacterium in the same experiment. Serially diluted genomic DNA of *L. sobrius* strain 001^T was used as real-time PCR control for total bacteria quantification.

RESULTS

Outline of the experiment. A feeding trial was performed in triplicate in which a total of 48 piglets (21 days of age) were fed 14 days one of two daily diets: a basic diet (control diet), and the same basic diet containing 10^{10} CFU of *L. sobrius* strain 001^T (LAB diet). The experiment consisted of two phases: an adaptation period of a week (day 0 till day 7), and a challenge period of a week during which the piglets were experimentally infected with ETEC (day 7 till day 14) (Fig. 1). During the entire trial the body weight of the piglets, as well as

their health status, and microbial and immune parameters were periodically determined (Table 3).

Animal observations. Piglets fed the LAB diet were found to grow significantly faster (+ 74%, P<0.05), while they did not show different feed intake, than the piglets fed on the control diet (Table 3). An increased number of days with diarrhea score > 2 was observed in the piglets fed the LAB diet (+1.9 days, P<0.05), while the average diarrhea score was not significantly changed. Faecal excretion of total *E. coli* and ETEC was not affected on day 2 of the challenge period. On day 3 post- infection a higher faecal shedding of ETEC, however not statistically significant, was observed in the piglets fed the LAB diet (Table 3).

TABLE 3. Effect of dietary supplementation with *L. sobrius* strain 001^T on growth, feed intake, diarrhoea score (1 = faeces very consistent; 5 = watery diarrhoea), total *E. coli* and ETEC shedding of challenged pigs (least squares means±SEM).

		Control	LAB	SEM
Daily live weight gain (g)		101.3	176.2	21.8*
Daily feed intake (g)		241.7	257.1	11.9
Average diarrhoea score		2.46	2.84	0.18
Days with diarrhoea score > 2		2.83	4.74	0.61*
Faecal shedding:				
Total *E.coli*				
- 2nd day	Log10 (CFU g^{-1})	7.08	7.46	0.31
- 3rd day	Log10 (CFU g^{-1})	7.87	8.05	0.29
ETEC (faeces)				
- 2nd day	Log10 (CFU g^{-1})	4.56	4.68	0.47
- 3rd day	Log10 (CFU g^{-1})	4.97	6.17	0.55

The pH in stomach, duodenum, jejunum, cecum and colon was not significantly different between the two dietary groups (Table 4). The morphology of small intestine, as measured by villous height and crypt depth, was also not modified by the diet (data not shown).

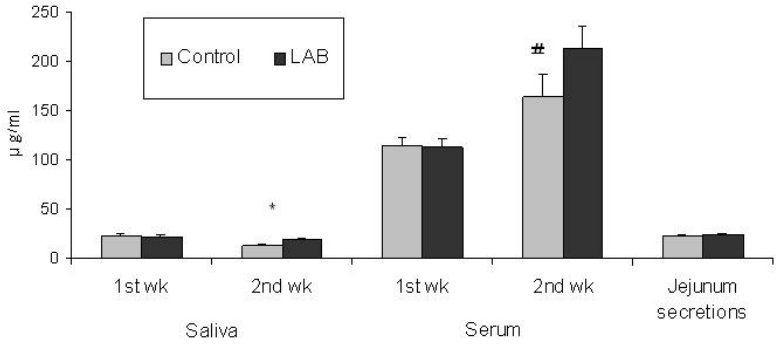
At the time of challenge (day 7), total contents of sIgA in saliva and blood serum in the piglets was not affected by the dietary addition of *L. sobrius* strain 001^T (Fig. 2). One week later, total content of sIgA in saliva was maintained in the piglets fed the LAB diet, while values decreased significantly in the control diet (p<0.05). For blood serum, total sIgA

increased in piglets, but the *L. sobrius* strain 001^T supplemented group tended to have more sIgA, compared to the control (p=0.10). Total sIgA contents in jejunum secrete was not affected.

TABLE 4. Effect of dietary supplementation with *L. sobrius*_001^T strain on pH of different gastrointestinal tract segments of ETEC challenged pigs (least squares means±SEM)

	Control	LAB	SEM
Stomach	3.27	3.64	0.190
Duodenum	5.70	5.41	0.205
Jejunum	6.91	7.10	0.151
Cecum	5.88	5.74	0.088
Colon	6.15	6.11	0.065

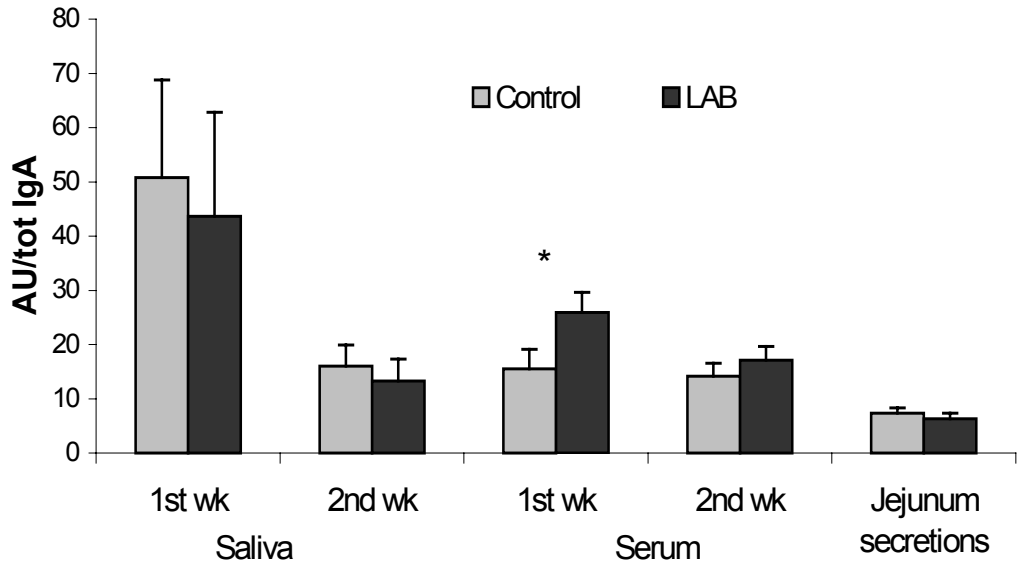
Figure 2. Effect of dietary supplementation with *L. sobrius* strain 001^T on total IgA in saliva, blood serum and jejunum secretions of ETEC challenged pigs (least squares means ±SEM).
Effect of diet: p=0.10.
*Effect of diet: p<0.05.



The effect of dietary supplementation with *L. sobrius* strain 001^T on sIgA against this strain in saliva, blood serum and jejunum secretions of ETEC challenged pigs is presented in Fig. 3. sIgA antibodies against *L. sobrius* strain 001^T were detected in nearly all pigs irrespective of the diet. Its concentration (expressed on total sIgA) was significantly higher in piglets fed the LAB diet only in the case of blood serum collected after 1 week of supplementation

($p<0.05$). In general, without considering the diet, sIgA against *L. sobrius* strain 001^T was still present after 2 weeks in intestinal secretion and a small reduction in blood was seen. However, a very strong reduction was observed for *L. sobrius* strain 001^T recognizing sIgA in saliva, changing from first week to second week sample. ETEC-specific sIgA's did not statistically vary between the LAB and control groups (data not shown).

Figure 3. Effect of dietary supplementation with *L. sobrius* strain 001^T on sIgA against *L. sobrius* strain 001^T in saliva, blood serum and jejunum secretions of *E. coli* challenged pigs (least squares means ±SEM).
*Effect of diet: $p<0.05$



Quantitative PCR detection of *L. sobrius* species in culture medium and in ileal lumen samples. The number of *L. sobrius* strain 001^T as added to the LAB diet was quantified by real-time PCR using species-specific primers. There were no significant differences between the results obtained by the real-time PCR assay, direct FISH counting of the DAPI stained cells or viable counts when samples were spiked with various amounts (10^9–10^5 cells) of *L. sobrius* strain 001^T (data not shown). Moreover, the real-time PCR efficiency was not affected when comparing DNA extracted from MRS grown cultures or ileal samples. Furthermore, real-time PCR analyses were performed to quantify *L. sobrius* species in ilea

samples of four piglets received LAB diet, and the results were compared with those obtained by FISH. For three samples both methods, quantitative PCR and FISH, detected the targeted species at the same levels (approximately 10^8/g). In the fourth sample, however, *L. sobrius* was assessed by quantitative PCR, but not FISH, due to the detection limit of the FISH technique (Table 5).

TABLE 5. Comparison of quantitative real-time PCR, and FISH for detection and quantification of *L. sobrius* species in four porcine ilea samples (A, B, C, D). Counts are expressed as mean cells. g^{-1}.

Sample	Real-time PCR	FISH
A	2.14×10^8	2.05×10^8
B	1.69×10^8	1.63×10^8
C	1.53×10^8	1.55×10^8
D	1.09×10^5	-

-, not detected, lower than 10^5 cells.g^{-1}.

Levels of *L. sobrius* strain 001^T and ETEC in ilea lumen samples of piglets examined by real-time PCR. To evaluate whether the ETEC number in ileal lumen was affected in a quantitative manner by the addition of the *L. sobrius* strain 001^T, both species- (*L. sobrius*) and strain-specific (*L. sobrius* strain 001^T or ETEC) real-time PCR assays were performed. The counts were determined in the ileal samples and compared between the piglets fed the LAB and control diet (Table 6). The administered *L. sobrius* strain 001^T was detected (>10^3 cells/g) in 12 out of 16 piglets fed the LAB diet with a population size of $0.66\pm1\times10^8$ cells/g ileal lumen (mean count ± SD). Within the LAB group, *L. sobrius* species was identified in significant numbers (>10^3 cells/g) in 13 out of 16 samples, and the population of *L. sobrius* per gram of ileal lumen was $1.6\pm0.9\times10^8$ cells. The species was detected at significantly lower level in the control group ($2.3\pm3\times10^4$; $p<0.05$). ETEC was detected in the ilea samples from 12 out of 16 piglets fed the control diet (>10^3 cells/g) and was present in a relatively larger population ($1\pm1\times10^6$ cells/g). In contrast, the population of this pathogen was significantly lower in the LAB group ($4.2\pm0.7\times10^4$; $p<0.05$). Interestingly, ETEC was not detected in ileal samples of four piglets of LAB group harboring large populations (>10^8 cells/g) of *L. sobrius* strain 001^T. The total number of bacteria was not significantly different between the two dietary groups (Table 6).

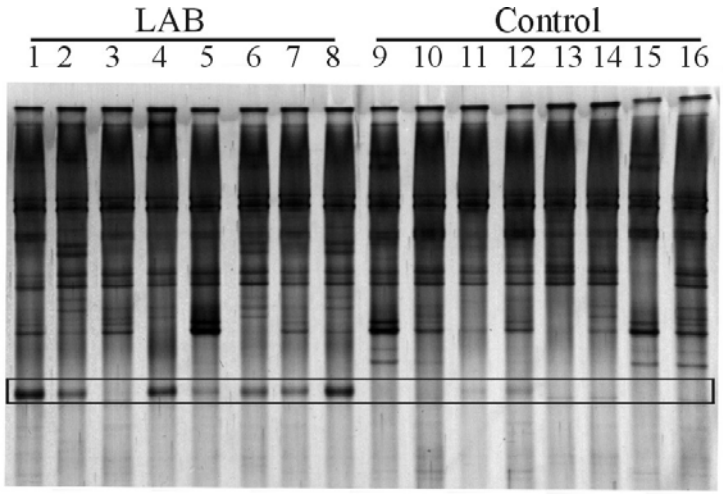
TABLE 6. Numbers of *L. sobrius* strain 001^T, *L. sobrius* species, ETEC, and total bacteria (BAC) in ileal samples, as determined by real-time PCR. Counts are expressed as mean ± SD cells g^{-1}.

	Control diet	LAB diet
BAC	$9.3\pm1.1\times10^9$	$5.4\pm0.9\times10^9$
L. sobrius species	$2.3\pm3\times10^4$	$1.6\pm0.9\times10^8$*
L. sobrius strain 001^T	$<10^3$	$0.66\pm1\times10^8$
ETEC	$1\pm1\times10^6$	$4.2\pm 0.7\times10^4$*

*, significant differences from the respective values compared at $p<0.05$.

DGGE analysis of the *Lactobacillus* community in the ileum of weaning piglets. Complimentary to real-time PCR detection, *Lactobacillus*-specific 16S rRNA gene-targeted amplification, in combination with DGGE fingerprinting analysis, was performed for piglets' ileal lumen samples. The analysis of one representative out of three experiments is shown (Fig. 4).

Figure 4. A representative DGGE analysis of the *Lactobacillus* communities in the samples from the third animal experiment replication. The specific position of the *L. sobrius* strain 001^T DGGE pattern is boxed; LAB, 1-8 samples of piglets fed the LAB diet; Control, 9-16 samples from the piglets fed the control diet.



However, a marked difference in the presence of a specific DGGE band outlining the administered *L. sobrius* strain 001^T was found in a majority (7 out of 8) of the ileal samples from piglets fed the LAB diet (Fig. 4). The amplicon was observed with lower frequency and intensity in the ileal samples from piglets fed the control diet. These data were consistent with the real-time PCR results, and indicated higher emergence of *L. sobrius* in LAB compared to the control group after the experimental ETEC challenge.

DISCUSSION

A placebo-controlled trial was carried out with piglets that received the dietary addition of a live culture of *L. sobrius* sp. nov. strain 001^T, and further challenged with ETEC. A significant reduction in ileal ETEC prevalence, an increase in animal body weight gain, and a modulation of sIgA development were evidenced in the piglets fed LAB compared to control diet. The data reported here suggest that *L. sobrius* strain 001^T has direct or indirect anti-ETEC activities and exerts positive effects on animal body growth and immunity at the time of weaning.

The real-time PCR assay reported in our study was proved as a valuable tool for the determination of population size and occurrence frequencies of *L. sobrius* strain 001^T, *L. sobrius* species and ETEC (Table 6). Previously, real-time PCR assays have been described for the quantification of total bacteria (35, 47) and archaea (47) present in various environments, as well as for the sensitive and accurate detection and quantification of pathogenic bacteria (50). Recently, bacterial groups typically present in human faeces, as well as the distribution of bifidobacterial species in the human intestinal tract have been investigated with real-time PCR (31, 32). Here the quantitative assessment of the porcine microbiota using real-time PCR technology unveiled that the persistence of *L. sobrius* strain 001^T in the porcine ilea microbiota correlated with a significant reduction in ETEC prevalence. Such a combination of strain- and species- specific real-time PCR quantification might be applied in further studies aiming to determine the fate of single strains in complex microbial communities, and the relation of such microdiversity to microbiota functionality.

The selection of *L. sobrius* strain 001^T used in our experiment was based on earlier studies on the GI tract microbial ecology of weaning piglets demonstrating a significant decrease in the number of previously predominant lactobacilli and an increase of coliforms in the GI-tract after the first week of weaning (13, 21, 24). Furthermore, the physiological and *in vitro*

characteristics of this specific bacterium were also examined before its *in vivo* application. *L. amylovorus* – like (phylotype OTU 171) was reported as one of the most abundant *Lactobacillus* phylotypes in the GI tract of pigs (27, 40). Recently, isolation of *L. amylovorus*-like strains from the intestine of weaning piglets and their characterization by physiological and genomic-based approaches was reported. These porcine isolates displayed marked differences compared to the closest *Lactobacillus* type species and the name *Lactobacillus sobrius* sp. nov. was proposed (Chapter 5). Moreover, *L. sobrius* strain 001^T significantly reduced the ETEC number *in vitro* using a porcine epithelial cell line IPEC-1 (42). Consisted with these *in vitro* findings, a significant decrease in the ileal ETEC prevalence in piglets fed the *L. sobrius* strain 001^T was observed in the current experiment. Several mechanisms, such as production of various anti-microbial factors such as acidic pH, H_2S, bacteriocins, fatty acids, deconjugated bile acids, and competition for nutrients or for adhesion receptors on the gut wall may be involved. The pH values measured in the intestinal lumen samples of the piglets fed LAB or control diet did not differ significantly, suggesting a minor role of the lumen pH as a factor behind the observed reduction of the ETEC prevalence. However, other mechanisms such as receptor-binding specificities shared between *L. sobrius* strain 001^T and ETEC or *L. sobrius* strain 001^T bacteriocin production, may have specifically contributed to this finding. In fact, a reduced ETEC invasion was observed upon *L. sobrius* strain 001^T attachment (42). In addition, *Lactobacillus* species phylogenetically closely related to the administered strain are known for their potential to suppress other bacterial populations within the intestine via production of bacteriocins or fatty acids (18). To what extent any of these mechanisms were involved in the significant decrease of ETEC reported here, is still not known.

Prolongation of diarrhoea observed in the LAB group, might be explained with a possible increase in the mitotic index of the epithelial cells of the piglets fed *L. sobrius* strain 001^T. Such stimulation has been reported after a mono-association with *Lactobacillus rhamnosus* GG and rotavirus infection in ex-germ free rodents (5). That study demonstrated that the rate of mitosis in the upper part of the small intestine (jejunum) increased as compared to the rates in germfree and conventional counterparts, respectively. The authors hypothesized that such excretion of the epithelial cells after infection may represent a reparative event for the mucosa and could possibly be one of the mechanisms behind the observed pathogenic reduction at the end of the current experiment. The diarrhoea observed in the LAB group did not significantly affect the piglets' weight gain. This is in line with some case reports from the French animal husbandry practice where piglets receiving skimmed milk maintained a

normal body weight gain through a short period of diarrhoea (Dr. B. Sève, INRA, France, personal communication).

To determine to what degree the piglet immune system was stimulated by the administered *L. sobrius* strain 001^T, the concentration of total sIgA was measured in saliva, blood serum and intestinal jejunal secrete. A week after the ETEC challenge, the saliva sIgA was significantly higher for the LAB group compared to the control group (Fig. 2, week 1 and week 2; $p<0.05$). Moreover, the LAB group tended to have elevated concentrations of sIgA, compared to the control ($p=0.10$) in the blood serum samples. Given the fact that sIgA is an efficient immunogenic barrier in the mucosa (17, 29, 34, 46), a higher sIgA production during the infection may potentially prevent the adherence of bacterial toxins or pathogens to epithelial cells. However, in knockout mice, which lack secretory antibodies, it has recently been shown that sIgA is crucial for the protection of gastrointestinal surfaces against secreted bacterial toxins, and may inhibit early pathogenic colonization by *Citrobacter rodentium*, but is not essential for protection against re-infection with *Salmonella typhimurium* or *C. rodentium* (51). Therefore, we anticipate that several mechanisms, besides the induction of protective IgA, might have been involved in the ETEC reduced prevalence reported in this study.

Here we demonstrated that *L. sobrius* strain 001^T is is recognized and can stimulate the immune response of the animal. sIgA antibodies against *L. sobrius* strain 001^T were detected in the saliva and jejunum secretions samples, indicating that at least some of the stimulus of the immune system by the appropriate antigens was a recent event. Furthermore, in control animals sIgA recognizing *L. sobrius* strain 001^T antibodies were detected as well, a finding suggesting their lower specificity upon antigenic induction, but not prior feeding, in 3-5 weeks old piglets. After 2 weeks of supplementation with *L. sobrius* strain 001^T, a very strong reduction for anti-*L. sobrius* strain 001^T sIgA in saliva was observed, compared to the earlier sampling. The data might be an indication that, after earlier humoral response against *L. sobrius* strain 001^T, an adaptation or tolerance occurs at the site of continuous contact with the administered bacterium. In contrast, the concentration of sIgA reactive with *L. sobrius* strain 001^T and detected in blood was similar in the two groups at the end of the experiment. The long persistence of anti-*L. sobrius* strain 001^T sIgA in blood can be explained by the turnover of immunoglobulins that might be higher than 2 weeks (41).

The influence of luminal antigens and particularly the intestinal microbiota on postnatal development of sIgA has been highlighted in animal studies demonstrating that monoassociation of germfree animals with certain commensal bacteria can restore normal

development of the sIgA system (9, 34). *L. sobrius* sp. nov. and other phylogenetically closely related *Lactobacillus* species are components of the normal microbiota of pigs (25), rodents (43) and humans (14). Hence, it is likely that the neonatal intestinal colonization by this bacterium could drive the immune system development in the direction of a humoral response, evidence for which is provided by this study, rather than a cell-mediated response. Interestingly, serum antibodies have been positively associated with weight gain (28), while delayed-type hypersensitivity reactions to food antigens were negatively associated; i.e. cellular responses were most damaging, while antibody responses might be protective. This observation is consistent with studies on inflammatory bowel disease in rodents and man, where cellular responses in the intestine are mostly associated with pathology (4).

Since probiotics are discussed as possible alternatives to antimicrobial growth promoters, the positive effect of *L. sobrius* strain 001^T on the performance of ETEC-challenged weaning piglets is of prime interest. According to a recent literature review no significant positive effects on the weight gain and feed conversion ratio could be found in peer-reviewed studies (reviewed in (44)). However, thus far even statistically non-significant trends were also considered as positive effects (15).

The improved animal growth reported here may be linked to the potential of lactobacilli for modulation of the expression of cytokine-encoding genes in enterocytes (10, 17, 20, 34). Furthermore, it has been demonstrated that mixtures of lactobacilli, bifidobacteria and streptococci, or *L. acidophilus* alone, were able to improve host resistance to infections (42). Moreover, the inhibition of ETEC adhesion to piglet ileal mucus by *Lactobacillus* spp. has also been reported (6). Cytokines are important mediators of normal gut immune responses and play a key role in the inflammatory process. Recently, it has been demonstrated that weaning is associated with a significant up regulation in gene expression of many inflammatory cytokines in the gut during the first 2 days post weaning (38). Given the fact that this inflammatory process occurred at a time of marked structural and functional small intestinal change (33, 45), this early and acute inflammatory response was suggested as primary factor in post-weaning gut disorders in piglets (26). Interestingly, when a pig cell line was co-cultured with ETEC and *L. sobrius* strain 001^T, a reduction in inflammatory cytokines (IL-1β and IL-8) was demonstrated (Chapter 6). Furthermore, the *in vitro* study showed that ETEC induced a disruption of cytoskeleton and tight junction. In contrast, *L. sobrius* strain 001^T was able to protect the cytoskeleton and tight junction organization by maintaining the correct localization of occludin and β-actin (Chapter 6). Hence, we

hypothesize that the ability of *L. sobrius* strain 001^T to modulate the cytokine expression in enterocytes and to exert a protective effect against the tight junction lesions promoted by ETEC are two likely mechanisms behind the observed body growth enhancement reported here. In our further research, the bacterial cues required for the local regulation of the epithelial barrier functions will be examined.

Clearly, the observations made during the course of this study have raised many fundamental questions concerning the complex relationships existing between the host and the administered bacterial strain. Opportunities are demonstrated, for reducing the ETEC levels and enhancement of animal body growth, without addition of antibiotics, but through the administration of *L. sobrius* strain 001^T to early weaning piglets.

ACKNOWLEDGEMENTS

This work has been carried out with financial support of the European Community specific RTD programme "Quality of Life and Management of Living Resources" research project HEALTHYPIGUT (QLK5-LT2000-00522).

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GENERAL DISCUSSION

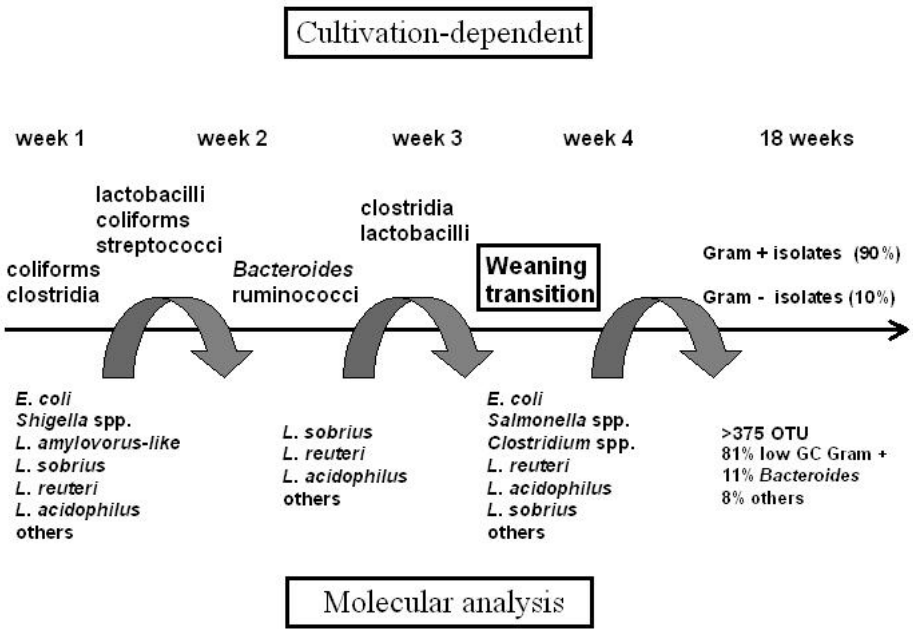
I. Development of the neonatal porcine microbiota: the stability hypothesis and its implication for the process of weaning

Ecophysiological studies over the past decades have indicated the importance of the GI tract microbiota during the neonatal and the weaning period for the mammalian host health and performance (9, 10, 19). Not without a cost, the animal gains numerous benefits by carrying a diverse and dense microbiota. The development of intestinal epithelium, lymphoid tissue, and the contribution to host nutrition and the phenomena of colonization resistance to pathogens are only a few of the ways in which resident bacteria contribute to the host (4, 14, 39, 44). The diverse GI tract microbiota provides the important stimuli for the host immune system and physiological development, while remaining in a truly mutualistic relationship with the host (2, 20). Studies in neonatal and weaning piglets, however, revealing when and how the intestinal microbial communities are shaped in relation with animal host immunology and physiology, are rare.

Porcine GI tract microbiota may be seen as an additional organ acquired in the postnatal host development. The proper function of such organ is thus only possible when a homeostasis with the host is established. The bacterial colonization starts at birth and follows a rapid succession during the first few weeks of life. The trend of the microbiota development is remarkably similar in the alimentary tract of most mammals (38). Using conventional cultivation-based techniques it has been shown that the first porcine intestine colonizers are lactic acid bacteria, enterobacteria and streptococci (63). The neonatal microbiota is relatively stable and dominated by lactic acid bacteria throughout the first week of age. Recent application of molecular techniques to study the microbial ecology of human babies and neonatal piglets, however, unveiled new trends in bacterial colonization (10) (Chapter 2) (Fig. 1).

As revealed by sequence analysis of 16S rRNA amplicons, the ileal samples of two days old piglets harbored a consortium of *Escherichia coli*, *Shigella flexneri*, *Lactobacillus amylovorus*-like, *L. reuteri,* and *L. acidophilus* related sequences (Chapter 2).

Figure 1. Establishment and succession of the porcine microbial GI tract community. OTU-operative taxonomic unit or bacteria identified after phylogenetic analysis of molecular markers such as 16S rRNA gene. The data is compiled from (35, 63) and Chapters 2, 3 and 4 in the thesis.



Remarkable stability was encountered in the populations of the early colonizers such as *Lactobacillus amylovorus*-like (most probably *L. sobrius* sp. nov., later defined in Chapter 5), *L. reuteri*, and *L. acidophilus* related populations, as long as the piglets remained with their sows. In comparison, the first bacterial colonizers in faecal samples of human babies were often found to belong to *Escherichia coli* or *Clostridium* spp. Rapid subsequent colonization with *Bifidobacterium*, *Ruminococcus*, *Enterococcus* was also demonstrated (10). After weaning, the DGGE fingerprints of 16S rRNA gene PCR amplicons derived from human faecal samples became more complex and several groups of amplicons were replaced by other ribotypes of *Clostridium*, *Ruminococcus*, *Enterococcus* and *Enterobacter* spp. As children grew older, the profiles became more complex and more constant in time (10). In contrast to the late and gradual weaning process of human babies, piglets experience an early and abrupt separation from the sow. After weaning, the predominant lactobacilli

became significantly less abundant compared to their levels in unweaned piglets (Chapter 2). In the immediate post-weaning period, shifts in the microbiota composition and metabolic activities were found for both ileal and the colonic microbiota. When individual faecal samples were examined in time, daily fluctuations in the predominant bacterial communities were also observed after weaning (32). Taken together, the data suggest that the early post-weaning period of piglets is characterized by instability in the predominant gut microbiota and its metabolic activities. Moreover, the populations of some potentially beneficial lactobacilli are significantly suppressed during the weaning transition. Consistent with reports from other groups that the porcine lactobacilli composition is age dependent and changes successively (59, 64), our study revealed a decrease in specific *Lactobacillus* populations after weaning. The observed decline may have been caused by several dietary, environmental, or host factors. Lactobacilli are strictly fermentative microorganisms that have complex nutritional requirements (17). Hence, their population is likely to be affected by the restricted feed intake after weaning. Markedly decreased numbers of lactobacilli and bifidobacteria were found when the animals were deprived of food and water for 72 h, while numbers of *E. coli* and *Bacteriodes* increased in the ileum (29). Furthermore, other groups of bacteria can grow more quickly compared to lactobacilli, and hence are able to compete more efficiently for limiting nutrients (11). While sow's colostrum does have a protective effect against diarrhoea caused by pathogenic *E. coli* (29), its role as a controlling factor of the *Lactobacillus* population during the neonatal period and weaning of piglets has to our knowledge not been fully elucidated. Specific receptors for the adhesion of lactobacilli to intestinal enterocytes are yet to be identified. Furthermore, the role of the mucus gel layer, suggested as a key contributor to the structural and functional stability of the microbiota (62), remain to be defined in the porcine GI tract.

Changes in the predominant microbiota, reported in some human studies, were found to contribute to the initiation of the inflammatory response and may have implications for the process of pathogenesis (50). Conditions such as diarrhoea and inflammatory bowel diseases have also been associated with changes in the intestinal composition (16), whereas the microbiota of healthy adult individuals remains stable over time (72). In piglets, it has often been hypothesised that an allergy to dietary proteins (34) and / or post-weaning anorexia (41) may play a significant role in the GI

tract disturbances during weaning. Based on our findings we anticipate that the microbiota instability followed by the rapid declination of previously predominant lactobacilli may have greater contributions to the porcine intestinal problems in the immediately post-weaning period than previously appreciated. Hence, the microbiota can be seen as important potential target in therapeutic and preventive interventions targeting porcine GI tract disturbances. Specifically, dietary strategies aiming to support the predominant microbiota stability while maintaining the levels of the indigenous lactobacilli communities, need to be developed.

II. Effect of fermentable carbohydrates on the microbiota of weaning piglets

It has been hypothesised that addition of fermentable carbohydrates to the diet of weaning piglets is a comparatively straightforward way to improve the microbial balance in both the small and large intestines of piglets (69). Established almost a decade ago, the concept of dietary modulation of the microbiota composition through the addition of nondigestable but fermentable carbohydrates (NDC), introduced as prebiotics, has been firstly applied to human studies (54). Prebiotics have been defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and / or activity of one of a limited number of bacteria in the colon (15). Using various techniques, it has been shown that some NDC can indeed selectively stimulate the growth and / or activity of lactic acid bacteria (54), with concomitant improvement of host colonization resistance (23). *In vitro* fermentation experiments showed that short-chain carbohydrates are fermented faster than long-chain carbohydrates (56). Similarly, linear chains are fermented faster than branched chains and soluble carbohydrates are fermented more rapidly than insoluble carbohydrates (3, 56). From these data it can be concluded that the fermentation rate of different NDC depends on their molecular structure; polysaccharides like cellulose and wheat fibre are non- or low-fermentable NDC, resistant starch is well-fermentable and inulin, fructooligosaccharides (FOS) and lactulose are rapidly fermentable (65).

Using cultivation-based approaches, the effects of NDC on the colonic microbiota in humans (58) and piglets (25) have been reported with respect to the specific stimulation of lactic acid bacteria (54). Further characterization of the stimulated lactobacilli, however, has not been achieved beyond the genus level (54). The recent

application of molecular ecological tools revealed that the porcine microbiota composition is susceptible to dietary interventions (36, 61). A strong diet effect was also found in weaning piglets consuming a diet supplemented with sugar beet pulp (SBP) alone or mixed with fructooligosaccharides (FOS) (Chapter 3). An increase in diversity and stability of the faecal bacterial community, as measured by DGGE analysis of 16S rRNA gene PCR amplicons, was found for the piglets fed NDC enriched diets compared to controls. Furthermore, the bacterial community composition in the guts of weaning piglets was significantly affected by the dietary addition of SBP, inulin, lactulose, and wheat starch (Chapter 4). Such a diet containing a mixture of fast (inulin, lactulose) and slow fermentable ingredients (wheat starch and SBP components) (3) was specifically designed to stimulate the fermentation along the entire GI tract (69). Inulin and lactoluse are two prebiotic oligosaccharides that have previously been reported to stimulate that part of the human colonic microbiota related to lactobacilli (54). However, dietary addition of FOS that can be derived from inulin has been also reported to increase the colonization of caecal mucosa and lumen of rats by *Salmonella* in challenge studies (66). Moreover, studies on the pathogen survival in healthy animals fed NDC show beneficial, inconsistent, or adverse effect (65). Using *in vitro* techniques, SBP has been shown to be readily fermentable by porcine faecal bacteria (1, 3, 71). It has been demonstrated to stimulate the bacterial diversity, and result in higher short chain fatty acid and lower ammonia concentrations compared to some other potential feed ingredients (1, 3) Moreover, the addition of SBP to the diet of pigs was reported to reduce the populations of coliforms (55). Other studies, however, suggested an increased proliferation of pathogenic *Escherichia coli* when the piglets were fed with a fiber-enriched diet (43). Overall, the reports about the role of dietary fiber on the development of swine dysentery are scarce (28). As shown by some research groups, diets with low fiber and resistant starches prevent the pigs from infection with *Brachyspira hyodysenteriae* (7, 52), while others were not able to confirm these findings (36, 37). Hence, the response of the bacterial community *in vivo* needs to be thoroughly examined in respect to the species-specific response to the dietary NDC at particular intestinal compartments. Our results suggest that the combination of fermentable dietary fiber and oligosaccharides may specifically stimulate the *L. amylovorus*-like population along the gut of weaning piglets. In addition, we found

that the dietary addition of these fermentable carbohydrates can lead to a higher bacterial diversity in the colon (30).

Animal parameters such as growth and feed intake were not altered by the inclusion of fermentable carbohydrates in the diet (25, 30, 32). This is in discrepancy with the usual expectation that such inclusion will negatively affect growth of the animal, as it is supposed to reduce the proportion of enzymatically degradable ingredients. Until recently, any part of the diet, which was fermented, was considered to have been wasted (69). The dietary addition of NDC was found to affect the bacterial community diversity and stability along with the stimulation of specific members of the *Lactobacillus* community (30). Since a stable and complex commensal bacterial community is a prerequisite of a healthy gut ecosystem (31), the promotion of colonization resistance through the addition of fermentable carbohydrates (prebiotics) may be a considered in further studies aiming to ensuring normal animal growth and nutrition of weaning piglets at stressful times, such as early and abrupt weaning.

III. *Lactobacillus sobrius*, a member of the resident porcine microbiota, protects against pathogenic challenges in newly weaned piglets

The remarkable response of a novel and abundant *L. amylovorus*-like phylotype to the dietary addition of fermentable carbohydrates (30) and the weaning process (Chapter 8) has brought up the question about the taxonomic position of the strains belonging to that phylotype. Using a combination of cultivation on MRS media followed by phylotype specific screening by FISH analysis, several *L. amylovorus*-like isolates have been cultured from the porcine intestine (Chapter 5). Their detailed genotypic and phenotypic characterization has revealed that they belong to a new *Lactobacillus* species, for which the name *Lactobacillus sobrius* sp. nov. was proposed (Chapter 5). The protective effect of *L. sobrius* strain 001^T against *Escherichia coli* K88 (ETEC), a common etiological agent of the post-weaning morbidity and mortality in piglets, was further examined using a combination of comprehensive *in vitro* and *in vivo* approaches (Chapters 6 & 8). Attachment of *L. sobrius* to an epithelial cell line, derived from the intestine of a neonatal piglet, was found to reduce ETEC adhesion, and to maintain the tight junction integrity of the epithelium. Moreover, ETEC proinflammatory stimuli were inhibited by yet unidentified *L. sobrius* determinants. These *L. sobrius* cues are likely to be cell wall components, including lipoteichoic

acids (LTAs), since the protective effect was exerted upon attachment of *L. sobrius* to the epithelium. Moreover, LTAs alone or in association with peptidoglycan fragments from a variety of Gram-positive bacteria are known for their immunogenicity (47). LTAs from two *Lactobacillus* strains appear to be involved in the downregulation of cytokine expression after bacterial stimulation (68). Shedding of epithelial receptors induced by these cell wall components might be one of the major mechanisms responsible for the observed effects of *L. sobrius*. Among the candidate receptors are members of the Toll-like receptor family (TLRs) (53) or other pattern recognition receptors (PRR), including mannose receptor (6), and different C-type lectin receptors (67). TLRs have been shown to mediate effects induced by microbial compounds (70) and signaling through these receptors reduced *E. coli*- and *Salmonella dublin*-induced proinflammatory cytokines secretion in a similar manner as observed in our study (49). Moreover, LTAs from gram-positive microorganisms, and present in *L. sobrius*, have been identified as ligands for TLR2 (70). TLR2 may activate protein kinase C in response to specific bacterial ligands, with the physiologic consequence of directly enhancing intestinal epithelial integrity (48). At what extend the protective effect of *L. sobrius* against ETEC induced damages involved a signalling through any of the PRRs remains to be determined. Nevertheless, it is likely that sustained inflammatory activation is required for immunological surveillance at the major portal of entry of pathogens (26). Hence, the anti-inflammatory effect demonstrated by *L. sobrius in vitro* may be a prerequisite to prevent the ETEC invasion and improve the epithelial integrity *in vivo*.

Phylogenetic analysis of *L. sobrius* and related lactic acid bacteria indicated a high degree of 16S rRNA similarity (>98%) between the type strains of *L. sobrius* and *L. amylovorus*, *L. crispatus*, and *L. kitasatonis*. Their DNA-DNA relatedness, however, was below 49% (Chapter 5). Such microdiversity is often reported among different *Lactobacillus* species (46). Furthermore, a combination of cultivation-based methods with a molecular biological approach has demonstrated that bacterial strains with identical 16S rRNA gene sequences can represent distinct eco- and genotypes (27). The current 16S rRNA-targeting molecular ecological techniques are only of limited value for the assessment of such microdiversity that can have significant functional impact (Chapter 7). Although previous reports have successfully quantified the bifidobacterial species diversity in the human intestinal tract by 16S rRNA-targeted real-time PCR (40), such approaches may not provide sufficient degree of

resolution between the lactobacilli closely related to *L. sobrius*. Thus, a novel technique have been developed based upon the isolation of strain specific genomic fragments by representative differences analysis (RDA), and their further detection using real-time PCR allowed selective detection, enumeration and identification of closely related *L. sobrius* isolates *in vivo* (Chapters 7 & 8). The approach is widely applicable in future studies aiming at strain-level identification and further insight in the occurrence or/and activity of functional bacterial genes in the particular environment (Chapter 7).

The development of the strain-specific detection tool also enabled us to study the protective effect of *L. sobrius* against ETEC challenges *in vivo* (Chapter 8). At the end of the challenge experiment, the persistence of *L. sobrius* at higher levels ($>10^8$ cells/g) was accompanied by a significantly lower number of ETEC in the pocine ileum. ETEC excretion, however, was not significantly influenced by the *L. sobrius* supplementation in the first 3 days after the infection (Chapter 8). As demonstrated in Chapter 5, FOS and SBP are two non-digestable but fermentable NDC utilized by *L. sobrius*. Hence, we anticipate that a further synbiotic application of a *L. sobrius* culture together with these NDC may enhance the ETEC excretion in the early post-weaning period. Animal performance and health parameters such as body weight gain and development of the secretory IgA were positively correlated with the abundance of *L. sobrius* ($p<0.05$). An exogenous supplementation of live microbials, defined as probiotics (13), is discussed as possible alternatives to antimicrobial growth promoters. According to a recent literature review, however, no significant positive effects on the weight gain and feed conversion ratio could be found in peer-reviewed studies (reviewed in (12, 60)). Hence the positive effect on the performance of weaning piglets reported above is of prime interest and requires further insight into the mechanisms underlying the *L. sobrius*-host relationships.

Several mechanisms may be involved in the improved host immunity and growth reported in this study (Chapter 8), including host- and / or microbiome-*L. sobrius* relationships (Fig. 2). As mentioned earlier, the persistence of *L. sobrius* was followed by a significant reduction of the number of ETEC in the ileum. Hence, direct-antagonistic activities of *L. sobrius* may have been executed in the gut *in vivo*. Lactobacilli phylogetically related to *L. sobrius* are known for their ability to secrete certain bacteriocines and suppress the populations of other bacteria, including ETEC, both *in vitro* and *in vivo* (5, 17). Moreover, as demonstrated *in vitro,* the bacterium

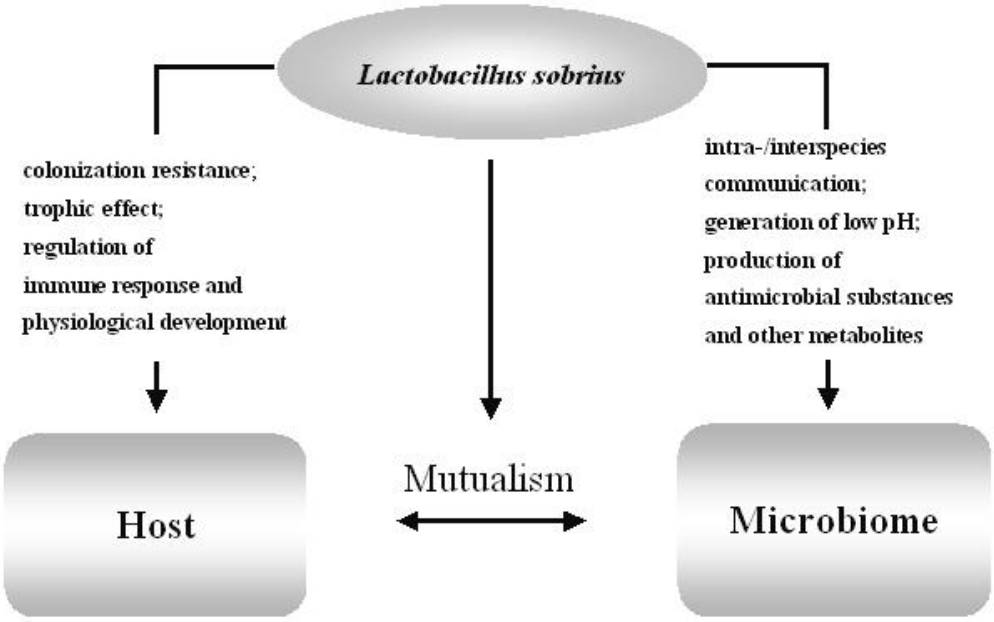
possesses the potential of modulating the expression of cytokine-encoding genes in enterocytes and to improve the barrier function of the intestine during ETEC challenge (Chapter 6). A variety of reports demonstrated that lactobacilli are potent immunomodulators in epithelial cells (8, 14, 18, 42) and dendritic cells (45), whereas others may improve host resistance to infections (57). To our knowledge, *L. sobrius* is the first isolate which dietary addition was found to improve the host immunity *in vitro* and *in vivo*, while stimulating the body weight gain upon ETEC challenges in weaning piglets. Furthermore, the two studies (Chapters 6 & 8) suggested the beneficial role of the *L. sobrius*-related population in the intestine of piglets and possibly in other mammals. Recently, it has been demonstrated that weaning is associated with a significant up-regulation in gene expression of many inflammatory cytokines in the gut during the first 2 days post weaning (51). Our study indicated a rapid declination in the number of *L. sobrius*-like populations in the post-weaning period (Chapter 2). At what extend the early and acute inflammatory response, suggested as primary factor in post-weaning gut disorders in piglets (33), is related to the disturbance of the *L. sobrius* population remain to be determined. Moreover, the *in vitro* and *in vivo* properties of *L. sobrius* shed a light upon a common observation from the microbial ecology in piglets, namely that presence of pathogenic *E. coli* strains is not sufficient to cause diarrhoea. *E. coli* may even predominate in the coliform microflora of early-weaned pigs that are healthy (24). Thus, the disturbances of the population of *L. sobrius*, or other beneficial commensals, may well be among the factors predisposing the animal host to intestinal GI disturbances after weaning. Furthermore, *L. sobrius* can be a key member of the porcine microbiota due to its ability to prevent an inflammatory response in the intestinal mucosa, which would otherwise disrupt the commensals' luminal habitat.

The direction of relationship between *L. sobrius* and its host may be symbiotic (both partners benefit) or commensal (neither is harmed) in regard to the presence or absence of pathogenic challenges in the porcine intestine. The host could benefit in several ways from filling its intestinal habitat with *L. sobrius* at the neonatal period and weaning. During the time of weaning characterized by great compositional flux in the microbiota, *L. sobrius* could provide colonization resistance. Additional benefits may include provision of critical metabolic activities and shaping of the underlying mucosal immune system. The paradigm illustrated earlier to *Bacteroides thetaiotaomicron* (2, 21, 22), a commensal isolate from the GI tract of mice and

humans, may in parts be applied to *L. sobrius* and other members of the beneficial porcine microbiota. Thus, a comparative analysis between the effects of different commensals on the mammalian host is clearly important in respect to the understanding of the bacterial contributions to the establishment of the GI tract homeostasis.

Early weaning of piglets is characterized with a high risk of GI tract disorders. In such crucial period of piglets' life, well-defined changes in the microbiota compostion were demonstrated using cultivation-independent approaches. Following the weaning, a transient instability was observed in the predominant microbiota and its metabolic activities. Furthermore, a reduction in the number of some previously abundant lactobacilli was also evidenced. Diets containing different fermentable carbohydrates were found to improve the intestinal balance of weaning piglets but had no effect on the animal body weight gain. Moreover, the dietary addition of fermentable carbohydrates stimulated the growth of some porcine commensals related to *L. amylovorus*.

Figure 2. Hypothetical model of the host- and microbiome-*L. sobrius* relationships in the porcine gut. Mutualism: commensalism and symbiosis are presented as part of a continuum, distinguished by the identification of specific benefits derived by one or both members of a host-bacterial partnership (2,20).



IV. Conclusions and perspectives

Their detailed characterization, however, revealed that they represent a previously unknown member of the porcine bacterial community defined as *L. sobrius* sp. nov. The *L. sobrius*-like population was encountered among the first bacterial colonizers in the porcine gut. During the weaning transition, however, its population was significantly diminished. Furthermore, the protective role of *L. sobrius* against a specific porcine pathogen was demonstrated *in vitro* and *in vivo*. Its dietary addition to weaning piglets was found to modulate the porcine immunity and stimulate the animal weight gain during the pathogenic challenge. These findings may potentially have major implications for development of dietary strategies aiming to improve the animal health during the weaning process. Furthermore, it is probable that even within the complex ecosystem of the gut, the neonatal and the weaning population of *L. sobrius* could protect against pathogens, while directing and determining important functions of the host development. Further, establishing the impact of such commensal on GI tract homeostasis is noticeably important. Moreover, the unraveling of the specific bacterial cues mediating the microbiota interactions with the intestinal epithelium and the local immune system is urgently needed for the understanding of the host-microbial beneficial relationships.

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SUMMARY

After birth, the porcine intestinal microbiota follows a rapid development from a simple and unstable community into a complex and balanced one influenced by dietary, environmental, and host factors. Piglets weaned within a production environment experience major changes in the intestinal microbiota predisposing them to severe intestinal diseases and reduced body weight gain. In order to overcome the intestinal complications and increase the production efficiency, antibiotic growth promoters have been fed to weaning piglets for more than four decades. The forthcoming ban on in-feed antibiotics in the EU have accelerated the search for alternative dietary strategies. In the present thesis, we hypothesize that prebiotics or probiotics may be used as alternatives to in-feed antibiotics for weaning piglets.

Chapter 1 provides a comprehensive overview of our current knowledge on the cultivation-independent methods to analyse the diversity of the mammalian GI tract microbiota. The new insights into GI tract microbiota composition are also highlighted. The advantages and the pitfalls of these techniques are further discussed.

Chapter 2 describes the development of bacterial community and its metabolic activities in the intestine of the neonatal and weaning piglets. After a molecular microbiological analysis, the early colonizing bacteria were identified. When the effect of weaning was examined, we found that the populations of previously abundant lactobacilli were significantly suppressed. The weaning process had also a profound effect on the stability in the predominant porcine microbiota and its metabolic activities.

The hypothesis that the dietary addition of fermentable carbohydrates may lead to more stable microbiota and stimulate the growth of some potentially beneficial bacteria was tested in **Chapters 3 and 4**. In **Chapter 3** diets containing sugar beet pulp alone or mixed with fructooligosaccharides were fed to piglets and the faecal bacterial communities were analysed using 16S rRNA-based approaches. After weaning, piglets fed with fermentable carbohydrates showed a higher bacterial diversity and a more rapid stabilisation of the microbiota compared with that of the animals fed with the control diet. The results indicate that bacteria related to *Ruminococcus* spp. may play a significant role in the utilisation of dietary fibres in the porcine intestine.

Chapter 4 describes the response of ileal and colonic bacterial communities of weaning piglets to dietary addition of four fermentable carbohydrates (inulin, lactulose, wheat starch and sugar beet pulp). The results revealed that the addition of fermentable carbohydrates (= prebiotics) lead to a higher bacterial diversity in the colon and an enrichment of lactobacilli in the ileum. Furthermore, the last study suggested a significant role of *L. amylovorus*-like phylotype in the metabolism of fermentable carbohydrates. The taxonomic position of porcine *Lactobacillus* isolates related to *L. amylovorus* was further examined in **Chapter 5**. Six bacterial isolates defined as *L. amylovorus*-like were obtained from porcine faecal specimens and further characterized by phenotypic and molecular taxonomic methods. Comparative 16S rRNA gene sequence analysis demonstrated that the novel isolates shared high similarity to *L. amylovorus* (DSM30531^T), but their DNA relatedness was found to be lower than 49%. After using physiological and biochemical tests, we unravelled that

the strains merit species status, and the name *Lactobacillus sobrius* sp. nov. was proposed.

Chapter 6 describes the protective effect of the novel porcine *L. sobrius* sp. nov. against enterotoxigenic *Escherichia coli* strain K88 (ETEC)-induced intestinal damages *in vitro*. The data suggest that *L. sobrius* isolated may reduce ETEC adhesion, and protects against the tight junction lesions of epithelial cells and inflammation promoted by this pathogen.

In an attempt to quantify the fate of *L. sobrius in vivo*, a set of strain- and species-specific real-time PCR primers was developed (**Chapter 7**). The detection specificity was validated by using genomic DNA of additional *L. sobrius* sp. nov. isolates and other phylogenetically closely related *Lactobacillus* spp.

To determine whether ETEC infection can be prevented by dietary supplementation with *Lactobacillus sobrius* sp. nov. strain 001^T and animal experiment was done (**Chapter 8**). Piglets (n=48) were subjected to a control diet and a diet containing *L. sobrius*, and further experimentally infected with ETEC. After the application of the *L. sobrius* single-strain detection and quantification approach, we observed that the persistence of *L. sobrius* was accompanied by a significant reduction of ETEC prevalence in the porcine ileum at the end of the experiment. Moreover, an improved daily weight gain was also evidenced in the piglets that received *L. sobrius*. The data indicate that the dietary addition of *L. sobrius* may provide the first line of defence against the ETEC infection. Studies on the mechanism behind the reported stimulation of the animal body growth following the *L. sobrius* supplementation are clearly needed.

Chapter 9 summarizes the knowledge gained during the course of the study. Moreover, the hypothesis that a stable and complex microbiota in weaning piglets is prerequisite of a healthy GI tract ecosystem was put forward. The current work specifically shows that in the presence of specific fermentable carbohydrates the growth of *Lactobacillus* spp. was stimulated, particularly in the small intestine. The outgrowth of lactobacilli is likely to enhance the colonisation resistance. Moreover, the protective role of the novel and abundant *Lactobacillus sobrius* against ETEC challenges were demonstrated *in vitro* and *in vivo*. The potential for development of novel dietary strategies aiming to improve the animal health during the weaning process are also discussed.

In conclusions

- A stable and complex commensal bacterial community is a prerequisite of a healthy intestinal ecosystem.
- Addition of fermentable carbohydrates to the diet of weaning piglets leads to an enrichment of lactobacilli in the small intestine and a significantly faster increase in diversity of the microbiota in the colon.
- Specific strains selected from the commensal porcine microbiota, and defined as *Lactobacillus sobrius* sp. nov. are working as a probioticum confer a resistance to intestinal diseases.

SAMENVATTING

De microbiële gemeenschap in de darm van het varken ontwikkelt zich na de geboorte van een eenvoudige en onstabiele gemeenschap in een complexe en stabiele gemeenschap die beïnvloed wordt door de voedings-, omgevings- en gastheerfactoren. Onder praktijkomstandigheden treden na het spenen van de biggen grote veranderingen op in de microbiële samenstelling van de darm. Deze maken hen vatbaar maken voor ernstige darmstoornissen en kunnen leiden tot groeidepressie. In de praktijk wordt dit voorkomen door toevoeging van antibiotica aan het voer. Daar binnenkort in Europa een verbod komt op het gebruik van antimicrobiële groeibevorderaars is onderzoek naar alternatieve methoden dringend gewenst. In dit promotieonderzoek is onderzocht of toevoeging van prebiotica en/of probiotica aan het voer als alternatief kan gelden.

Hoofdstuk 1 geeft een uitgebreid overzicht van de huidige kennis van het analyseren van microbiële gemeenschappen (microbiota) bij zoogdieren met behulp van methoden zonder de microorganismen vooraf te kweken. Tevens worden de nieuwe inzichten in de samenstelling en de funktie van de microbiota in de darm nader toegelicht. De voor- en nadelen van deze moleculaire methoden worden besproken.

In **hoofdstuk 2** wordt een beschrijving gegeven van de ontwikkeling en de metabole activiteit van de bacteriële gemeenschap in de darm van de biggen na de geboorte en na het spenen. Met behulp van moleculair microbiologische technieken kon de identiteit van de eerste koloniserende bacteriën worden vastgesteld. Vastgesteld werd dat lactobacilli, die aanvankelijk prominent aanwezig waren, na het spenen sterk in aantal terugliepen. Dit had een sterk effect op de stabiliteit en de metabole activiteit van de darmmicrobiota tot gevolg.

In de **hoofdstukken 3 en 4** is de hypothese getest dat toevoeging van fermenteerbare koolhydraten aan het voer kan leiden tot een meer stabiele microbiota en stimulatie van de groei van enkele potentieel nuttige bacteriën. In **hoofdstuk 3** worden de resultaten vermeld van een voedingsproef waarbij suikerbietenpulp (SBP) alleen of in combinatie met fructooligosacchariden (FOS) aan het voer van biggen werd toegevoegd. Hierbij werd de samenstelling van de bacteriële gemeenschap in de feces onderzocht met behulp van 16S rDNA analysemethoden. Biggen die na het spenen gevoerd werden met fermenteerbare koolhydraten vertoonden een grotere bacteriële diversiteit en een snellere stabiliteit van de microbiota in vergelijking tot biggen die een controledieet ontvingen. De resultaten tonen aan dat bacteriën verwant aan *Ruminococcus* spp. Mogelijk een belangrijke rol spelen bij de afbraak van voedingsvezels in de darm van de biggen.

In **hoofdstuk 4** wordt het effect van toevoeging van vier fermenteerbare koolhydraten (inuline, lactulose, tarwezetmeel en suikerbietenpulp) beschreven op de bacteriële gemeenschappen in het ileum en colon van gespeende biggen. De resultaten laten zien dat toevoeging van fermenteerbare koolhydraten als *prebiotica* leidt tot een grotere bacteriële diversiteit in de dikke darm (colon) en een ophoping van lactobacilli in de dunne darm (ileum). Verondersteld wordt dat een *Lactobacillus amylovorus*-achtige phylotype een belangrijke rol speelt in het metabolisme van fermenteerbare koolhydraten. De taxonomische identificatie van de *Lactobacillus* isolaten die verwant waren aan *L. amylovorus* werd nader beschreven in **hoofdstuk 5**. Zes *L. amylovorus* isolaten afkomstig uit feces van biggen werden nader gekarakteriseerd met behulp van fenotypische en moleculair taxonomische methoden. Vergelijkbare 16S rRNA gensequentieanalyse toonde aan dat de nieuwe isolaten sterk verwantschap vertoonde met die van *L. amylovorus* (DSM30531), maar dat de DNA verwantschap

minder dan 49% was. Na uitvoering van fysiologische en biochemische testen kon worden geconcludeerd dat de isolaten tot een aparte soort (species) gerekend moeten worden. Voorgesteld werd de naam *Lactobacillus sobrius* sp. Nov.
In **hoofdstuk 6** wordt in een *in vitro* model het beschermend effect van *L. sobrius* sp. nov. beschreven tegen de door enterotoxigene *Escherichia coli* K88 (ETEC) geïnduceerde darmbeschadiging. De resultaten wijzen er op dat *L. sobrius* mogelijk de aanhechting van ETEC aan de epitheelcellen vermindert en deze beschermt tegen beschadiging van de hechte verbindingen tussen de epitheelcellen en ontstekingen die door de pathogeen worden veroorzaakt.
In een poging het lot van *L. sobrius in vivo* te kwantificeren, werden stam- en species-specifieke real-time PCR primers ontwikkeld (**hoofdstuk 7**). De specificiteit van deze detectiemethode werd bevestigd door genomisch DNA te testen van een aantal *L. sobrius* sp. nov. isolaten en andere fylogenetisch sterk verwante *Lactobacillus* spp.
Teneinde aan te kunnen tonen of een ETEC infectie kan worden voorkomen door toevoeging van *Lactobacillus sobrius* sp. nov. strain 001^T aan het voer, werd een dierproef uitgevoerd (**hoofdstuk 8**). In een voedingsproef met biggen (n=48) werd aan de helft een controle dieet gegeven terwijl aan de andere helft *L. sobrius* was toegevoegd. De dieren worden experimenteel met ETEC geïnfecteerd. Met behulp van de *L. sobrius* stam-specifieke kwantitatieve detectiemethode werd aangetoond dat aan het eind van het experiment de aanwezigheid van *L. sobrius* gepaard ging met een significantie reductie van ETEC in de dunne darm. Bovendien werd aangetoond dat de biggen die *L. sobrius* kregen toegediend een verhoogde dagelijkse gewichtstoename hadden. De resultaten tonen aan dat toevoeging van *L. sobrius* een eerste stap in de verdediging tegen ETECT infecties zou kunnen betekenen. Vervolgonderzoek naar de oorzaak van de waargenomen stimulatie van de groei van de dieren na toedienen van *L. sobrius* in het voer is duidelijk noodzakelijk.
In **hoofdstuk 9** worden de resultaten van dit onderzoek samengevat. Aandacht wordt besteed aan de hypothese dat een stabiele en complexe bacteriële gemeenschap in de darm van gespeende biggen een voorwaarde is voor een gezond darmecosysteem. Het uitgevoerde onderzoek laat zien dat in de aanwezigheid van specifieke fermenteerbare koolhydraten de groei van *Lactobacillus* spp. Gestimuleerd wordt, in het bijzonder in de dunne darm. Vermoed wordt dat de uitgroei van lactobacilli de kolonisatieresistentie verhoogd. Bovendien kon aangetoond worden zowel in *in vitro* als in *in vivo* experimenten de nieuw beschreven en predominante *Lactobacillus sobrius* een beschermende rol speelt tegen ETEC. Deze resultaten kunnen belangrijke gevolgen hebben voor de ontwikkeling van voedingsstrategiën die tot doel hebben de gezondheid van het dier na het spenen te verbeteren.

Conclusies

* Toevoeging van fermenteerbare koolhydraten aan het dieet van gespeende biggen leidt tot een toename van lactobacilli in de dunne darm en een significant snellere toename van de diversiteit van de microbiota in de dikke darm.
* Een stabiele en complexe commensale bacteriële gemeenschap is een voorwaarde voor een gezond darmecosysteem.
* Specifieke stammen, afkomstig uit de commensale darmmicrobiota van biggen en beschreven als *Lactobacillus sobrius* sp. nov. zijn als probioticum te beschouwen en verlenen resistentie tegen darmziekten.

РЕЗЮМЕ

Стомашно-чревният тракт на бозайниците е населен от богата и разнообразна микрофлора. Повечето от микроорганизмите са полезни за гостоприемника, тъй като осгуряват необходимите хранителни вещества или предпазват от развитието на патогени. Процеса на микробиологична колонизация започва непосредствено след раждането и търпи бързо развитие през първите месеци на живот. Отбиването от майката и преминаването към самостоятелено хранене е критичен период в развитито на всеки организъм. В продължение на повече от 50 години в селското стопанство на Европа и Америка, като хранителни добвки се използват антибиотици, тъй като те предотвратят развитието на патогенни микроорганизми и стимулират растежа на животните. Акумулирането на антибиотична резистенност в микрофлората на стомашно-чревния тракт и търсенето на алтернативни методи за предпазване от инфекции наложи извършването на изследванията описани в тази десертация.

Развитието на микрофлората в стомашно-чревния тракт на прасето беше изследвано чрез разнообразни молекулярни методи на базата на анализ на нуклеотидните последователности на гена, кодиращ малката рибозомална субединица. Резултатите от нашето изследване показаха, че стабилна микрофлора, характерезираща се с богато видово разнообразие, е показател за стомашно чревна микробиална екосистема, способна да предодврати развитието на патогенни микроорганизми. Въглехидратни хранителни добавки, потенциални заместители на антибиотиците, бяха изпитани върху прасета и показаха стимулиране на микроорганизми близки до *Lactobacillus amylovorus* в стомашно-чревния тракт. Фенотипното и молекулярно определяне на тези щамове доказа, че те принадлежат кам нов вид *Lactobacillus sobrius sp. nov*. Както беше установено *in vitro*, целостта на тъканна култура, нарушена под деиствието на патогенен щам *Escherichia coli*, може да бъде запазена при третиране с *L. sobrius*. Използвайки прасета като моделни животни за изучаването на стомашно чревния тракт при човека, *L. sobrius* показва предпазен ефект срещу развитието на зараза от *E. coli*, и стимулираше развитието на имунната система. На базата на тези резултати в бъдеще могат да бъдат създадени препарати, целящи да подотвратят развитието на патогенни микроорганизми по време на стрес при животните.

ACKNOWLEDGEMENTS

It this, one the most enjoyable part of the thesis, I would like to express my deep gratitude to a number of people I met, worked and made friendships with throughout the years of my research. Dear Willem, thank you for the support during the entire research. Your stimulating comments have shaped enormously my scientific interests. The ideas shared during our meetings are always alive in my mind. I learned a lot from you, and you were always open to read the manuscripts even when the time was a limiting factor.

Antoon, you were the one who accepted me in your group as an unexperienced graduate student. Kindly guiding me along the path of microbial ecology, you were and still are the one from who I received the vival optimism and support during the study. The meetings we had with you have always been more that scientific discussions, but also valuable life lessons. Thank you for the warm dinners at your place and all understanding of my problems, and your help when we moved to Amsterdam. This thesis would have not been started without your support. Many thanks also for the critical reading of all versions of this thesis. Special gratitude for translating the summary in Dutch. Your help in these final steps have added a lot on the overall outlook of the thesis, including its cover.

Hauke, I met you at the time when I did not know that you will be my present co-promotor and supervisor. After all this time, I am glad that I am still experiencing your friendship. Your precise stile is something I admired and tried to implement in my work. Your crucial ideas for the directions of the research were always on time. Thank you for all your help! Happily enough, our work together is ongoing.

Wilma, I am truly thankful for your support ever since I come to ork in MolEco. You showed me how to do some of the survival kit techniques in our group. Always willing to help me, we shared the opposite sides of one bench. Besides, you know all of my problems during the times of ups and downs. Kind regards.

Susana, thank you for the friendship and the generous support. The trip we had to your family in San Diego was one of the most enjoyable times after the submission of the thesis. I am glad I met and made friends with Pablo and Paloma as well.

To the co-authors of the chapters is given a special acknowledgement. The work described in the thesis would not have been done without the help of Ajay, Alberto, Barbara, Bevis, Chris, Christine, Elaine, Elena, Elisa, Jeannette, Luisa, Maurizio, Marianna, Nora, Paolo Bosi, Paolo Trevisi, Phil, Sara, Seerp, Serena, Wei-Yun, Wolfgang-Bernhard.

Here I want to acknowledge the people that came to work with me during this thesis research. Yao-Wen, Cornelia, Jana, Joel, Joan, thank you for the enthusiastic and nice work we did together.

I am grateful for the nice time we had with all people from the MolEco group Alcina, Anna, Arian, Carien, Cincia, Cristina, David, Diana, Eline, Erwin, Farai, Hans, Hoa, Jaroen, Jordan, Kauther, Kathrin, Kees, Maaike, Mahmut, Mark, Markus, Marianna, Meta, Muriel, Mirijana, Neslihan, Odette, Petia, Reetta, Rocio, Toshio, Vesela, Yao Wen, Yoyok. The cakes and the cheerful athmosphere in the lab during all these years were always bringing a good mood in me, and a gratitude that I am working in the MolEco group.

Bedankt Ans, Caroline, Dauwe, Francis, Fons, Gosse, Jannie, John, Leo, Nees, Reneé, Wim, en alle Micro's voor jullie hulp en de fantastische tijd in het lab.

Hey Ajay, the work and the friendships with you is wonderful! Your jokes were always bringing good mood. Dear Barbara, thank you for the stimulating discussions, the English writing lessons, and the support I had from you in my first steps in Wageningen. The dinner we had at your place was wonderful. I wish you a lot of happiness to you, Jan Kees and Pieter, and a very nice time in Australia. I hope to see you soon. Dear Martin, many thanks for your open door you had for my questions. I am glad for the contacts and discussions I have with people from Animal Nutrition Group, and want to express thanks to Huug, Jane-Martine, Marlou, Meijke, Tamme, Walter, Wilbert.

I would like to thank all colleagues in the Healthypigut project. I truly enjoyed the fruitful meetings and the friendly discussion with the people all over Europe. Once again special thanks to the colleagues from Italy, Roma: Elena, Marianna, Alberto, Selena, and Bologna: Paolo Bosi, Luisa, Paolo Trevisi, Maurizio, Sara. Without you this thesis would have been much thinner. Here I also want to express also my deep gratitude to the people from the Bristol group: Bevis, Chris, Mick, Karin, Phil**,** Martin, Dilip, Cecilia and rest of the group. I thank to Jean-Paul, Bernard, Christine and all other people from INRA group. Hermann-Joseph regards for the visit to Hannover in the early 2002. The work with you, Eveline, Andrea and the rest of the Hannover group was most enjoyable. Gratitude also to Isabelle, Sandrine, Wolfgang, Jeannette.

Here I also want to thank to Thomas for inviting me to visit the Royal Danish Veterinary Lab, and for his hospitality during my stay. Regards also to Joanna, and Katia for helping me out in the lab, and for the wonderful Copenhagen sightseeing.

Dear Hermie, thank you for giving me the opportunity to learn the FISH technique from one of the masters in this field. I will be always pleased for what I learned during my stay in your lab. On my research path I met also a number of colleagues from USA, with which we still keep contacts. Dear Rod, thank you for the excellent discussions we had during our meetings in Wageningen, and Lake Tahoe. For sure, I will never forget the ideas you shared with me on field of the microbial ecology. John, your sabbatical in our lab was the time we became involved in a common work. Later, in Banff, Canada, I had the chance to experience the rock climbing together with you. Quite extreme though, it was worth trying. Thanks a lot.

Special acknowledgements to my present colleagues from Prosearch project: Harro, Linda, Louis, Ger, Paul, for the excellent collaboration. My work in this project have evolved also in exciting research with people from VUMC to whom I also want to thank here, Satwinder, Yvette van Kooyk, Ben Appelmelk.

I won't forget to thank for the cozy dinners we had and the support I received from many friends I met in Wageningen and Amsterdam, Samir, Nikolay, Rumi, Elena, Arnold, Ilia, Najeli, Stefan, Asaph, Rafa, Ruud, Iansen, Albena, Diliana, Bob, Truus. Once again, I want to express my gratitude to all people that helped us while moving from one to another leaving place: Hans, Antoon, Vesela, Mark, Ivo. Without your help I would have been really difficult for me, Pavlina, and Rafael. Thank you very much!!

This work would not have been possible without the absolute support of my wife Pavlina. Your vital optimism and courage were always keeping my spirit up. Thank you for everything you did for me! I know, the time I spent in writing of the thesis was the time without you and our son Rafael. Thousands of regards for your patience and understanding!

Искам да изкажа специални благодарности на моите родители, майка ми Елена и баща ми Румен за тяхната дълбока подкрепа през цялото време на моето следване в Холандия. Вашето разбиране и обич ми даваше винаги кураж да следвам своят път дори, когато той води далеч от вас. Много благодарности изказвам още и на баба Мария за нейната всеотдайна помощ в последните месеци на моята работа по тази дисертация. Твоите грижи за Рафаел ми помогнаха много за да завърша навреме. Благодаря на брат ми Владимир, Стоян, Миро и Маринка, Петя и Вени, Сашо и Таня, за помощта, която ми оказаха, някои от тях със съвети, други с добри думи и весели гостувания.

Amsterdam, May 2005

ABOUT THE AUTHOR

Sergey Rumenov Konstantinv was born on May 7, 1974, in Burgas, Bulgaria. He completed secondary school in his home-town and received his diploma in 1992. From 1992 to 1998 he studied molecular biology at the Faculty of Biology, University of Sofia, Bulgaria. His MSc thesis entitled “Effects of some polyamines on the activity of the soluble acetylcholinesterase from rat brain” was defended in the Department of Human and Animal Physiology, Faculty of Biology, University of Sofia, under the supervision of Prof. Dr. I. Minkov. In 2000 he came to the Netherlands, where he worked till 2001 on a research project “The effect of diet on the pig large intestine microbial communities characterized using sequencing analysis of bacterial 16S rRNA genes” at the Laboratory of Microbiology, Wageningen University. This was a joint project between the Laboratory of Microbiology and the Animal Nutrition Group, and supervised by Dr. Antoon A.L. Akkermans and Dr. Barbara A. Williams. From 2001 to 2005 he worked as a PhD student in the same laboratory under the supervision of Dr. Antoon D. L. Akkermans, Dr. Hauke Smidt and Prof. Dr. Willem M. de Vos. As described in this thesis, his main interests were on microbial ecology in the gastrointestinal tract with a specific focus on the relationships between some beneficial, as well as pathogenic bacteria and the animal host. From February 2005 he started in the same lab. as a post-doctoral researcher on a project aiming to prevent the development of acute pancreatitis in rat models using novel dietary strategies.

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Konstantinov S.R., Fitzsimons N., Vaughan E.E., Akkermans A.D.L. 2002. From composition to functionality of the intestinal microbial communities. *In* Probiotics and Prebiotics: Where are we going?, p. 59-84. (ed.) G.W. Tannock, Caiser Academic Press, Wymondham, UK.

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Konstantinov S.R., Smidt H., and de Vos W.M. Novel probiotic. **Submitted patent, file number (P6001554EP)**.

Konstantinov S. R., Poznanski E, Fuentes S, Akkermans ADL, Smidt H, and de Vos WM. *Lactobacillus sobrius* sp. nov., a novel isolate abundant in the intestine of weaning piglets. *submitted for publication*.

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Konstantinov S.R., Smidt H., and de Vos W.M. Representative difference analysis and real-time PCR for strain-specific quantification of *Lactobacillus sobrius* sp. nov. *submitted for publication*.

Konstantinov S. R., Ajay A.A., Williams B. A., Miller B. G., Johns P., Stokes C. R., Akkermans A.D.L., Smidt H., and de Vos W. M., Postnatal development of the porcine microbiota composition and activities: implications for the weaning process. *in peparation*.

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R. O. Ball (ed.), Proceedings of the 9th International Symposium on Digestive Physiology in Pigs., vol. 1, Banff, Canada.

Awati, A., Konstantinov S.R., Williams B.A., Akkermans A.D.L., Bosch M.W., Smidt H., and Verstegen M.W.A., 2005. Effect of substrate adaptation on the microbial fermentation and microbial composition of faecal microbiota of weaning piglets studied in vitro. Journal of the Science of Food and Agriculture, *in press*.

Casini, L., Kostantinov S.R., Coloretti F., De Filippi S., Mazzoni M., Trevisi P., and Bosi P. 2005. Relevance of immune response against resident and not resident commensal strains for the definition of strategies of probiotic supply in the diet of weaning pigs. Italian Journal of Animal Science, *in press*.

Klüß, J., Akkermans A., Konstantinov S.R., Kuhla S., Kwella M., and Souffrant W. B. 2003. The microbial community and its metabolic activities in the small intestine of weaning piglets, p. 613-618. In W. B. Souffrant, and C. C. Metges (eds.), Progress in research on energy and protein metabolism., vol. 1. Wageningen Academic Publishers, Rostock, Germany.

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Lalles, J.-P., Konstantinov S.R., Smidt H., Rothkötter H.-J., and. Stokes C. R., 2004. Recent data on physiology, microbiology and immunology of the gut of piglets around weaning. Implications for alternatives to in-feed antibiotics., Animal production in Europe: The way forward in a changing world., Proceedings of the international congress, Saint-Malo - France, October 11 - 13, 2004.

Luyer, M. D. P., Hadfoune M.h.H., Jacobs J.A., Konstantinov S.R., Dejong C.H.C., Buurman W.A., and Greve J.W.M., 2004. High-fat nutrition reduces the inflammatory responce and preserves gut barrier function in rats early aftrer hemorragic shock. SHOCK. 24:65–71.

Roselli, M., Finamore A., Britti M.S., Konstantinov S.R., Smidt H., de Vos W.M., and Mengheri E., A *Lactobacillus amylovorus*-like strain 001T from the resident porcine gastrointestinal microbiota protects pig enterocytes from enterotoxigenic *Escherichia coli* strain K88 induced damages. *submitted for publication*.

Williams, B.A., Bosch M.W., Awati A., Konstantinov S.R., Smidt H., Akkermans A. D.L., Verstegen M.W.A., and Tamminga S., 2005. *In vitro* assessment of GIT fermentation: fermentable substrates and microbial activity. Animal Research, *in press.*

Zhu, W.-Y., Williams B.A., Konstantinov S.R., Tamminga S., de Vos W.M., and Akkermans A.D.L., 2003. Analysis of 16S rDNA reveals bacterial shift during in vitro fermentation of fermentable carbohydrate using piglet faeces as inoculum Anaerobe, 9:175-180.

Training and supervision plan

Courses	**Organized**	**Year**	**Credit points** [a)]
PhD week	VLAG	2001	1
Larenstein course 'Safe handling with radioactive materials and sources (article 5B)	WUR	2001	1
English Writing	WUR	2002	1
Ecophysiology of the GI-tract	VLAG	2003	1
Career Orientation	VLAG	2005	1
Dutch Language	WUR, UVA, ROC	2001-2004	6
Conferences			
Darmendag	RIVM *110*, Lelystad, Wageningen, Utrecht	2000-2004	1
ISME	Amsterdam, Nl	2001	1
Microbial Ecology in Health and Disease	Lunteren, Nl	2001	1
Beyond Antimicrobials-future of gut microbiology	INRA-RRI, Scotland	2002	1
NVVM	Papendal, Nl	2003 and 2005	0.4
9th International Symposium on digestive physiology of pigs	Banff, Canada	2003	1
Healthypigut Workshop	Rostock, Germany	2003	0.4
Gut flora in health and disease	KNAW, Nl	2004	0.4
Beneficial microbes conferences, ASM	Lake Tahoe, Nevada, USA	2005	1
Others			
Journal club	Lab. Microbiology	2004	0.4
Seminars, Group meetings, PhD and Posdoc meetings	Lab. Microbiology	2000-2004	3
Visit to Royal Danish Lab.	-	2002	2
Visit to Bristol University	-	2003	1
EU project Healthypigut (meetings every 6 months)	EU	2001-2004	2

VLAG AIO Rad meetings	VLAG	2002-2003	1
Writing of the training and supervision PhD plan	VLAG	2001	3
Microarray Platform Meetings	UMC	2002-2003	0.4
Microarray meeting	Switzerland	2002	0.4
total score			31.4

[a)] One credit point equals a study load of approximately 40hrs.

This work has been carried out with financial support of the European Community specific RTD programme "Quality of Life and Management of Living Resources" research project: HEALTHYPIGUT (QLK5-LT2000-00522) and the Product Board Animal Feed, The Netherlands".

Printing of the thesis is partly supported by:

Nutreco Nederland BV

Akkermansia Foundation (WUR)

Winclove Bio Industries BV