Research Paper

PCR monitoring of *Lactobacillus* and *Bifidobacterium* dynamics in fermentations by piglet intestinal microbiota

Patrícia Moura[1], **Fernanda Simões**[1], **Francisco Gírio**[1], **Maria C. Loureiro-Dias**[2] **and M. Paula Esteves**[1]

[1] Instituto Nacional de Engenharia, Tecnologia e Inovação, Departamento de Biotecnologia, Lisboa, Portugal
[2] Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisboa, Portugal

A new group-specific primer (Lact71R), targeting the 16S-23S rDNA intergenic spacer region of *Lactobacillus*, was tested in its specificity to amplify rDNA of lactobacilli from piglet intestinal origin by polymerase chain reaction (PCR). Lact71R and Lab0677F, a *Lactobacillus* group-specific primer targeting the 16S rDNA, generated a common amplicon by PCR with DNA from *Lactobacillus* and *Pediococcus* reference strains, but not from *Weissella* strains. Sequence analysis of clones obtained by PCR amplification with Lact71R and Lab0677F and total DNA isolated from the ileal, caecal and colonic contents of one piglet resulted in *Lactobacillus* and *Lactobacillus*-like sequences mainly retrieved from intestinal environments. The primer pair was further validated in a culture independent PCR-analysis to monitor broad fluctuations of lactobacilli populations in fructo-oligosaccharides (FOS) fermentations by piglet intestinal microbiota. *Bifidobacterium* genus-specific primers were also used for PCR titre determination throughout FOS fermentations, in parallel with lactate and short chain fatty acids (SCFA) quantification. Increases between PCR titres were correlated with lactate detection in early stages of fermentation. Based on the obtained results, a simple monitoring PCR approach is proposed, foreseeing its application to the study of the dynamics of specific bacterial populations in complex environments.

Keywords: *Lactobacillus* / Group-specific primers / 16S-23S rDNA intergenic spacer region / Pig intestinal microbiota / Fructo-oligosaccharides

Received: June 03, 2006; returned for modification: July 20, 2006; accepted: September 11, 2006

DOI 10.1002/jobm.200610210

Introduction

The potential effects of prebiotic ingredients and probiotics on the gastro-intestinal (GI) tract led to an increasing interest in characterising the intestinal microbiota and in monitoring diet-dependent bacterial shifts induced by the consumption of such compounds in humans (Blaut *et al.* 2002, Tannock *et al*. 2004) and animals (Leser *et al.* 2002, Houdijk *et al*. 2002, Mikkelsen and Jensen 2004). This interest is particularly relevant for bacterial groups which are generally assigned to intestinal health and thus considered as beneficial organisms from the intestinal microbiota, such as members of the genera *Bifidobacterium* and *Lactobacillus* (Matsuki *et al*. 2004, Simpson *et al.* 2000, Ohashi *et al*. 2004, Konstantinov *et al*. 2004, Tannock *et al*. 2000).

Conventional microbiology methods appear to be inadequate for extensive characterisation purposes of the intestinal microbiota due to the large diversity of microorganisms, which is difficult to assess in laborious and time-consuming culture based techniques. Alternatively, molecular-based techniques, in particular culture-independent PCR analysis, allow the fast, easy and highly sensitive detection of specific members of such complex communities (Walter *et al.* 2000, Matsuki *et al*. 2004). Particularly, the 16S rRNA gene constitutes a well-established standard method for the identification of bacterial species and genera, constituting an adequate region for specific primer targeting (Gürtler and Stanisich 1996). *Lactobacillus* and *Bifidobacterium*

Correspondence: M. Paula Esteves, INETI, Departamento de Biotecnologia, Unidade de Fisiologia Microbiana e Bioprocessos, Estrada do Paço do Lumiar, 22, 1649-038 Lisboa, Portugal.
E-mail: paula.esteves@ineti.pt

species-specific oligonucleotide primers, whose discriminatory power is based on targeting variable regions of the 16S rDNA sequence, have been used in the characterisation of human and animal intestinal microbiota (Walter *et al.* 2000, Song *et al*. 2000, Kwon *et al*. 2004). However, due to the extreme complexity of the intestinal ecosystem, the use of genus- and/or group-specific primers is advantageous when the objective is the global monitoring of changes in major bacterial genera and/or groups induced by specific substrates (Possemiers *et al.* 2004). Detection using genus- and group-specific can also be regarded as a first approach to screen broad variations in specific bacterial populations before further discriminative analysis (Ziemer *et al.* 2004).

The overall conservative nature of the 16S rRNA gene tends to limit the discriminatory power of 16S rDNA-based genus-specific primers among lactic acid bacteria (LAB) (Charteris *et al.* 1997). Conversely, the sequence information of the 16S-23S rDNA intergenic spacer region (ISR) exhibits considerable inter-species and intra-species variation, in both length and sequence (Gürtler and Stanisich 1996, Nour 1998). Gürtler and Stanisich (1996) suggested that homologous sequences based on short regions of the 16S-23S ISR might expose inter-species homologies, which would make them appropriate for grouping microorganisms at the genus level and simultaneously distinguish these from closely related bacteria. Based on the 16S-23S rDNA ISR, a lactobacilli genus-specific primer has been proposed (Dubernet *et al.* 2002), even though its specificity has not been evaluated in culture independent PCR analysis using samples from intestinal environments.

The first aim of this study was to design a *Lactobacillus* group-specific primer targeting the 16S-23S rDNA ISR capable of grouping members of the *Lactobacillus* genus and simultaneously narrowing the actual range of related LAB coamplified by PCR. The second objective was to demonstrate the validity of this primer when applied to samples from porcine intestinal microbiota in a culture independent PCR detection procedure. Furthermore, *Lactobacillus* and *Bifidobacterium* populations were PCR monitored throughout FOS fermentations by the piglet intestinal microbiota, with simultaneous analysis of lactate and short chain fatty acids (SCFA) production.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Bacteroides*, *Lactobacillus* and *Bifidobacterium* strains were grown anaerobically at 37 °C, under static conditions, in PYG-medium (Medium 104, DSMZ), MRS broth (Panreac, Barcelona, Spain) and *Bifidobacterium* medium (Medium 58, DSMZ), respectively. *Pediococcus* and *Weissella* strains were cultured aerobically in MRS broth at 30 °C, and *Escherichia coli* HB101 in Luria-Bertani broth (Duchefa, Harleem, The Netherlands) at 37 °C. *Enterococcus* and *Streptococcus* strains were cultured in Brain Heart Infusion broth (Oxoid, Hampshire, England) at 37 °C, while *Enterobacter cloacae* was grown on Tryptone Soya agar (Oxoid, Hampshire, England) at 30 °C.

Table 1. Bacterial strains assayed by PCR with the primer pair Lab0677F and Lact71R. The formation of specific 950-bp PCR products with 35 amplification cycles and an annealing temperature of 50 °C is indicated by + and the absence by –.

Bacterial strains	Strain Nº	PCR product
Lactobacillus acidophilus	DSM 20079[a]	+
L. brevis	DSM 20054[a]	+
L. delbrueckii	ATCC 7830[b]	+
L. farciminis	Food isolate[c]	+
L. fermentum	ATCC 9338[b]	+
L. gasseri	DSM 20243[a]	+
L. plantarum	ATCC 8014[b]	+
L. reuteri	DSM 20016[a]	+
L. sakei	CECT 980[d]	+
L. salivarius	ATCC 11741[b]	+
L. rhamnosus	ATCC 7469[b]	+
L. ruminis	DSM 20403[a]	+
Bifidobacterium adolescentis	DSM 20083[a]	–
Bif. catenulatum	DSM 20103[a]	–
Bif. infantis	DSM 20088[a]	–
Bif. longum	DSM 20097[a]	–
Bacteroides vulgatus	DSM 1447[a]	–
B. thetaiotaomicron	DSM 2079[a]	–
Enterobacter cloacae	Clinical isolate[e]	–
Enterococcus hirae	ATCC 8043[b]	–
E. faecalis	Clinical isolate[e]	–
E. faecium	NCIMB 8842[b]	–
E. faecium	ATCC 6569[b]	–
Escherichia coli	HB101[f]	–
Pediococcus acidilactici	ATCC 8081[b]	+
P. damnosus	DSM 20331[a]	+
P. inopinatus	DSM 20285[a]	+
Streptococcus pyogenes	NCIMB 11841[b]	–
Weissella confusa	DSM 20196[a]	–
W. paramesenteroides	DSM 20288[a]	–
W. viridescens	DSM 20410	–

[a] German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany.
[b] purchased from the Industrial Microorganisms Culture Collection (CCMI) of INETI, Lisboa, Portugal.
[c] purchased from Área de Nutrición y Bromatología, Dep. de Zootecnia, University of Extremadura, Spain.
[d] Spanish Type Culture Collection, Valencia, Spain.
[e] purchased from Instituto Superior de Saúde Egas Moniz, Monte de Caparica, Portugal.
[f] purchased from Promega, Mannheim, Germany.

Collection of intestinal samples

Intestinal samples were obtained from one 34-day-old Duroc × Landrace male piglet, fed with a commercial weaning diet based on barley and soybean meal. The animal was sacrificed after a 16 h fasting period, for removal of the entire gastrointestinal tract. Samples from the luminal contents of the terminal ileum (ca. 20 cm before the ileal-caecal valve), from the caecum and the distal colon (ca. 1 m away from the caecum) were collected into sterile vials. All the vials were kept refrigerated until arrival at the laboratory, for a period not exceeding 2 h after collection. The intestinal inocula for the fermentation assays were prepared immediately and aliquots were stored at −70 °C.

Fermentation medium

The nutrient base medium used in the fermentation experiments was modified from Jaskari *et al.* (1998): trypticase soya broth (TSB) without dextrose 5.0 g l^{-1}, bactopeptone 5.0 g l^{-1}, yeast nitrogen base (YNB) 5.0 g l^{-1} (prepared separately, as described below), cysteine hydrochloride 0.5 g l^{-1}, 1.0% (v/v) of salt solution A (NH_4Cl 100.0 g l^{-1}, $MgCl_2 \cdot 6\ H_2O$ 10.0 g l^{-1}, $CaCl_2 \cdot 2\ H_2O$ 10.0 g l^{-1}) and trace minerals solution, 0.2% (v/v) of salt solution B ($K_2HPO_4 \cdot 3\ H_2O$ 200.0 g l^{-1}) and 0.2% (v/v) of resazurin solution (0.5 g l^{-1}). The medium was deoxygenated with replacement of the gas phase by 80% nitrogen and 20% carbon dioxide using a gassing manifold system. Nine ml aliquots were dispensed into airtight anaerobic culture tubes (Bellco Biotechnology Inc., Vineland, USA), which were capped with butyl rubber stoppers and sealed with aluminium caps (Bellco Biotechnology Inc.) before autoclave sterilisation. Stock solutions (250 g l^{-1}) of YNB (Difco, Detroit, Michigan, USA) and FOS (Raftilose®P95, Orafti S.A., Belgium) were filter-sterilised (0.2 µm, Chromafil®, Düren, Germany) into airtight 100-ml serum bottles. These solutions were made anaerobic by repeated replacement of the inside gas phase for N_2. Before inoculation, YNB (5 g l^{-1}) and FOS (10 g l^{-1}) were distributed into the anaerobic culture tubes.

Fermentation experiment

Inocula for the fermentation experiments were prepared as follows: 1.0 mL of ileal content was diluted in 9 ml of reduced physiological saline (RPS), pH 6.8 (Hartemink 1999), and 3.0 g of caecal and colon contents were 100-fold diluted in RPS. Tubes containing the fermentation medium were inoculated in triplicate for each combination of strain/fermentation time (0, 11.5, 30.0 and 72.0 h) with 200 µl of each intestinal dilution and incubated at 37 °C for 72 h, under static conditions. At each predetermined time, the anaerobic culture tubes were opened and cells were harvested by centrifugation at 6.000 rpm, for 5 min. Supernatants were filtered through 0.2 µm pore size (Chromafil®, Düren, Germany) for HPLC analysis of the main fermentation products (lactic, acetic, propionic and butyric acids) against external standards. The HPLC system (Waters, Milford, USA) was equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA), a cation H^+-guard column (Bio-Rad) and a refractive index (RI) detector (Waters 2410). Elution took place at 50 °C with 5 mM H_2SO_4.

DNA isolation

NucleoSpin Tissue Kit (Mackerey-Nagel, Düren, Germany) was used to isolate DNA from overnight grown cultures of pure bacterial strains and from cells of the fermentation experiments. QIAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to isolate DNA from intestinal aliquots stored at −70 °C, being the cell lysis carried out at 95 °C.

Primers and PCR conditions

ClustalW (Thompson *et al.* 1994) (http://www.ebi.ac.uk/clustalw) was used to perform multiple sequence alignments of *Lactobacillus* 16S-23S rDNA ISR sequences retrieved from GenBank (www.ncbi.nlm.nih.gov). A potential PCR target site was chosen for designing a 20 mer primer, Lact71R (Table 2). This primer was used in PCR reactions with Lab0677F (Table 2), the reverse complementary sequence of S-G-Lab-0677-a-A-17 (Heilig *et al.* 2002). Amplification reactions with reference strains were performed in a total volume of 25 µl containing 10 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, 1.0 U of *Taq* DNA Polymerase (MBI Fermentas, St.Leon-Rot, Germany) on a MJResearch PTC-200 thermal cycler (MJ Research Inc., Waltham, USA) according to the following cycling program: an initial denaturation step at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, and a final extension cycle of 72 °C for 10 min. An iCycler thermal cycler (BioRad, Hercules, USA) was used for optimising the PCR procedure with FOS fermentation samples. The optimised procedure comprised 40 amplification cycles, an annealing temperature of 54 °C and PCR conditions as described above, except that 3.0 mM $MgCl_2$ and 1× PCR buffer with $(NH_4)_2SO_4$ were used. Total DNA isolated from one fermentation tube correspondent to each predetermined time was serially diluted starting from 10 ng (correspondent to 1/1 dil.), to be used as template in PCR reactions. *Bifidobacterium* genus-specific primers were used according to Matsuki *et al.* (2002). Values of PCR titres were determined in

duplicates of independent PCR reactions. Final values corresponded to the highest DNA dilution which produced two positive results. Electrophoresis was performed for one hour at 90 V, on 1% agarose gels in 1× TAE buffer in a Midicell® Primo EC-330 electrophoresis apparatus (Holbrook, New York). A fixed volume of sample (8 μl) was loaded in each lane. PCR products were visualised using ethidium bromide staining.

Cloning and sequencing

PCR products from the intestinal samples were cloned using InsT/Aclone PCR Product Cloning kit (MBI Fermentas, St. Leon-Rot, Germany). Plasmids from transformants were purified with the QIAprep spin miniprep kit (QIAgen, Hilden, Germany), insertion was confirmed by EcoRI/HindIII total digestion and 1% agarose gel electrophoresis analysis. Thirty-nine purified plasmids were partially sequenced in an ABI Prism 310 DNA Sequencer (PE Applied Biosystems, Weiterstadt, Germany). The sequences, corresponding to *E. coli* 16 rDNA positions 677 to 1047, were compared with GenBank nucleotide sequences database using BLAST algorithm to determine the percentage of identity with the most similar sequences (Altschul *et al.* 1990).

Nucleotide sequence accession numbers

The thirty-nine 16S rDNA sequences were deposited in GenBank database under accession numbers from DQ445052 to DQ445090.

Results and discussion

Group-specific primers for *Lactobacillus*

In order to design a primer that would specifically hybridise with members of the *Lactobacillus* genus, potential PCR binding sites were identified based on the highest homology regions within alignments made with 16S-23S rDNA ISR sequences of lactobacilli. The reverse primer Lact71R (Table 2), targeting *L. acidophilus* ATCC 4356^T positions 52-71 of the 16S-23S rDNA ISR sequence was designed. The potential target site of the primer is a conserved region among lactic acid bacteria (Tilsala-Timisjärvi and Alatossava 1997), flanking the end of the 16S rRNA gene sequence, and located upstream to tRNA genes eventually present in the 16S-23S rDNA ISR of the *rrn* operons of lactobacilli (Gürtler and Stanisich 1996, Nour 1998, Massi *et al*. 2004).

Primer Lact71R was paired with Lab0677F (Heilig *et al.* 2002) and the specificity of the primer pair was evaluated using DNA from reference strains (Table 1). Amplification reactions produced a common 950-bp PCR product that was obtained for all the *Lactobacillus* reference strains tested. Among related lactic acid bacteria tested, only *P. acidilactici*, *P. damnosus* and *P. inopinatus* produced a PCR product comparable in terms of size and intensity to the amplicons obtained with *Lactobacillus* strains. In accordance with the probable occurrence of mismatches within the sequence targeted by Lact71R, PCR amplification with DNA from *L. brevis*, *L. fermentum* and *L. salivarius* resulted in a slightly fainter 950-bp amplicon, while using *Weissella* DNA no amplification was detected (Table 1).

The specificity of Lab0677F and Lact71R was further evaluated when applied to the analysis of complex microbial communities in a culture independent PCR protocol. Total DNA was isolated from the ileal, caecal and colonic contents of one 34-day-old piglet and used for PCR amplification with Lab0677F and Lact71R. Amplicons obtained consisted in one major 950-bp product, in accordance with the fragment size obtained with *Lactobacillus* reference strains. To confirm the correlation between a positive PCR signal and the presence of bacteria belonging to the *Lactobacillus* genus, randomly chosen PCR products were cloned in *E. coli*. A total of 39 clones were used for sequencing and comparing with sequences held in GenBank, using the BLAST algorithm. The highest identity percentages obtained corresponded to *Lactobacillus* reference strains or to uncultured bacterial clones disclosed in studies on microbiota diversity from animal intestinal origin, from the cloaca and vagina of laying hens, from the rumen, and from foodstuff origin (Table 3). Sequences from known *Lactobacillus* strains were always present in the alignments showing higher homology scores with unknown bacterial clones, suggesting a correspondence

Table 2. Primers used in this study.

Primer	Primer sequence (5′ to 3′)	Targeted rDNA		Source or reference
		position	region	
Lab0677F	CTCCATGTGTAGCGGTG	677–693[a]	16S rDNA	Heilig *et al*. (2002)
Lact71R	TCAAAACTAAACAAAGTTTC	52–71[b]	16S-23S rDNA ISR	This work

[a] according to the 16S rDNA of E. coli.
[b] according to the 16S-23S rDNA intergenic spacer region (ISR) sequence of *L. acidophilus* ATCC 4356^T (Accession N°. U32971).

with *Lactobacillus*-like sequences. The most abundant *Lactobacillus* phylotypes found in the GI tract of pigs are *L. amylovorus*-, *L. johnsonii*- and *L. reuteri*-like phylotypes (Leser *et al*. 2002, Pryde *et al*. 1999, Konstantinov *et al*. 2004). Although the number of clones analysed in this study was limited and originated from a single piglet, *L. johnsonii*- and *L. reuteri*-like sequences were well represented among the 39 clones. *L. kitasatonis*-like sequences were also retrieved from all the three intestinal contents and *L. ruminis*-like sequences were exclusively obtained from the colon (Table 3). *L. kitasatonis* had firstly been isolated from chicken small intestine (Mukai *et al*. 2003), while strains of *L. ruminis* had already been retrieved from pig intestinal contents in a culture-dependent study of the *Lactobacillus* populations from the GI tract of adult pigs performed under strictly anaerobic conditions (Yin and Zheng 2005).

Monitoring fermentations with group- and genus-specific primers

The primer pair Lab0677F and Lact71R, together with bifidobacteria genus-specific primers from the literature (Matsuki *et al*. 2004), were used for PCR detection in *in vitro* fermentations of FOS, a prebiotic oligosaccharide. The luminal contents from the ileum, caecum and colon of one piglet were used as inocula. The aim was to monitor changes in the PCR titres along the fermentation.

Table 3. Percentage of identity (% ID), GenBank accession number and origin of the closest relative sequences held in GenBank of clones retrieved from the piglet ileal, caecal and colonic contents by using the primer pair Lab0677F and Lact71R. In the BLAST results that corresponded to unknown sequences, the identity of the closest related known sequence is indicated immediately below, between parentheses.

Intestinal content	Nº of clones	Blast result	Accession Nº	Origin	Clone identification and % ID
Ileum	3	*L. kitasatonis*	AY841164	Pig faeces	SP147IL (99%), SP160IL (100%), SP161IL (99%)
	1	*L. johnsonii*	AJ853329	Cloaca and vagina of laying hens	SP127IL (99%)
Caecum	4	*Lactobacillus* sp.	AJ853342	Cloaca and vagina of laying hens	SP117CA (98%), SP128CA (99%), SP163CA (98%), SP185CA (99%)
	3	*L. kitasatonis*	AY841164	Pig faeces	SP124CA (100%), SP138CA (100%), SP178CA (99%)
	2	*L. johnsonii*	AJ853329	Cloaca and vagina of laying hens	SP112CA (100%), SP136CA (100%)
	2	*L. pontis*	AJ422032	Foodstuff origin	SP115CA (99%), SP206CA (99%)
	2	*L. reuteri*	AY845204	Pig faeces	SP123CA (99%), SP139CA (100%)
	1	Uncultured bacterium clone (*L. johnsonii*; AJ853329)	AY994039	Murine intestine	SP137CA (100%)
Colon	5	Uncultured bacterium clone (*L. ruminus*; M58828)	AF371499	Pig GI tract	SP98COL (100%), SP101COL (100%), SP119COL (99%), SP120COL (100%), SP129COL (99%)
	4	*L. johnsonii*	AJ853329	Cloaca and vagina of laying hens	SP114COL (100%), SP140COL (99%), SP143COL (100%), SP159COL (100%)
	3	*Lactobacillus* sp.	AJ853342	Cloaca and vagina of laying hens	SP153COL (99%), SP165COL (99%), SP189COL (99%)
	3	*L. reuteri*	AY845204	Pig faeces	SP113COL (99%), SP142COL (100%), SP203COL (99%)
	2	*L. pontis*	AJ422032	Foodstuff origin	SP131COL (100%), SP154COL (99%)
	1	*L. kitasatonis*	AY841164	Pig faeces	SP93COL (100%)
	1	*Lactobacillus* sp.	AY445129	Equine GI tract	SP92COL (100%)
	1	Uncultured rumen bacterium (*L. ruminus*; M58828)	AB034090	Rumen	SP99COL (99%)
	1	Uncultured bacterium clone (*L. salivarius*; AJ853341)	AF371497	Pig GI tract	SP188COL (99%)

The amount of rDNA targeted sequences in a sample varies directly with the copy numbers of the *rrn* operon, which depends on the overall number of targeted cells and the species present (von Wintzingerode *et al.* 1997). The maximum dilution of total DNA still capable of producing a positive PCR amplicon (PCR titre) can be determined by serially diluting the total DNA isolated at each fermentation time. Assuming that variations in PCR titres correspond to different proportions of rDNA sequences in total DNA, it should be possible to screen fluctuations of targeted bacterial groups along fermentations (Wang *et al*. 1996). An increase in the PCR titre reflects a higher proportion of targeted template rDNA in total DNA. This higher proportion is related with an increase in cell number and genomic replication throughout the fermentation. On the other hand, a decrease in PCR titre suggests a lower proportion of targeted template rDNA in total DNA. However, such a decrease observed throughout fermentation cannot be directly ascribed to cell death since dead bacterial cells can also be detected by PCR (Satokari *et al*. 2003). A decrease in PCR titre should result from an increase in the number of non-targeted sequences, which exerts a "dilution effect" on the targeted DNA towards unspecific DNA.

Major increases in the PCR titres with both primer sets (Lab0677F + Lact71R and Bif.F + Bif.R) were detected from 0 up to 11.5 h, but considerable differences were observed (Fig. 1). The expected 950-bp amplicon obtained by PCR with the primer pair Lab0677F and Lact71R was present in all the samples, from 0 to 72 h. With this primer set, no remarkable increase was observed in the PCR titres from the ileal fermentations, whilst in the caecal and colonic fermentations the PCR titres increased 100- and 10-times, respectively, from 0 to 11.5 h. The PCR titres remained high and not drastically different in subsequent fermentation times. This feature suggests that the bacterial groups targeted by Lab0677F and Lact71R were able to maintain a sufficient replication capability to assure a representative proportion of sequences in total DNA throughout the fermentation. In fact, lactobacilli constitute a predominant group of microorganisms inhabiting the porcine GI tract (Yin and Zheng 2005).

Conversely, bifidobacteria were not detected in any sample at the initial time (t_0) (Fig. 1). Since detection of bifidobacteria was possible in the fermentation samples at different times from t_0, it may be assumed that the bifidobacterial template was present in t_0 samples in a concentration below the detection limit of the primers (10^6 cells/g faeces) (Matsuki *et al*. 2004). In studies aimed at profiling the pig intestinal microbiota community,

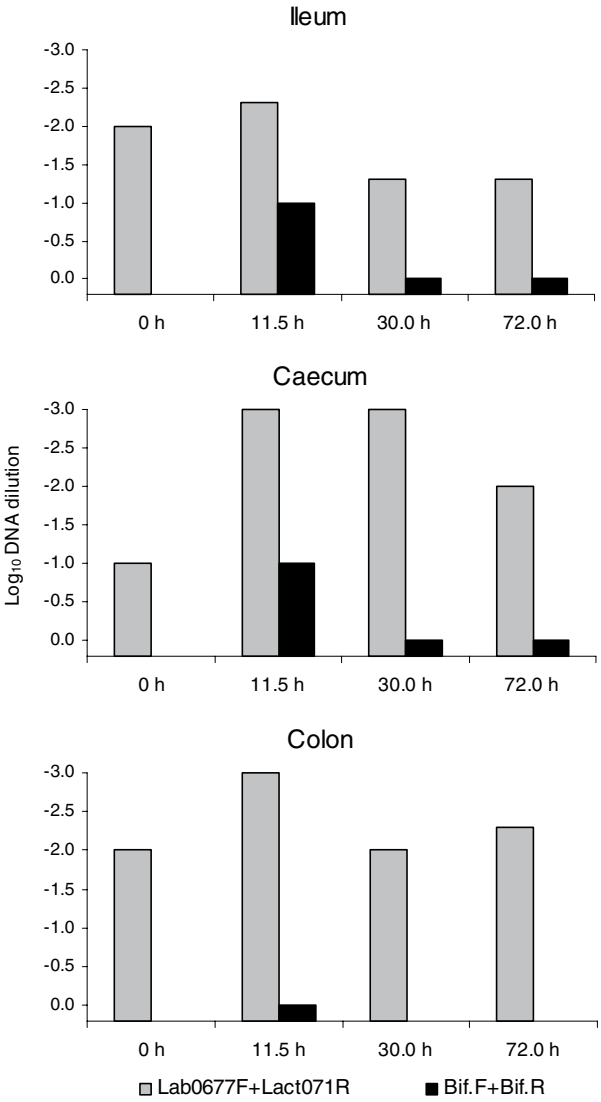

Figure 1. Log_{10} values of the maximal dilutions of total DNA (isolated at 0, 11.5, 30.0 and 72.0 h from FOS fermentation trials by the microbiota of the piglet ileum, caecum or colon) capable of producing an expected amplicon by PCR using the primer pairs Lab0677F + Lact71R or Bif.F + Bif.R. Values were determined in duplicate from independent positive PCR reactions.

sequences similar to bifidobacteria were only infrequently retrieved (Hill *et al.* 2002, Leser *et al.* 2002). In a culture-based study with bifidobacterial isolates, it was found that the population of bifidobacteria represented a minor proportion of less than 1% of the intestinal microbiota in suckling pigs (Mikkelsen *et al.* 2003). References to the recovery of *Bifidobacterium* strains from the pig GI tract are generally obtained after isolation in selective media and/or after diet-dependent shifts that affect positively the growth of bifidobacteria in specific intestinal compartments of piglet's gut (Simpson *et al*.

2003, Shim *et al*. 2005). In our study, major increases in PCR titres with Bif.F and Bif.R were obtained with the ileal and caecal inocula up to 11.5 h, reflecting the capability of bifidobacteria from the piglet foregut to replicate in FOS-containing media. In *in vivo* assays, FOS were described as exerting a particular effect proximally in the pig intestinal tract, being almost entirely fermented pre-caecally (Houdijk *et al*. 1999). Additionally, diets supplemented with Neosugar, a prebiotic FOS-based mixture, increased the number of bifidobacteria in the ileum of suckling pigs (Shim *et al*. 2005). More distally in the pig gut, bacterial growth is more extensive and its diversity increases (Hill *et al*. 2002). Accordingly, PCR detection using Bif.F and Bif.R in fermentations with the inocula from the colon was only possible at 11.5 h, suggesting a quick underrepresentation of bifidobacterial DNA towards unspecific DNA. Although the most marked variations in PCR titres were observed for bifidobacteria, the time to achieve the highest PCR titres with Bif.F and Bif.R, and with Lab0677F and Lact71R, was the same for the three inocula (up to 11.5 h). We considered this period as a first stage of the fermentation, where the populations targeted by the group- and genus-specific primers exhibit their capability to replicate rapidly in FOS-containing media. In a second stage, after 11.5 h, all bifidobacteria PCR titres decreased, as well as lactobacilli PCR titres in the ileal and colonic fermentations. This decrease is illustrated in Fig. 2.

The culture independent PCR detection procedure was further validated with the quantification of lactate and short chain fatty acids (SCFA) throughout FOS fermentations. Lactate was mainly detected up to the first 11.5 h and remained measurable up to 30.0 h only in fermentations with the ileal inocula (Fig. 3). The maximum amount (2.2 ± 0.1 mmol l^{-1}) and the highest rate (0.19 mmol l^{-1} h^{-1}) of lactate accumulation were obtained with the caecal inocula up to 11.5 h. This was coincident with the most pronounced increase in PCR titre of both bifidobacteria and lactobacilli (Fig. 1). After 11.5 h, acetate and especially butyrate increased strongly in the media, suggesting replication of other intestinal bacteria, not targeted by Lab0677F and Lact71R or Bif.F and Bif.R. As an intermediate product of the global fermentation process, lactate can be metabolised to SCFA to varying extends, by cross-feeding of lactate-utilising species commonly found in the pig intestinal tract (Macfarlane and Macfarlane 2003, Duncan *et al*. 2004). The concentration of lactate in the media should result from the balance between production and consumption throughout the fermentation. Besides detection of death bacterial cells by PCR, this balance explains divergences between no lactate detection and the still positive PCR amplification. The concentration of lactate in the media at 11.5 h ranged from 1.3 mmol l^{-1} to 2.2 mmol l^{-1} in the fermentations with the ileal and caecal microbiota, respectively. In the second stage of fermentation, the high SCFA production at 30.0 h, rang-

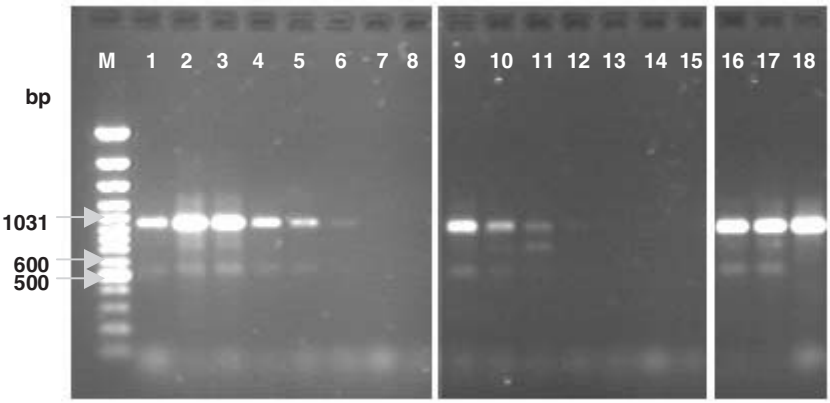

Figure 2. Agarose gels showing PCR products obtained with primer pair Lab0677F + Lact71R and serial dilutions of total DNA isolated at 0, 11.5 and 72.0 h from FOS fermentation trials by the the microbiota of the piglet ileum.

Lanes	DNA sample or dilution and respective fermentation time
lane M	DNA size marker (sizes are shown on the left)
lane 1	no dil., from t_0
lanes 2 – 8	1/1 dil., 1/2 dil., 1/10 dil., 1/20 dil., 1/100 dil., 1/200 dil. and 1/1000 dil., from 11.5 h
lanes 9 – 15	1/1 dil., 1/2 dil., 1/10 dil., 1/20 dil., 1/100 dil., 1/200 dil. and 1/1000 dil., from 72.0 h
lane 16	1/1 dil., from 11.5 h + 10 ng of DNA from *L. casei* (positive control)
lane 17	1/1 dil., from 72.0 h + 10 ng of DNA from *L. casei* (positive control)
lane 18	10 ng of DNA from *L. casei* (positive control)

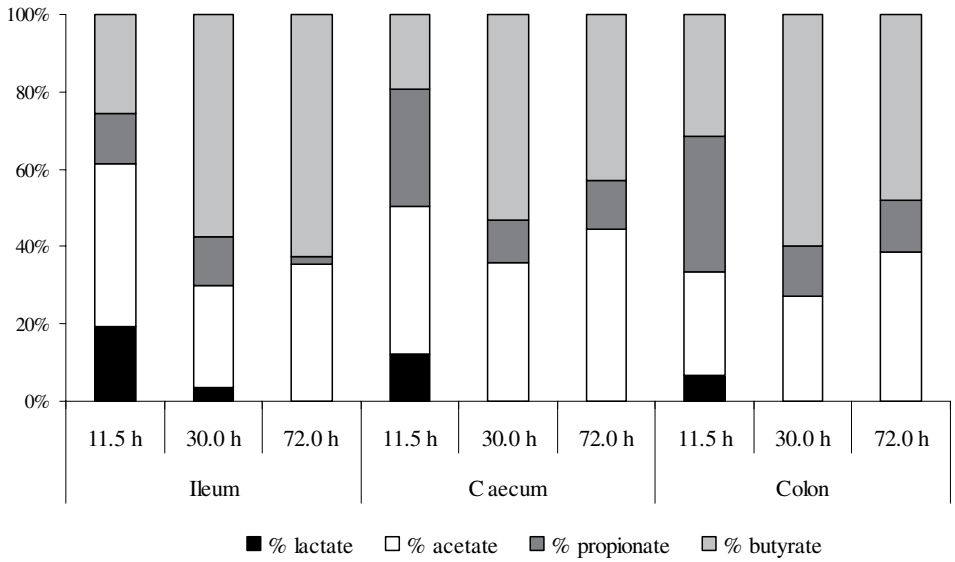

Figure 3. Lactate, acetate, propionate and butyrate production (% relative concentration) at 11.5, 30.0 and 72.0 h of FOS fermentation by the microbiota of the piglet ileum, caecum or colon.

ing from 49.8 mmol l^{-1} to 53.7 mmol l^{-1} with the ileal and caecal microbiota, respectively, certainly conduced to lactate decline and/or depletion. In *in vitro* fermentation studies with pig intestinal microbiota, lactate is seldom detected or only detected at low levels even in short fermentation periods (Smiricky-Tjardes *et al*. 2003). Although the piglet intestinal tract harbours a multiplicity of lactate producing bacteria besides bifidobacteria and lactobacilli, (Pryde *et al*. 1999, Duncan *et al*. 2004), the correlation between increases in PCR titres and lactate accumulation provides a strong evidence that bifidobacteria (targeted by Bif.F and Bif.R) and members of *Lactobacillus* and *Pediococcus* genera (targeted by Lab0677F and Lac071R) take part in the first stage of FOS fermentation. This constitutes an important trait when applied to *in vivo* trials, due to bifidobacteria and lactobacilli capacity to decrease the pH on the gut and to excrete natural antibiotics, making competition of microbial pathogens more difficult within intestinal microbiota at an early stage of oligosaccharides fermentation (Manning and Gibson 2004).

Conclusions

The primers used in this study made possible to implement a simple PCR-based approach for monitoring lactobacilli and bifidobacteria fluctuations in fermentations by piglet intestinal microbiota. The primer pair Lab0677F and Lac071R was validated for the specific detection of *Lactobacillus* and *Pediococcus* in a culture independent PCR procedure with samples from the porcine GI tract. In FOS fermentations by piglet intestinal microbiota, the increases in PCR titres produced by specific amplification using Lab0677F and Lac071R or Bif.F and Bif.R were in good agreement with fermentation metabolite data, as revealed by quantification of lactate and SCFA. The PCR-based strategy developed should be applicable to more extensive studies on bacterial groups' dynamics either on natural or defined complex environments.

Acknowledgements

The authors are grateful to the Commission of the European Communities and the FEDER Funds for financial support of this work (Project POCTI/CVT/37545/2002). We would also like to thank Diogo Mendonça for his technological support in sequencing, Professor Bengala Freire for providing the piglet intestinal samples, and Ana Lança, Ricardo Pires, Dr. Isabel Fernandes and Dr. F. Pérez Nevado for providing us several bacterial strains.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol., **215**, 403–410.

Blaut, M., Collins, M.D., Welling, G.W., Dore, J., van Loo, J. and de Vos, W., 2002. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. Brit. J. Nutr., **87**, S203–S211.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K., 1997. Selective detection, enumeration and identification of po-

tentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol., **35**, 1–27.

Dubernet, S., Desmasures, N. and Gueguen, M., 2002. A PCR-based method for identification of lactobacilli at the genus level. FEMS Microbiol. Lett., **214**, 271–275.

Duncan, S.H., Louis, P. and Flint, H.J., 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl. Environ. Microb., **70**, 5810–5817.

Gürtler, V. and Stanisich, V.A., 1996. New approaches to typing and identification of bacteria using the 16S- 23S rDNA spacer region. Microbiology, **142**, 3–16.

Hartemink, R., 1999. Prebiotic effects of non-digestible oligo- and polysaccharides. PhD Thesis, Wageningen Agricultural University, the Netherlands.

Heilig, H.G.H.J., Zoetendal, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D.L. and de Vos, W.M., 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microb., **68**, 114–123.

Hill, J.E., Seipp, R.P., Betts, M., Hawkins, L., van Kessel, A.G., Crosby, W.L. and Hemmingsen, S.M., 2002. Extensive profiling of a complex microbial community by high-throughput sequencing. Appl. Environ. Microb., **68**, 3055–3066.

Houdijk, J.G.M., Bosch, M.W., Tamminga, S., Verstegen, M.W.A., Berenpas, E.B. and Knoop, H., 1999. Apparent ileal and total-tract nutrient digestion by pigs as affected by dietary nondigestible oligosaccharides. J. Animal Sci., **77**, 148–158.

Houdijk, J.G.M., Hartemink, R., Verstegen, M.W.A. and Bosch, M.W., 2002. Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. Arch. Animal Nutrition – Arch. Tierernährung, **56**, 297–307.

Jaskari, J., Kontula, P., Siitonen, A., Jousimies-Somer, H., Mattila-Sandholm, T. and Poutanen, K., 1998. Oat beta-glucan and xylan hydrolysates as selective substrates for *Bifidobacterium* and *Lactobacillus* strains. Appl. Microbiol. Biot., **49**, 175–181.

Konstantinov, S.R., Awati, A., Smidt, H., Williams, B.A., Akkermans, A.D.L. and de Vos, W.A., 2004. Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the guts of weaning piglets. Appl. Environ. Microb., **70**, 3821–3830.

Kwon, H.S., Yang, E.H., Yeon, S.W., Kang, B.H. and Kim, T.Y., 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol. Lett., **239**, 267–275.

Leser, T.D., Amenuvor, J.Z., Jensen, T.K., Lindecrona, R.H., Boye, M. and Moller, K., 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microb., **68**, 673–690.

MacFarlane, S. and MacFarlane, G.T., 2003. Regulation of short-chain fatty acid production. Proc. Nutr. Soc., **62**, 67–72.

Manning, T.S. and Gibson, G.R., 2004. Prebiotics. Best. Pract. Res. Cl. Ga., **18**, 287–298.

Massi, M., Vitali, B., Federici, F., Matteuzzi, D. and Brigidi, P., 2004. Identification method based on PCR combined with automated ribotyping for tracking probiotic *Lactobacillus* strains colonizing the human gut and vagina. J. Appl. Microbiol., **96**, 777–786.

Matsuki, T., Watanabe, K., Fujimoto, J., Kado, Y., Takada, T., Matsumoto, K. and Tanaka, R., 2004. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl. Environ. Microb., **70**, 167–173.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H. and Tanaka, R., 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microb., **68**, 5445–5451.

Millelsen, L.L., Bendixen, C., Jakobsen, M. and Jensen, B.B., 2003. Enumeration of bifidobacteria in gastrointestinal samples from piglets. Appl. Environ. Microb., **69**, 654–658.

Milleösen, L.L. and Jensen, B.B., 2004. Effect of fructo-oligosaccharides and transgalacto-oligosaccharides on microbial populations and microbial activity in the gastrointestinal tract of piglets post-weaning. Anim. Feed Sci. Tech., **117**, 107–119.

Mukai, T., Arihara, K., Ikeda, A., Nomura, K., Suzuki, F. and Ohori, H., 2003. *Lactobacillus kitasatonis* sp nov., from chicken intestine. Int. J. Syst. Evol. Microbiol., **53**, 2055–2059.

Nour, M., 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res. Microbiol., **149**, 433–448.

Ohashi, Y., Umesaki, Y. and Ushida, K., 2004. Transition of the probiotic bacteria, *Lactobacillus casei* strain Shirota, in the gastrointestinal tract of a pig. Int. J. Food Microbiol., **96**, 61–66.

Possemiers, S., Verthe, K., Uyttendaele, S. and Verstraete, W., 2004. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol. Ecol., **49**, 495–507.

Pryde, S.E., Richardson, A.J., Stewart, C.S. and Flint, H.J., 1999. Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. Appl. Environ. Microb., **65**, 5372–5377.

Satokari, R.M., Vaughan, E.E., Smidt, H., Saarela, M., Matto, J. and de Vos, W.M., 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. Syst. Appl. Microbiol., **26**, 572–584.

Shim, S.B., Verstegen, M.W.A., Kim, I.H., Kwon, O.S. and Verdonk, J.M.A.J., 2005. Effects of feeding antibiotic-free creep feed supplemented with oligofructose, probiotics or synbiotics to suckling piglets increases the preweaning weight gain and composition of intestinal microbiota. Arch. Anim. Nutr., **59**, 419–427.

Simpson, J.M., McCracken, V.J., Gaskins, H.R. and Mackie, R.I., 2000. Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microb., **66**, 4705–4714.

Simpson, P.J., Stanton, C., Fitzgerald, G.F. and Ross, R.P. 2003. Genomic diversity and relatedness of bifidobacteria isolated from a porcine cecum. J. Bacteriol., **185**, 2571–2581.

Smiricky-Tjardes, M.R., Flickinger, E.A., Grieshop, C.M., Bauer, L.L., Murphy, M.R. and Fahey, G.C., 2003. *In vitro* fermentation characteristics of selected oligosaccharides by swine fecal microflora. J. Animal Sci., **81**, 2505–2514.

Song, Y.L., Kato, N., Liu, C.X., Matsumiya, Y., Kato, H. and Watanabe, K., 2000. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol. Letters, **187**, 167–173.

Tannock, G.W., Munro, K., Bibiloni, R., Simon, M.A., Hargreaves, P., Gopal, P., Harmsen, H. and Welling, G., 2004. Impact of consumption of oligosaccharide-containing biscuits on the fecal microbiota of humans. Appl. Environ. Microb., **70**, 2129–2136.

Tannock, G.W., Munro, K., Harmsen, H.J.M., Welling, G.W., Smart, J. and Gopal, P.K., 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. Appl. Environ. Microb., **66**, 2578–2588.

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., **22**, 4673–4680.

Tilsala-Timisjärvi, A. and Alatossava, T., 1997. Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Int. J. Food Microbiol., **35**, 49–56.

Von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev., **21**, 213–229.

Walter, J., Tannock, G.W., Tilsala-Timisjärvi, A., Rodtong, S., Loach, D.M., Munro, K. and Alatossava, T., 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ.Microb., **66**, 297–303.

Wang, R.F., Cao, W.W. and Cerniglia, C.E., 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microb., **62**, 1242–1247.

Yin, Q.Q. and Zheng, Q.H., 2005. Isolation and identification of the dominant *Lactobacillus* in gut and faeces of pigs using carbohydrate fermentation and 16S rDNA analysis. J. Biosci. Bioeng., **99**, 68–71.

Ziemer, C.J., Cotta, M.A. and Whitehead, T.R., 2004. Application of group specific amplified rDNA restriction analysis to characterize swine fecal and manure storage pit samples. Anaerobe, **10**, 217–227.

