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Survival and Persistence of *Lactobacillus plantarum* 4.1 and *Lactobacillus reuteri* 3S7 in the Gastro-Intestinal Tract of Pigs

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Abstract

Lactobacillus **sp. are important inhabitants of the intestines of animals. They are also largely used as probiotics for both humans and animals. To exert beneficial effects, lactobacilli have to survive through the gastrointestinal transit. Based on bile-salt resistance, pH tolerance, antimicrobial activity and heat resistance, *Lactobacillus plantarum* 4.1 and *Lactobacillus reuteri* 3S7 were previously selected and used as probiotic additives in pelleted feeding trials. Both strains were fed to pigs (sows and piglets) at a cell number of ca. 10^{10} viable cells per days. A polyphasic approach, comprising growth on selective media, Biolog system analysis, 16S rRNA gene sequencing and RAPD-PCR typing, was used to identify and differentiate *L. plantarum* 4.1 and *L. reuteri* 3S7 from other faecal *Lactobacillus* sp. *L. plantarum* 4.1 and *L. reuteri* 3S7 had the capacity to survive the gastrointestinal transit and were found in the feaces at a cell number of 6 to 8 log cfu/g. Their persistence was shown after six days from the administration. Compared to untreated pigs, the administration of *L. plantarum* 4.1 and *L. reuteri* 3S7 significantly ($P < 0.05$) decreased the population of *Enterobacteriaceae*. Besides, the β-glucuronidase activity of all pigs decreased of ca. 23.0% during administration. The findings of this study showed that *L. plantarum* 4.1 and *L. reuteri* 3S7 have the potential to be used as probiotic additives in pelleted feed for pigs.**

Keywords: Lactobacillus; Probiotics; β-glucuronidase activity; *Enterobacteriaceae;* Pigs

1. Introduction

Oedema and diarrhea caused by pathogenic *Escherichia coli* are common diseases of piglets, especially during early weaning (Beutin et al., 1998). These diseases may result in considerable economic losses for the pig rearing industry. Intestinal carriage of enteropathogens (e.g., *Salmonella* and *Yersinia*) by farm animals might determine carcass contamination during slaughtering and, subsequently, their transmission to humans. An important concern in pig breeding is represented by *Salmonella* infection from sows to piglets (Osterberg et al., 2001; Nollet et al., 2005). Overall, treatments with antibiotics (e.g. enrofloxacin) failed to prevent shedding by *Salmonella enterica* serovar *Typhimurium* in pig farms, causing the further infection of piglets (Roesler et al., 2005). Pregnant sows and young pigs may be subjected to stressful physiological and environmental conditions which promote the proliferation of pathogens in the digestive tract. Since most of the antibiotics used as growth promoters in pig feeding have been banned by European laws, alternative strategies to favor the prevalence at gastrointestinal tract (GIT) of useful indigenous bacteria towards pathogens are needed (Wegener et al., 1999). As a consequence, the pig diet is considered as a crucial point to prevent enteric bacterial infections (Bach Knudsen, 2001).

Probiotics are described as 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host'. The prevention of GIT pathogen colonization through competitive exclusion, and/or synthesis of inhibitory compounds (Kaur et al., 2002; Isolauri et al., 2004) is the primary mechanism mediated by probiotics (Forestier et al., 2001; Lu and Walker, 2001). However, commercially available probiotic-based products have been of variable efficacy, and in most cases retailing has preceded scientific validation. Probiotic bacteria may be effective in promoting good health when they reach the small and large intestines without loss of viability. Overall, it has been shown that probiotic bacteria vary considerably in the tolerance of bile and other GI conditions. Therefore, *in vivo* challenges are indispensable to show the capacity to survive after the GI transit (Lick et al., 2001). As shown in several reports (Alander et al., 1999; Fujiwara et al., 2001; Ohashi et al., 2001; Gardiner et al., 2004), the survival of administered probiotics is

determined based on their recovery as viable cells from faeces (Alander et al., 1999; Fujiwara et al., 2001; Ohashi et al., 2001; Gardiner et al., 2004).

This study aimed at investigating the *in vivo* performance of *L. plantarum* 4.1 and *L. reuteri* 3S7 orally administered to pigs. The two strains were previously isolated from porcine faeces and *in vitro* characterized for their technological (resistance to freeze-drying and heating) and probiotic (bile-salt and low pH tolerance, inhibition of pathogen *E. coli* causing diarrhea and edema disease, and *S. enterica* serovar *Typhimurium*) potentialities (De Angelis et al., 2006).

2. Materials and methods

2.1. Micro-organisms and growth conditions

The lactic acid bacteria used in this study were *L. plantarum* 4.1 and *L. reuteri* 3S7 and belong to the Culture Collection of Department of Plant Protection and Applied Microbiology of the Agriculture Faculty, University of Bari, Italy. The strains, isolated from pig faeces of Large White sows, were previously characterized by *in vitro* assays for bile-salt resistance, pH tolerance, antimicrobial activity, freeze-drying and heat resistance (De Angelis et al., 2006). Bacteria were anaerobically grown over night at 37 °C in MRS (Oxoid Ltd., Basingstoke, Hampshire, England) medium (4% inoculum) from glycerol (20% v/v) stocks stored at -80 °C. After growth, lactic acid bacteria were harvested by centrifugation (8000 × *g* for 10 min), washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0), lyophilized and used as additives for pellet diet.

2.2. Experimental diet

The commercial pig diet had the following composition (w/w): 55% maize, 27% soya, 8.4% barley, 4% wheat bran, 3% fat, 1.2% $CaPO_4$, 1% $CaCO_3$ and 0.4% NaCl. The feed (1.5 kg) was mixed and gently homogenized with 5% (w/w) of water and 1% (w/w) of lyophilized cells of

lactobacilli mix (10 log cfu/g). The mixture was pelleted by a Baby–IEMME equipment (IEMME, Verona, Italy) at 55-60 °C for 30-40 s. Final moisture of the pelleted diet was ca. 16% (w/w).

2.3. Pig–feeding trials

Seven pregnant sows and four piglets (two males and two females) of the Large White breed were used for feeding trials. Four additional animals (two sows and two piglets) were included in the study as the control and were not subjected to treatment with lactobacilli. The pig-feeding trial complied with European Union Council Directive 91/630/EEC, which lays down minimum standards for the protection of pigs. In order to avoid cross-contaminations, each animal was individually penned. All animals had unrestricted access to water. Diets used for 15 days before starting the trial did not contain antimicrobials, performance enhancers, probiotics or acidifiers. The feeding trial consisted of three consecutive periods: baseline (10 days), lactobacilli administration (15 and 70 days for piglets and sows, respectively), and post-administration (6 days). During baseline each sow and piglet received 500 g of pelleted diet per day and other un-pelleted feed. During lactobacilli administration, a total of ca. 10 log cfu of lactobacilli were provided daily to each pig by supplying 500 g of pelleted feed which contained ca. 7.1 log cfu/g of each *Lactobacillus* strain. During post-administration, pigs did not ingest lactobacilli. Control pigs were fed with a basal diet without *Lactobacillus* strains.

2.4. Microbiological analysis of pig faecal samples, and identification and typing of probiotic strains

Rectal samples of faeces were obtained for each animal prior, during and after lactobacilli administration. After collection, faeces (ca. 5 g) were mixed with Amies Transport medium (Oxoid) under anaerobic conditions. Subsequently, samples were suspended in quarter-strength Ringer's solution, homogenized with a classic blender (PBI International, Milan, Italy) and plated on MRS agar at 30 °C for 48 h. MRS, containing 150 µg/ml of chloramphenicol or streptomycin as selective

agents, was also used (De Angelis et al., 2006). Representative colonies were randomly picked up from plates of MRS or MRS, containing chloramphenicol or streptomycin, and were subjected to 16S rRNA, PCR–RAPD and Biolog system analyses (Biolog, Inc., Hayward, CA, USA). Faecal bacteria of the family *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBA, Oxoid) at 37 °C for 24 h under anaerobic conditions (Gardiner et al., 2004).

2.4.1. Genotypic identification of lactic acid bacteria. Total DNAs from Gram-positive, catalase-negative and non-motile presumptive lactobacilli were extracted from 2 ml of overnight cultures grown in MRS and incubated anaerobically at 37 °C. The final concentration of lysozyme used for cell lysis was 2 mg/ml. The concentration and purity of DNA were assessed by determining the absorbance at 260 and 280 nm. Primers used to amplify 16S rRNA gene fragment of lactobacilli are listed in Table 1. Fifty microliters of each polymerase chain reaction (PCR) mixture contained: 5 µl of 10 x PCR buffer, 200 µM of each 2'–deoxynucleoside 5'–triphosphate (dNTPs), 1 µM of both forward and reverse primer, 2 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Milan, Italy), ca. 50 ng of DNA and enough bidistilled water to reach the final volume. PCRs were performed using the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) as reported by De Angelis et al. (2006). PCR products were separated by electrophoresis on 1.5% (w/v) agarose gel (Gibco BRL, Eragny, France) stained with ethidium bromide (0.5 µg/ml), and the DNA was detected by UV transillumination. The expected amplicons were excised from the gel and purified by the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA). DNA sequencing reactions were performed by MWG Biotech AG (Ebersberg, Germany). The identities of the sequences, obtained after analysis of amplified PCR products, were verified by a BlastN search against the NCBI non-redundant sequence database located at http://www.ncbi.nlm.nih.gov. Strains showing homology of at least 97% were considered to belong to the same species.

144 *2.4.2. Genotypic characterization by RAPD.* Isolates from pig faeces were characterized genotypically
145 by RAPD-PCR analysis. Four primers (Table 1) with arbitrarily chosen sequences were used. The
146 reaction mixture for primer M13 contained: 25 ng of DNA, $MgCl_2$ 1.5 mM , 20 µM of primer, 200 µM
147 for each 2'-deoxynucleoside 5'-triphosphate, 2.5 µl of PCR buffer (10x), 1.25 U of *Taq* DNA
148 polimerasi (Invitrogen Life Technologies) and enough sterile bidistilled water to bring the volume to 25
149 µl. The PCR program comprised 40 cycles of denaturation for 1 min at 94 °C, annealing for 20 sec at 35
150 °C, and extension for 2 min at 72 °C. The cycles were preceded by denaturation step at 94 °C for 2 min
151 and followed by extension step at 72 °C for 10 min. The other primers were used in the same conditions
152 described by De Angelis et al. (2001). PCR products were separated by electrophoresis at 120 V for 3 h
153 on 1.5% (w/v) agarose gel as above reported. Molecular sizes of the amplified DNA fragments were
154 estimated by comparison with a 1 Kb plus ladder DNA (Invitrogen Life Technologies). Gels were
155 acquired using a UNIsave gel documentation system camera, Model GAS9200/1/2/3, Version 11
156 (UVItec Limited, Cambridge. United Kindom). Electrophoretic profiles were compared using Quantity
157 One software (Biorad, Milan, Italy).
158
159 *2.4.3. Fermentative profile by Biolog System.* Three days before the inoculation of Biolog AN plates
160 (Biolog, Inc.), the strains were streaked twice on MRS agar plates. The plates were incubated at 30
161 °C for 24 h and thereafter cells were used for Biolog assays. Wells of Biolog AN plates were
162 inoculated with 150 µl of bacterial suspensions adjusted to 65% transmittance as recommended by
163 the manufacturer. Positive reactions were automatically recorded using a microplate reader with
164 590 nm wavelength filter.
165
166 *2.5. β-glucuronidase activity*
167 β-glucuronidase activity (EC 3.2.1.31) was determined as described by Haberer et al. (2003),
168 using *p*-nitrophenyl-β-D-glucuronide (Diffchamb, Sweden) as substrate. The assay mixture,
169 containing 0.085 M potassium phosphate buffer at pH 7.5, 0.015 M substrate, 0.031 M 3,4-

dichloronitrobenzene, and 0.5 ml of faecal suspension, was incubated at 30 °C for 48 h under anaerobic and stirring conditions (150 rpm). After incubation, the absorbance of the mixture was measured at 402 nm. One unit (U) of activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per 10 min under condition assay. Data obtained were compared to standard curves set up by using *p*-nitrophenol.

2.6. Statistical analyses

Data from three independent replicates were subjected to one–way ANOVA (SAS, 1985); for multiple comparison the Tukey test was used and the alpha value for all experiments was set at 0.05, using a statistical software, Statistica for Windows (Statistica 6.0 per Windows 1998).

3. Results

3.1. Performance of animals

No antibiotics were fed during the period of investigation period as well as no particular feed composition was needed. All the sows and piglets remained healthy throughout the duration of the experiments and no cases of diarrhea were observed.

3.2. Enumeration and identification of lactobacilli in pig faeces

As determined by plate counts, the pig faeces of the 7 Large White sows had similar cell numbers of presumptive lactobacilli which ranged from 8.88 to 9.11 log cfu/g (Fig. 1). Presumptive lactobacilli were isolated at the same order of magnitude (8.67 to 9.11 log cfu/g) from the faeces of the control group during 70 days. No significant changes in the cell number of presumptive lactobacilli were found throughout the whole feeding period: it was constant in the range 8.99 – 9.13 log cfu/g.

Lactobacilli were detected in piglets (5.85 – 6.25 log cfu/g) at a cell number 2 – 3 log cycles lower than adult animals (8.88 – 9.11 log cfu/g). During the 15 days of treatment, lactobacilli raised to

195 7.87 log cfu/g within 3 days and further increased to ca. 8.5 log cfu/g for the remaining time (Fig.
196 2). After 3 and 6 days of post-administration the cell number of lactobacilli decreased of ca. 1 log
197 cycle (7.84 and 7.54 log cfu/g, respectively) but was still significantly (P<0.05) higher than the
198 value determined in the control group. Fifteen Gram-positive, catalase-negative, non-motile and
199 acidifying isolates of presumptive lactobacilli were randomly picked up from the plates of the
200 highest sample dilution and subjected to preliminary group differentiation based on several
201 physiological characters. All isolates with different characters (Biolog and/or RAPD profiles) were
202 identified based on partial sequencing of the 16S rRNA gene. All sequences were deposited in
203 GenBank database and the accession numbers were: EF439669 – EF439675; EF439676 –
204 EF439684; EF439685 – EF439689; EF439690 – EF439696; EF439697 – EF439704 and EF439705
205 – EF439706 for *L. reuteri*, *L. plantarum*, *Lactobacillus crispatus*, *Lactobacillus mucosae*,
206 *Lactobacillus amylovorus* and *Lactobacillus intestinalis* strains, respectively. The partial 16S rRNA
207 gene sequences of *L. reuteri* 3S7 (AY823503) and *L. plantarum* 4.1 (AY845198) were deposited
208 previously. The endogenous species of *Lactobacillus* identified in the pigs subjected to lactobacilli
209 administration did not differ from those detected in the control groups. The *Lactobacillus*
210 microbiota did not differ between sows and piglets.
211
212 *3.3 Survival and persistence of administered strains*.
213 As previously described (De Angelis et al., 2006), *L. reuteri* 3S7 was sensitive to chloramphenicol
214 and resistant to streptomycin while *L. plantarum* 4.1 was sensitive to streptomycin but resistant to
215 chloramphenicol. As a consequence, MRS containing chloramphenicol or streptomycin was used to
216 differentiate in faecal samples the administrated strains. Prior to lactobacilli administration, 4.5 –
217 5.0 and 5.1 – 6.0 log cfu/g of lactobacilli resistant to chloramphenicol or streptomycin, respectively,
218 were determined in faecal samples. After 3 and 7 days, all pigs subjected to lactobacilli
219 administration excreted 6.4 to 7.0 log cfu of chloramphenicol and streptomycin resistant lactobacilli
220 per gram of faeces and similar cell densities (6.8 – 8.2) were found throughout the remaining period

(data not shown). Fifteen Gram-positive, catalase-negative, non-motile and acidifying isolates of presumptive lactobacilli were randomly picked up from the plates of the highest sample dilution and subjected to RAPD-PCR analysis by using single primers P1, P4, P7 or M13. The reproducibility of RAPD fingerprints was assessed by comparing the PCR products obtained from three separate cultures of the same strain. The patterns showed ca. 92 – 95% similarity, indicating the reproducibility of the technique under the conditions used (data not shown). RAPD-PCR analysis allowed the identification of the two lactobacilli administrated once re-isolated from faecal samples. Fig. 3 shows the RAPD-PCR fingerprints, generated with primer MI3, of most of the representative faecal isolates which differed from the administrated strains. To further confirm the presence of *L. plantarum* and *L. reuteri* in faecal samples of treated pigs, the isolates were analysed by Biolog System also. Table 2 shows the fermentative profiles of some of the isolates of the *L. plantarum* and *L. reuteri* species. Prior to culture administration, none of the two administrated strains was detected in the faeces of any pigs (Table 3). In the same way, *L. plantarum* 4.1 and *L. reuteri* 3S7 were not detected in the control groups (sows and piglets numbered 1-2). During administration, the recovery of *L. plantarum* 4.1 and *L. reuteri* 3S7 was relatively high (ca. 4 strains out of 15 total lactobacilli). After 6 days from the treatment, the two lactobacilli strains were still detected in the sow faeces. The recovery of *L. plantarum* 4.1 and *L. reuteri* 3S7 in the piglet faeces was higher than that in sow faeces; a maximum of 7 strains out of 15 total lactobacilli was detected after twelve days. Re-isolation of *L. plantarum* 4.1 and *L. reuteri* 3S7 from faeces of both sows and piglets was less frequent during the washout period.

3.4. Effects on intestinal microbiota

The enumeration of *Enterobacteriaceae* in faeces was carried out since this group of Gram-negative bacteria is indicative of the presence of pathogenic species such as *E. coli* and *Salmonella* sp. (Gardiner et al., 2004). The cell number of *Enterobacteriaceae* excreted in sow and piglet faeces during lactobacilli administration and post-administration is shown in Figs. 4 and 5. The averaged

number of *Enterobacteriaceae* in the sow feaces of the control group was 7.58 log cfu/g throughout the challenge without significant difference with respect to the pre-administration period (day 0). On the contrary, the excreted faecal *Enterobacteriaceae* of sows subjected to feeding with *L. plantarum* 4.1 and *L. reuteri* 3S7 was in the range of 5.33 to 4.88 log cfu/g. The population of *Enterobacteriaceae* increased to 6.0 log cfu/g only after 7 days of post-administration but it was always lower than the number of baseline (7.64 log cfu/g) (Fig. 4). The averaged number of *Enterobacteriaceae* in the piglet feaces of the control group was 6.25 log cfu/g with slight variations (less than 0.5 log cycle) found during the challenge (Fig. 5). The population of *Enterobacteriaceae* detected in the faeces of piglets (5.70 log cfu/g) before administration of lactobacilli linearly decreased to 3.65 log cfu/g at the end of administration (15 days). This cell number of *Enterobacteriaceae* persisted until 3 days of post-administration to further increase only after 6 days.

3.5 β-glucuronidase

The rate of *p*-nitrophenyl-β-D-glucuronide degradation by faecal enzymes during treatment with lactobacilli is shown in Figure 6A and B. β-glucuronidase activity of control pigs was limited in a very narrow range, 27.9 – 30.1 U and 18.7 – 23.3 U for sows and piglets, respectively. During lactobacilli administration a significant ($P<0.05$) decrease of the enzyme activity was found in all the pigs. Compared to the control group, the β-glucuronidase activity of sows fed with lactobacilli decreased of ca. 28.9% after 7 days and of 36.4 - 43.3% during the remaining 63 days (Fig. 6A). A similar decrease of the faecal enzyme activity (23.0 – 37.4%) was found in piglets fed with lactobacilli (Fig. 6B). After 6 days of post-administration, the β-glucuronidase activity had still a lower value than the baseline.

4. Discussion

Before human or animal application, probiotic bacteria must be thoroughly tested. Overall, pigs are chosen as experimental animals since the comparability of their digestive and circulatory systems to those of humans (Muralidhara et al., 1977; Singer et al., 2003). *L. plantarum* 4.1 and *L. reuteri* 3S7 were previously *in vitro* characterized for their bile-salt and low pH resistance (De Angelis et al., 2006). Nevertheless, laboratory testing may only provide useful evidences for selection of potentially effective probiotic strains, but performances in the gut and effects on intestinal microflora need to be accurately determined *in vivo* (Gardiner et al., 2004). The complexity of the intestine may lead to variation among animals and, therefore, probiotic strains may have different effects. From this perspective mixtures of strains are recommended (Gardiner et al., 2004). Due to their survival during the pelletting process (De Angelis et al., 2006), pig faecal isolates of *L. plantarum* 4.1 and *L. reuteri* 4S7 were used for an *in vivo* trial based on the administration of pelleted feed containing selected lactobacilli. Microbiological analyses of faeces from animals fed with a diet without additives revealed usual numbers of lactic acid bacteria in the range 8.8 – 9.11 log cfu/g (Gardiner et al., 2000). In this study, the population of faecal lactobacilli in piglets was ca. 2 log cycles lower than that of adults, but the same species were identified in sows and piglets (*L. reuteri*, *Lactobacillus crispatus, L. plantarum, Lactobacillus mucosae, Lactobacillus amylovorus* and *Lactobacillus intestinalis*). As already shown by du Toit et al. (1998), no increases of the faecal *Lactobacillus* cell number was found in sows when fed with lactobacilli. On the contrary, the cell number of lactobacilli significantly ($P<0.05$) increased in piglets feed with lactobacilli (Chang et al., 2001). After 3 or 7 days of administration, all treated pigs excreted 6.0 – 7.5 log cfu/g of the ingested strains. The cell number of administrated strains in pig faeces was similar to that found in other studies (Gardiner et al., 2004). After 3 and 6 days of post-administration, the faecal population of lactobacilli in treated piglets was higher than that of the control group. The findings of this study suggested that the increased cell density of *Lactobacillus* in piglets was not only due to the administrated strains but also to the increased cell number of

297 endogenous lactobacili. A stimulatory effect on the growth/survival of endogenous lactobacilli in
298 the pig intestine has been shown for *Lactobacillus casei* SHIROTA (Ohashi et al., 2001).

299 Pelleting is a process that eliminates problems for pig diets with small particle sizes, decreases
300 dustiness and segregation of ingredients, and increases bulk density. Pelleting diets improved feed
301 efficiency of gain compared to the same diet in meal form. The selected strains used in this study
302 could be used as additives in pelleted diet.

303 *L. plantarum* 4.1, and *L. reuteri* 3S7 were recovered after 6 days of post-administration,
304 indicating that these strains persisted in the GIT of pigs. These results agreed with previous studies
305 showing the persistence of other probiotic *Lactobacillus* strains for 3 to 10 days (Rogelj et al., 2002;
306 Gardiner et al., 2004). To our knowledge, none of the human probiotics actually colonises the
307 intestinal tract but are washed out after a couple of days / a few weeks post-administration.

308 Previously, *L. plantarum* 4.1, and *L. reuteri* 3S7 were *in vitro* characterized for the
309 antimicrobial activity towards *Salmonella enterica* serovar *Typhimurium* and *E. coli* ED36 and
310 ED38 (De Angelis et al., 2006). The two *E. coli* strains produced a verocytotoxin 2e (VT2e) variant
311 causing vascular lesions in the intestine, subcutis and brain, and leading to edema and neurological
312 symptoms (Imberechts et al., 1992; Bertschinger et al. 1999). This study aimed at *in vivo* showing
313 the antimicrobial activity of the selected lactobacilli. Jørgensen et al. (1999) demonstrated a clear
314 positive correlation between the incidence of *Salmonella*-positive pigs and the density of coliform
315 bacteria in the GIT of pigs. The findings of this study showed that selected lactobacilli inhibited in
316 part the faecal *Enterobacteriaceae* microbiota. After 7 – 15 days of lactobacilli administration, the
317 averaged cell number of faecal *Enterobacteriaceae* in sows and piglets was decreased of 2 – 2.6 log
318 cycles. The decrease of the *Enterobacteriaceae* persisted during 6 days of post–administration. Also
319 other studies (Chang et al., 2001; Gardiner et al., 2004) showed decreases of the intestinal coliform
320 and *Enterobacteriaceae* numbers after administration of lactic acid bacteria, even though other
321 authors (De Cupere et al., 1992; Gardiner et al., 1999) did not show the same effect.

Haberer et al., (2003) showed that the administration of lactobacilli to pigs caused a marked decrease of the faecal β-glucuronidase activity. Several investigations also showed an influence of the intake of lactobacilli and fermented milk products on the gut flora enzyme activities associated with colon carcinogenesis (Wollowski et al., 2001). Bacterial β-glucuronidase seems to play an important role in initiating colon cancer due to its wide substrate specificity and the hydrolysis of many different glucuronides (Hawksworth et al., 1971; Lidbeck et al., 1992). As a consequence of β-glucuronidase activity, toxic compounds already detoxified in the liver by conjugation are regenerated by the release of toxic aglycones, thus delaying their excretion (Wollowski et al., 2001). Enterobacteria and clostridia show the highest level of bacterial β-glucuronidase activity (Hawksworth et al., 1971). The findings of this study are consistent with those of Goldin and Gorbach (1984) which used an oral supplement of *Lactobacillus acidophilus* to partially replace the human intestinal microbiota and to significantly decrease the level of β-glucuronidase activity. At this purpose pigs provide a suitable model system since their GIT is physiologically and anatomically similar to that of humans (Ayebo et al., 1980).

The results of this study showed that *L. plantarum* 4.2 and *L. reuteri* 3S7 have the capacity to: (i) survive in the sows' and piglets' intestine; (ii) stimulate the growth of endogenous *Lactobacillus* in piglets; (iii) inhibit the enterobacteria population in GIT; and (iv) to inhibit β-glucuronidase activity in faecal samples. The use of *L. plantarum* 4.2 and *L. reuteri* 3S7 in pelletted feed for pigs will be further investigated. In particular, pigs with spontaneous diarrhoeal syndrome and pigs infected by certain pathogenic such as *S. enterica* serovar *Typhimurium* or *E. coli* strains will be considered. In future studies, *L. plantarum* 4.2 and *L. reuteri* 3S7 will be also assayed for their possible human applications.

Legends to figures

Fig. 1. Faecal excretion of total viable lactobacilli in sows during feed administration with or without the mixture of *L. plantarum* 4.1 and *L. reuteri* 3S7. Each determination was carried out in triplicate. Values are the means of the results of five pigs (fed with culture mix) or two pigs (control) with standard error indicated by vertical bars.

Fig. 2. Faecal excretion of total viable lactobacilli in piglets during feed administration with or without a mixture of *L. plantarum* 4.1 and *L. reuteri* 3S7. Each determination was carried out in triplicate. Values are the means of the results of two piglets (fed with culture mix) or two piglets (control) with standard error indicated by vertical bars.

Fig. 3. Randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) profiles (generated with primer M13) of faecal isolates from sows and piglets fed with lactobacilli. Lanes: S, DNA molecular size standards; lane 1, *Lactobacillus reuteri* 3S7 parental strain; lanes 2-15, cloramphenicol or streptomycin resistant isolates which differed from strains 3S7 and 4.1; lane 16, *Lactobacillus plantarum* 4.1 parental strain.

Fig. 4. Faecal excretion of total viable *Enterobacteriaceae* in sows during feed administration with or without a mixture of *L. plantarum* 4.1 and *L. reuteri* 3S7. Each determination was carried out in triplicate. Values are the means of the results from five pigs (fed with lactobacilli) or two pigs (control) with standard error indicated by vertical bars.

Fig. 5. Faecal excretion of total viable *Enterobacteriaceae* in piglets during feed administration with or without a mixture of *L. plantarum* 4.1 and *L. reuteri* 3S7. Each determination was carried out in triplicate. Values are the means of the results from two piglets (fed with culture mix) or two piglets (control) with standard error indicated by vertical bars.

Fig. 6. Faecal β-glucuronidase activity of piglets (A) and sows (B) during feed administration with or without a mixture of *L. plantarum* 4.1 and *L. reuteri* 3S7. Each determination was carried out in triplicate. Values are the means of the results with standard error indicated by vertical bars.

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Table 1. Nucleotide sequences, microbial species and gene targeted, of the primers used for PCR amplifications

Primer	**Specificity /gene target**	**Oligonucleotide sequence (5'→3')**	**Reference / NCBI GenBank accession number (of reference sequence)**
LacbF	*L. brevis*/16S rRNA	TGCCTAATACATGCAAGT	AF515220
LacbR	*L. brevis* /16S rRNA	CTTGTTACGACTTCACCC	AF515220
LpigF	*L. mucosae*/16S rRNA	TACGGGAGGCAGCAGTAG	AF126738
LpigR	*L. mucosae*/16S rRNA	CATGGTGGACGGGCGGT	AF126738
M13		GAGGGTGGCGGTTCT	Stendid *et al.* 1994
P1		ACGCGCCCT	De Angelis et al., 2001
P4		CCGCAGCGTT	De Angelis et al., 2001
P7		AGCAGCGTGG	De Angelis et al., 2001

1 **Table 2.** Main carbon sources fermented by some of the *Lactobacillus plantarum* and *Lactobacillus*
2 *reuteri* isolates from faecal samples of pigs fed with lactobacilli

Carbon sources	*Lactobacillus plantarum* 4.1	*Lactobacillus reuteri* 3S7	*Lactobacillus reuteri* 31B	*Lactobacillus reuteri* 42Z	*Lactobacillus plantarum* LT3	*Lactobacillus plantarum* SV47
N-acetyl-D-glucosamine	+*	-	+	-	+	+
N-acetyl-D-mannosamine	+	-	-	-	+	+
Amygdalin	+	-	-	-	+	+
Arbutin	+	-	-	-	-	-
D-Cellobiose	+	+	+	+	-	-
Dextrin	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
D-Galacturonic acid	-	+	+	-	-	-
Gentiobiose	+	-	-	-	-	+
α-D-Glucose	+	+	+	+	+	+
Glucose-6-Phpsphate	-	+	-	-	-	-
Glycerol	-	+	-	-	-	-
α-D-Lactose	-	+	+	+	+	+
Lactulose	-	+	+	+	+	+
Maltose	+	+	+	+	+	+
Maltotriose	-	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	-
D-Melibiose	-	+	+	+	+	+
3-methyl-D-Glucose	+	-	+	+	-	-
α-methyl-D-Galactoside	-	+	-	+	+	-
β-methyl-D-Galactoside	-	+	+	-	+	+
β-methyl-D-Glucoside	+	-	-	-	+	+
Palatinose	+	+	+	+	-	-
D-Raffinose	-	+	+	+	-	+
L-Rhamnose						
Salicin	+	-	-	-	-	-
D-Sorbitol	+	-	-	-	-	-
Stachyose	-	+	+	+	+	+
Sucrose	-	+	+	+	+	+
D-Trehalose	+	-	-	-	-	-
Turanose	+	+	+	+	-	-
α-Hydroxy-butyric-acid	+	+	+	+	+	+
D,L-Lactic acid	+	+	+	+	+	+
L-Lactic acid	+	+	+	+	+	+
D-Lactic acid methyl ester	-	-	+	+	-	-
Pyruvic acid	+	+	+	+	-	-
Pyruvic acid methyl ester	-	+	+	-	-	-
2'-Deoxy adenosine	-	+	-	+	-	-
Inosine	-	+	+	+	-	-
Thymidine	-	+	+	+	-	-
Uridine	-	+	+	+	-	-

3 *-, Negative reaction; +, positive reaction.

Table 3. Number of post-administration pig faecal isolates identified by partial 16S gene sequences, RAPD fingerprints and Biolog analysis as the administrated strains and presented as a portion of the total number of isolates

	Cultured administered Days 1-70							Post-administration Days 71-76
	0	7	14	21	28	56	70	76
Sows								
1	ND[a]	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	2,3/15[b]	4,2/15	4,4/15	4,4/15	4,4/15	4,4/15	1,0/15
4	ND	4,4/15	4,2/15	4,3/15	4,3/15	4,3/15	4,3/15	1,1/15
5	ND	5,2/15	3,3/15	3,5/15	3,5/15	3,5/15	3,5/15	2,1/15
6	ND	4,1/15	5,2/15	4,4/15	4,4/15	4,4/15	4,4/15	2,2/15
7	ND	4,2/15	5,5/15	5,3/15	4,4/15	4,4/15	4,4/15	2,3/15

Piglets	Cultured administered Days 1-15						Post-administration 16-21	
	0	3	6	9	12	15	18	21
1	ND	ND	ND	ND	ND	ND	ND	ND
2	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	5,3/15	6,5/15	5,6/15	6,7/15	7,5/15	2,3/15	1,2/15
4	ND	5,3/15	5,3/15	6,5/15	6,6/15	4,7/15	4,1/15	1,1/15

[a] ND, not determined in the corresponding pig.
[b] The first and second numbers indicate *L. plantarum* 4.1 and *L. reuteri* 3S7 isolates, respectively.