Relative Ability of Orally Administered \textit{Lactobacillus murinus} To Predominate and Persist in the Porcine Gastrointestinal Tract

Gillian E. Gardiner,\textsuperscript{1} Pat G. Casey,\textsuperscript{2,3} Garrett Casey,\textsuperscript{2} P. Brendan Lynch,\textsuperscript{4} Peadar G. Lawlor,\textsuperscript{4} Colin Hill,\textsuperscript{2} Gerald F. Fitzgerald,\textsuperscript{2} Catherine Stanton,\textsuperscript{1,3} and R. Paul Ross\textsuperscript{1,3,*}

\textit{Dairy Products Research Center} and \textit{Pharmabiotic Center}, Teagasc, Moorepark, Fermoy, County Cork, Ireland

\textit{Dairy Products Research Center} and \textit{Pig Production Department}, Teagasc, Moorepark, Fermoy, County Cork, Ireland; \textit{Department of Microbiology, University College Cork}, and \textit{Alimentary Pharmabiotic Center}, Cork, Ireland

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Five porcine-derived \textit{Lactobacillus} or \textit{Pediococcus} isolates administered to pigs (\(n = 4\)), either singly or as a combination at \(\sim 10^{10}\) CFU per day varied with respect to intestinal survival and persistence. Two \textit{Lactobacillus murinus} strains survived best and were excreted at \(\sim 10^7\) to \(10^8\) CFU/g of feces. In contrast, \textit{Pediococcus pentosaceus} DPC6006 had the lowest fecal count at \(\sim 10^5\) CFU/g and was excreted at a significantly lower level than both \textit{L. murinus} strains. Fecal \textit{L. murinus} DPC6003 counts were also significantly higher than both \textit{Lactobacillus salivaruis} DPC6005 and \textit{Lactobacillus pentosus} DPC6004 (\(\sim 10^6\) CFU/g). The \textit{L. murinus} strains persisted for at least 9 days postadministration in both the feces and the cecum. Animals fed a combination of all five strains excreted \(\sim 10^7\) CFU of the administered strains/g, with \textit{L. murinus} predominating, as determined by randomly amplified polymorphic DNA PCR. Postadministration, variation was observed between animals fed the strain combination, but in general, \textit{L. murinus} DPC6002 and DPC6003 and \textit{L. pentosus} DPC6004 predominated in the feces and the cecum while \textit{P. pentosaceus} DPC6006 was detected only in the cecum. Fifteen days after the start of culture administration, mean fecal \textit{Enterobacteriaceae} counts were significantly lower in some of the treatment groups. In addition, when mean preadministration counts were compared with those obtained after 21 days of culture administration, \textit{Enterobacteriaceae} counts were reduced by \(\sim 87\) to \(98\%\) in pigs fed \textit{L. salivaruis} DPC6005, \textit{P. pentosaceus} DPC6006, \textit{L. pentosus} DPC6004, and the culture mix. In conclusion, the porcine intestinal isolates have potential as probiotic feed additives for pigs, with differences in strain performance highlighting the advantages of using culture combinations.

Intestinal carriage of enteropathogens, such as \textit{Salmonella}, \textit{Escherichia coli} O157, \textit{Yersinia}, and \textit{Campylobacter}, in farm animals is a cause for concern, as carcass contamination at slaughter can lead to pathogen transmission to humans. Antibiotics are generally unsuitable for the treatment of carrier animals (1, 29), and the use of antibiotics in animal production, for growth promotion and treatment or prevention of disease is controversial, as it has been associated with the emergence of antibiotic-resistant pathogens. Multiresistant strains of \textit{Salmonella} and \textit{E. coli}, vancomycin-resistant enterococci, and fluoroquinolone-resistant \textit{Campylobacter} are being isolated from pigs and poultry in particular at increasing frequencies (1). In light of these concerns and the implementation of European legislation banning the addition of certain antibiotic growth promoters to animal feed, alternatives to antibiotics in animal production are highly desirable (1).

Probiotics, aimed at restoration and maintenance of a healthy gut microflora, are live microorganisms which when administered in adequate amounts exert a health benefit on the host (9). As microbial feed additives, they offer potential as an alternative to antibiotics, both as a means of controlling pathogen carriage and improving growth rate and feed conversion. The cultures most commonly used include lactic acid bacteria, \textit{Bacillus} organisms, and yeasts such as \textit{Saccharomyces boulardii} (36). Data from animal trials have shown that probiotics can improve growth performance in pigs (4, 39), chickens (19), and lambs (21). Competitive exclusion cultures have been applied successfully to animals for the reduction of enteropathogen carriage, with probably the best example being a commercially available Food and Drug Administration-approved 29-strain mix that significantly reduces \textit{Salmonella} carriage in chickens (27). Inoculation with probiotic bacteria can also reduce fecal shedding of \textit{E. coli} O157:H7 in both cattle (44) and lambs (21). Probiotics offer potential for use in pigs as a means of improving performance and health, particularly in light of the fact that pig rearing has become more intensive in recent years (40). Reducing pathogen carriage is also a potential target, considering that pigs are the predominant carriers of \textit{Salmonella}, with 23% of pig cecal samples in one study identified as \textit{Salmonella} positive at slaughter compared with only 0.2 and 0.1% of cattle and sheep rectal samples, respectively (6).

Although the exact mode of action of probiotics is unknown, suggested mechanisms include immunomodulation, suppression of pathogens through competitive exclusion, and/or the production of inhibitory compounds such as organic acids, hydrogen peroxide, and bacteriocins (40). The use of high levels of viable microorganisms selected in vitro on the basis of these criteria may improve probiotic efficacy in vivo. Other selection criteria considered important include survival and persistence in the host, safety, and technological suitability (8, 12). Many studies have reported the isolation and selection of potential probiotic strains for use in pigs (4, 17, 25). However,
the results of in vivo feeding trials can be variable (36). This is perhaps understandable, given that the complexity of the intestine can lead to variation between individual animals. Furthermore, commercial animal probiotic products may not contain the strains or species listed on the label at an adequate probiotic dose or may have no indication that the strains used possess any of the recommended probiotic properties (2, 41). On the other hand, while undefined cultures used as competitive exclusion products are particularly effective in pigs (10, 15), uncertainty regarding their exact composition has led to concerns that they may result in pathogen transmission. Therefore, there is a need for rational selection and characterization of strains intended for use as probiotic feed additives. Given that the performance of probiotic strains can vary between individual animals, a good case can be made for the use of mixtures, but the most appropriate approach may be to isolate and characterize individual strains prior to their combination in a probiotic product.

Although there is increasing interest in the use of probiotics as prophylactic and therapeutic agents in pig production, relatively few strains have demonstrated efficacy in vivo. The objective of the present study was to investigate the performance in pigs of five cultures when administered orally both individually and as a part of a strain combination. These cultures were previously isolated from the porcine cecum and characterized in vitro with regard to their probiotic potential, based on a number of selection criteria, including the ability to inhibit Salmonella enterica serovar Typhimurium (3).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Lactobacillus murinus DPC6002 and DPC6003, Lactobacillus pentosus DPC6004, Lactobacillus salivarius DPC6005, and Pediococcus pentosaceus DPC6006, previously isolated from pig cecal contents, were selected from a bank of porcine intestinal isolates on the basis of molecular typing and properties such as antimicrobial activity, bile tolerance, and growth in milk (3). Spontaneous rifampin-resistant (RifR) variants of these porcine isolates required to facilitate subsequent enumeration in the pig intestine were isolated by spread plating ~10^9 CFU from an overnight culture onto MRS agar containing 100 µg of rifampin (Sigma Chemical Co., Poole, Dorset, United Kingdom)/ml. Following anaerobic incubation at 37°C for 3 days, the colonies that had grown were selected and stocked in MRS broth containing 40% (vol/vol) glycerol. To confirm that the rifampin-resistant variants were identical to the parent strains, molecular fingerprinting by randomly amplified polymorphic DNA (RAPD) analysis was employed, as outlined below. Both parent and variant strains were routinely cultured at 37°C in MRS broth (Difco Laboratories, Detroit, Mich.) in anaerobic jars with CO2-generating kits (Anaerocult A; Merck, Darmstadt, Germany).

Genetic fingerprinting by RAPD PCR. RAPD PCR analysis was performed on each administered Lactobacillus or Pediococcus strain and on intestinal isolates recovered from pig fecal and cecal samples. Initially, genomic DNA was isolated from 1.5 ml of overnight MRS broth cultures according to the method outlined by Coakley et al. (5). The extracted DNA was then used as a template in PCR amplifications, which were performed with either R1 (5'-ATGTAACGCC-3') or R2 (5'-GTGATGTCGTTGTTAGTATGTA-3') random primers (manufactured by MWG Biotech, Ebersberg, Germany) as outlined by Gardiner et al. (11) with the following modifications. PCR amplifications were performed in a total volume of 50 µl in an Eppendorf DNA thermal cycler (Eppendorf Scientific Inc., Westbury, N.Y.) with 1.25 U of Taq DNA polymerase (Bioline, London, United Kingdom) added to the reaction mix. The PCR products (10 µl of each reaction mixture) were analyzed on a 1.5% (wt/vol) agarose (Sigma) gel, with a 100-bp ladder (New England Biolabs, Hitchin, Hertfordshire, United Kingdom) as a molecular size standard.

Survival of porcine isolates in gastric juice. Gastric contents collected from 12 porcine isolates (selected from a local slaughter (pigs) and ranging in pH from 3.1 to 4.0 were pooled and filtered through glass wool. Porcine gastric juice was obtained by centrifugation twice at 13,000 × g for 30 min and filtered through a Whatman no. 113 filter. The gastric juice was then checked for sterility by pour plating on brain heart infusion agar (Merck) and incubating the plates aerobically at 30°C for 2 days. The pH was adjusted to 3.0 with 1 N HCl, and the gastric juice was stored at ~20°C until use. For each of the porcine cultures, overnight MRS broth cultures were diluted 10-fold in 1 ml of one-fourth strength Ringers solution (Lab M Ltd., Bury, Lancashire, United Kingdom) and cells were harvested from this dilution, washed twice in one-fourth strength Ringers solution, and resuspended in 1 ml of gastric juice to achieve a final cell concentration of ~10^10 to 10^11 CFU/ml. Inoculated gastric juice was incubated in a shaking water bath at 37°C. Samples (100 µl) were taken at 0, 5, 15, 30, 45, and 60 min and immediately added to 900 µl of Ringers solution. Six independent cultures were processed by further diluting 10-fold in Ringers solution, spread plating 5-µl volumes of appropriate dilutions in quadruplicate on MRS agar, and incubating the plates anaerobically at 37°C for 2 days. The experiment was performed in triplicate with three separate overnight cultures for each porcine isolate.

Preparation of skim milk cultures for pig-feeding trial. To investigate the optimal skim milk medium for growth and survival of the porcine strains, each of the five rifampin-resistant variants was inoculated at a rate of 1% (vol/vol) from an overnight MRS broth culture into both 100 ml of heat-treated (121°C for 5 min) 10% (wt/vol) reconstituted skim milk (RSM) and 100 ml of heat-treated 10% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract (Merck). Inoculated milks were then incubated at 37°C for 18 h, and the fermentation was terminated by cooling on ice. The pH was measured with an MP220 pH meter (Mettler-Toledo GmbH, Greifensee, Switzerland), and the cultures were stored at 4°C for 5 days. Viable plate counts were performed on both freshly grown and stored cultures by serially diluting samples in maximum recovery diluent (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and pour plating on MRS agar which was incubated anaerobically at 37°C for 3 days. Based on the results of these experiments, 10% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract was chosen as the optimal skim milk medium for growth and survival of all of the strains except for L. salivarius DPC6005, which was grown without the addition of yeast extract. For administration to pigs, the isolates were grown in 450- to 900-ml volumes of the relevant skim milk medium. The resultant culture fermentates were then aliquoted into 100-ml volumes, stored at 4°C, and used within 5 days. In addition, a culture mix was prepared by mixing equal volumes of fermentates of each of the five porcine isolates. Throughout the pig-feeding trial, bacterial numbers were routinely checked in each batch of culture prepared by plating as outlined above.

Pig-feeding trial. The pig-feeding trial complied with European Union Council Directive 91/629/EEC, which lays down minimum standards for the protection of pigs, and European Union Council Directive 98/58/EC, which concerns the protection of animals kept for farming purposes. A total of 28 crossbred (Large White × Landrace) pigs (14 males and 14 females) were weaned at 24 to 28 days and blocked by sex and weight. Pigs within each block were assigned at random to one of seven treatment groups (n = 4) as follows: group A, control; group B, L. salivarius DPC6005; group C, P. pentosaceus DPC6006; group D, L. pentosus DPC6002; group E, L. pentosus DPC6002; group F, L. salivarius DPC6005; group G, culture mix containing all five strains. Each animal was penned individually, with control animals penned in isolation from culture-fed animals to prevent cross-contamination. In addition to the cultures of skim milk administered throughout the trial as outlined below, all animals had unrestricted access to water and nonmedicated creep feed manufactured in the Moorpark feed mill. The creep feed was formulated to contain 14.5 MJ of digestible energy per kg and 1.29% (wt/wt) total lysine by using the following ingredients: full fat soy (29% wt/wt), dried whey (20% wt/wt), barley (15.5% wt/wt), wheat (15% wt/wt), maize (17.5% wt/wt), and minerals and vitamins (1.8% wt/wt) with synthetic amino acids added.

The feeding trial consisted of three consecutive periods: baseline period (10 days), culture administration period (21 days), and postadministration period (9 days). During the baseline period, each pig received 100 ml of sterile 10% (wt/vol) RSM supplemented with 0.5% (wt/vol) yeast extract per day. During the administration period, 100 ml of skim milk culture fermentate containing on average ~3 × 10^8 CFU of the relevant porcine strain/ml (prepared as outlined above) was administered daily to each of the pigs in groups B, C, D, E, and F, giving a total average daily intake of ~3 × 10^9 CFU. Pigs in group G received 100 ml of skim milk culture fermentate (~3 × 10^7 CFU/ml) containing a mixture of all five strains in approximately equal proportions daily, providing a total dose of ~3 × 10^9 CFU/day. Pigs in the control group (A) each received 100 ml of sterile skim milk adjusted to pH 4.7 with food-grade lactic acid (Parac Biochem, Gorrinchem, The Netherlands) per day. Theretofore, during the postadministration period neither culture fermentate nor skim milk was administered to the animals.

Nine days after culture administration had ceased, two pigs per treatment...
group were sacrificed by electrical stunning followed by bleeding. Immediately after slaughter, the eecum was removed and the eecal contents were collected and stored on ice during transport to the laboratory.

Microbiological analysis of pig fecal and cecal samples. Fecal samples were obtained from each animal prior to (day −5), during (days 3, 8, 15, and 22), and 5 days after (day 26) culture administration, where day 1 was the first day of culture administration. Fecal samples were stored at 4°C and analyzed within 24 h. We have previously demonstrated no difference in bacterial counts between fresh and stored samples (data not shown). Fecal samples were homogenized in maximum recovery diluent as 10-fold dilutions with a stomacher (Lab-Blender 400; Seward Medical, London, United Kingdom) and further diluted, and the appropriate dilutions were pour plated. The administered strains were enumerated on MRS-RIF agar, i.e., MRS agar containing 100 μg of rifampin/ml as a selective agent and 50 U nystatin (Sigma)/ml to inhibit yeasts and molds, following anaerobic incubation for 2 days at 37°C. Fecal bacteria in the family Enterobacteriaceae were enumerated on violet red bile glucose agar (Merck) incubated at 37°C for 24 h. Total Lactobacillus counts were obtained on Lactobacillus-selective agar (35) (Becton Dickinson, Cockeysville, Md.) following anaerobic incubation at 37°C for 5 days. In the same way, the administered cultures, Enterobacteriaceae, and total lactobacilli were enumerated in the pig cecal content samples collected at slaughter. The pH of the cecal contents was also measured with a Mettler Toledo MP220 pH meter.

In addition, representative colonies randomly selected from MRS-RIF plates from day 15 and 26 fecal samples and cecal content samples were analyzed by RAPD PCR, as outlined above, and fingerprints were compared with those of the relevant administered strain(s).

Statistical analyses. For all pig fecal and fecal counts, mean values were calculated for each treatment and the results are presented with standard errors of the means or standard deviations (SD). Analysis of variance (split-plot in time design), used to compare treatments and time points, was performed by using Genstat (16), and Tukey's test was used for separation of means.

RESULTS AND DISCUSSION

The pig-derived strains used in the present study have been characterized by a range of in vitro probiotic tests, including assays for acid and bile tolerance, anti-Salmonella activity, and adherence to intestinal epithelial cells (3). However, while laboratory testing can provide information useful for selection of potentially effective probiotic strains, performance in the gut and effects on intestinal microflora can only be accurately determined in vivo. In the present study, following preliminary gastric transit simulation studies, five different strains were administered to pigs both individually and as a culture mix to investigate survival and persistence in the porcine gastrointestinal tract (GIT) and to assess any impact on the resident gut microflora.

DNA fingerprinting of porcine cultures by RAPD PCR. RAPD, a molecular technique which employs PCR with primers of arbitrary sequence, is important as an adjunct to the use of rifampin-resistant variants to facilitate tracking of the strains in the porcine GIT and has previously been used in human trials (14). It enables differentiation of the administered strains from the indigenous gut microflora and is useful for identification of the predominating strain(s) in animals fed a strain combination. In this study, RAPD was used to discriminate each of the five porcine isolates, with L. salivarius DPC6005, L. pentosus DPC6004, and P. pentosaceus DPC6006 yielding unique reproducible DNA fingerprints with the R1 random primer (data not shown). However, the L. muriinus strains DPC6002 and DPC6003 could not be differentiated from each other with this primer. A range of additional primers was investigated, and one (R2) was found to successfully differentiate these closely related strains (data not shown). The RAPD method was also used to demonstrate that the rifampin-resistant variants yielded identical fingerprints to those of the corresponding parent strains (data not shown).

Survival of porcine isolates in porcine gastric juice. Among the desirable properties recommended for a probiotic microorganism is the ability to tolerate the acidic conditions encountered in the stomach so that bacterial viability is maintained during gastric transit (8, 12). The potential of each of the porcine cultures to resist gastric transit was initially investigated by means of an in vitro simulation which determined the survival of the strains in porcine gastric juice (pH 3.0) over a 60-min period. While the viability of all isolates was dramatically reduced under these conditions, L. pentosus DPC6004 was the most tolerant, with 4.6 × 10^3 CFU/ml of viable cells remaining following the treatment (10,000-fold reduction) (Fig. 1). In comparison, the other four strains tested survived for up to 45 min in gastric juice (pH 3.0), with numbers of P. pentosaceus DPC6006 and L. muriinus DPC6002 and DPC6003 reduced to between 2.9 × 10^2 and 7.4 × 10^4 CFU/ml at this time point (Fig. 1). L. salivarius DPC6005 was the least tolerant to simulated gastric transit conditions, and only 1.7 × 10^2 CFU/ml could be recovered after 30 min of exposure, although it was also still detectable at low levels after 45 min of exposure (Fig. 1). Porcine lactobacilli previously investigated by in vitro gastric transit studies also demonstrated considerable strain variation, with an Lactobacillus fermentum strain declining only 10-fold after 60 min of exposure to rabbit gastric juice (pH 2.0), whereas Lactobacillus acidophilus declined 100,000-fold under the same conditions (43). While many studies have investi-
gated \textit{L. acidophilus} strains of human origin (26, 32), Dunne et al. (8) compared the survival of a range of \textit{Lactobacillus} spp. in human gastric juice (pH 2.5) and found that all but one survived well for 60 min. In comparison, an \textit{Enterococcus faecium} strain previously investigated in our laboratory was undetectable after only 8 min of exposure to porcine gastric juice (pH 2) (13). However, comparing the different studies is complicated by the different types of gastric juice, different pH values, and various starting cell numbers employed.

\textbf{Pig-feeding trial.} Having demonstrated that the porcine intestinal isolates survived, albeit to various degrees, in porcine gastric juice in vitro, a pig-feeding trial was performed to monitor the fate of these strains in vivo in the porcine gut. During the 21-day administration period (days 1 to 22, where day 1 represents the first day of culture administration), each group of pigs ingested \(\sim 10^{10}\) CFU per day of a different \textit{Lactobacillus} or \textit{Pediococcus} strain or the culture mix containing all five strains, also at a mean daily intake of \(\sim 10^{10}\) CFU per day. Selective enumeration of rifampin-resistant variants, in combination with DNA fingerprinting of selected colonies by RAPD PCR, was used to track the fate of the administered strains. A control group received skim milk acidified with lactic acid to pH 4.7, i.e., approximately the same pH as the culture fermentates. Although rifampin-resistant colonies were recovered from the fecal and cecal samples of some control animals during the trial at low counts, RAPD PCR confirmed that these were not any of the administered cultures (data not shown).

\textbf{Survival and persistence of administered strains.} Prior to culture administration (day –5), none of the test strains was detected in the feces of any of the animals, although low background counts of rifampin-resistant bacteria were detected in some animals. By day 3, all culture-fed animals excreted \(10^6\) to \(10^7\) CFU of rifampin-resistant cultures/g of feces and continued to do so at between \(10^7\) and \(10^8\) CFU/g during the remainder of the 21-day administration period (Fig. 2A). For the treatment groups fed individual cultures, RAPD PCR analysis confirmed all rifampin-resistant colonies selected from the fecal samples at day 15 as the administered culture (Fig. 2B). However, even with four animals per group, considerable differences could be seen in excretion rates of the administered cultures during the 21-day administration period. Of all of the individually administered strains, the \textit{L. murinus} strains showed superior intestinal transit, with mean fecal excretion rates of \(1.3 \times 10^8\) and \(4.7 \times 10^7\) CFU/g observed for strains DPC6003 and DPC6002, respectively (Fig. 2A). Both were excreted at significantly higher numbers (\(P < 0.05\)) than \textit{P. pentosaceus} DPC6006 (which had the lowest mean fecal count, \(5.9 \times 10^5\) CFU/g). Furthermore, throughout the administration period, the \textit{L. murinus} strains represented on average 23.7 and 20.8\% of the total fecal \textit{Lactobacillus} population, respectively, which is a higher percentage of total fecal lactobacilli than the other \textit{Lactobacillus} strains administered (Fig. 3). \textit{L. murinus} DPC6003 was also excreted at a significantly higher (\(P < 0.05\)) level than \textit{L. salivarius} DPC6005 and \textit{L. pentosus} DPC6004, both of which were intermediate in their survival ability, being detected at \(5.3 \times 10^5\) to \(5.6 \times 10^6\) CFU/g of feces and representing on average only 1.1 to 1.3\% of total fecal lactobacilli (Fig. 2A and 3). Interestingly, these results correlate to some extent with in vitro findings for survival in gastric juice, where \textit{L. pentosus} DPC6004 demonstrated superior survival, \textit{L. murinus} DPC6002 and DPC6003 and \textit{P. pentosaceus} DPC6006 were intermediate in their survival abilities, and \textit{L. salivarius} DPC6005 was the least tolerant (Fig. 1). Mean total fecal excretion of the administered cultures in the animals fed the 5-strain combination was \(8.2 \times 10^7\) CFU/g, which on average constituted 13\% of the total fecal \textit{Lactobacillus} population and was significantly higher (\(P < 0.05\)) than mean excretion of \textit{P. pentosaceus} DPC6006 (Fig. 2A; Fig. 3). Interestingly, for those animals fed the culture mix, the predominant culture excreted at day 15 by all animals was \textit{L. murinus}, albeit that in some animals \textit{L. murinus} DPC6003 predominated and in others both strains DPC6002 and DPC6003 were detected (Fig. 2C).

The administered cultures could still be detected at day 5 postadministration in fecal samples of the majority of animals (Fig. 2A; Table 1), although none were detected in the two animals per group tested at day 66 postadministration (data not shown). However, the presence of the culture in the feces at day 5 postadministration and the level detected varied depending on the culture fed, and this correlated with the performance of the strain during the 21-day administration period (Table 1). For example, animals fed \textit{L. murinus} DPC6002 and DPC6003 still excreted a high mean level (\(2.4 \times 10^8\) to \(2.8 \times 10^9\) CFU/g) of the administered strains (Fig. 2A), except for animal 423, which was fed strain DPC6003 (Table 1). Animals fed the culture mix also harbored high levels of rifampin-resistant bacteria 5 days after culture administration had stopped, with a mean fecal count of \(2 \times 10^7\) CFU/g obtained for this group (Fig. 2A). Representative colonies isolated at this time point were identified by RAPD as either \textit{L. murinus} DPC6002 or DPC6003 or \textit{L. pentosus} DPC6004, depending on the animal (Fig. 4A and B). This suggests that there was a change in the dynamics of intestinal microbial populations when the strains were no longer administered, as in comparison, \textit{L. murinus} DPC6002 and DPC6003 were identified as the only predominant surviving strains at day 15. \textit{L. salivarius} DPC6005, \textit{L. pentosus} DPC6004, and \textit{P. pentosaceus} DPC6006 were detected at \(10^5\) to \(10^6\) CFU/g of feces at day 5 postadministration in only 2, 1, and 3 animals per group, respectively (Table 1), indicating that they had declined to levels that were too low to be detected in some animals. Interestingly, poor fecal persistence postadministration correlated with lower excretion rates during the administration period. Also, even in animals that harbored high levels of the administered strains at 5 days postadministration, the cultures constituted a lower portion of the total \textit{Lactobacillus} population at this time than during culture administration. Mean values were 0.5, 1.8, 0.02, 0.002, and 10.5\% for cultures DPC6002, DPC6003, DPC6005, DPC6004, and the culture mix, respectively, with large variations between individual animals. This observation that fecal excretion rates decline once culture administration has stopped is common and has been noted previously in pigs (30, 31). Nonetheless, the majority of the strains administered in the present study persisted for at least 5 days postadministration in pig feces, which compares well with previous reports of 3- to 10-day persistence for other \textit{Lactobacillus} strains (30, 34).

We also investigated the ability of the strains to establish themselves in the cecum, as this is one of the principal intestinal sites of \textit{Salmonella} colonization in pigs (42) and was the
FIG. 2. (A) Excretion of administered strains in pigs fed \( \sim 10^{10} \) CFU of each of the porcine cultures/day. *L. salivarius* DPC6005 Rifr (●), *P. pentosaceus* DPC6006 Rifr (■), *L. pentosus* DPC6004 Rifr (▲), *L. murinus* DPC6002 Rifr (X), *L. murinus* DPC6003 Rifr (♦), or a combination of all five cultures (●) was fed from day 1 to 22, where day 1 represents the first day of culture administration. Values are means of the results from 4 pigs (except for the *L. murinus* DPC6002 treatment, where values are the means of the results from 3 pigs), with standard errors of the means indicated by vertical bars. (B) RAPD fingerprints (generated with R1 primer) of representative fecal isolates obtained from selected pigs on day 15 of oral administration of these porcine cultures individually (lanes 2 to 6, 9 to 13, 15 to 18, 20 to 24, and 26 to 30) compared with RAPD fingerprints of the relevant administered strains (lanes 1, 8, 14, 19, and 25, respectively). Lanes 7 and 31 contain a 100-bp ladder. (C) RAPD fingerprints (generated with R2 primer) of representative fecal isolates obtained from selected pigs on day 15 of oral administration of a combination of all five porcine cultures (lanes 6 to 15) compared with RAPD fingerprints of each of the strains administered in the mixture (lanes 1 to 5). Lane 16 contains a 100-bp ladder.
original source of the strains (3). Cecal persistence of the administered cultures was investigated in 2 animals per treatment following slaughter 9 days after culture administration had stopped. Some animals still harbored the cultures at high levels in the cecum, but as at day 5 postadministration, this was very much dependent on the particular strain(s) used (Table 1; Fig. 5). Again the *L. murinus* strains performed well, persisting at the highest level of all the individually administered strains (10⁶ to 10⁷ CFU/g, 0.01 to 1.3% total lactobacilli) (Fig. 5), which was confirmed by RAPD analysis of selected colonies (Table 1). In the mixture-fed animals, the total counts for the administered strains were also high (10⁶ and 10⁷ CFU/g) (Fig. 5) and RAPD PCR identified the predominant persisting strains as *L. murinus* DPC6002 and DPC6003 in one of the animals sacrificed and *L. pentosus* DPC6004 and *P. pentosaceus* DPC6006 in the other animal (Fig. 4C). It is noteworthy that the strains detected in the cecum did not always correspond to those predominating in the feces at day 5 postadministration (Table 1; Fig. 4). When administered individually, *P. pentosaceus* DPC6006, *L. pentosus* DPC6004, and *L. salivarius* DPC6005 persisted poorly in the cecum, which correlates with findings for fecal persistence at day 5 postadministration (Table 1). These strains were detected as part of the predominant microflora in only one of the two animals slaughtered per treatment and then only as a small portion of the predominant colonies analyzed by RAPD PCR (Fig. 5; Table 1).

While further analyses are required to investigate the exact duration of strain retention in the intestine following cessation of administration, *L. murinus* DPC6002 and DPC6003 and *P. pentosaceus* DPC6006, when administered individually, were capable of persisting in the gut, at least for a limited period. In addition, all of the strains in the mixture except *L. salivarius* DPC6005 performed well in the porcine intestine. In comparison, in previous studies, strains of *L. acidophilus* or a 4-strain Lacto-
Shirota strain fed at the same rate was recovered at only 10^4 CFU/g of feces (28). However, it is not always possible to maintain at 10^4 to 10^5 CFU/g for 5 to 7 days postadministration (i.e., days 15, 22, and 26) than prior to or during the first week of culture administration (i.e., days 5, 3, and 8). However, fecal Enterobacteriaceae counts were highly variable throughout the trial, with large variance of values within individual treatment groups and fluctuations observed in counts at different time points.

**Effects on intestinal microflora.** Evaluating the effects of probiotic administration on intestinal microflora is limited by the unculturable nature of the majority of intestinal species and the bias introduced due to the unsuitability of conventional culture methods (37). Nonetheless, we measured total fecal Lactobacillus and Enterobacteriaceae in an attempt to assess any major effects of culture administration on these representative intestinal species.

Examination of Enterobacteriaceae in both the feces and cecum was performed, since this group of gram-negative microorganisms represents pathogenic indicator species, such as *E. coli* and *Salmonella*. The five porcine isolates administered have previously been shown to display in vitro anti-Salmonella activity (3) and these strains also inhibit representative fecal Enterobacteriaceae of pig origin in agar plate assays (data not shown). In the present study, there was evidence that some of the cultures may have influenced fecal Enterobacteriaceae counts in the pigs (Table 2; Fig. 6). On average, when all treatment groups were analyzed together, fecal Enterobacteriaceae counts were significantly lower (*P < 0.05*) towards the end of the culture administration period (i.e., on days 15, 22, and 26) than prior to or during the first week of culture administration (i.e., days 5, 3, and 8). However, fecal Enterobacteriaceae counts were highly variable throughout the trial, with large variance of values within individual treatment groups and fluctuations observed in counts at different time points.

**TABLE 1.** Number of postadministration pig fecal and cecal isolates identified by RAPD PCR as the administered strain(s) presented as a portion of the total isolates examined

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Porcine strain(s) administered</th>
<th>No. of fecal isolates identified as administered strain(s) on day 5 postadministration/total no. of isolates</th>
<th>No. of cecal isolates identified as administered strain(s) on day 9 postadministration/total no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td><em>L. salivarius</em> DPC6005</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>321</td>
<td><em>L. salivarius</em> DPC6005</td>
<td>NG</td>
<td>3/7</td>
</tr>
<tr>
<td>322</td>
<td><em>L. salivarius</em> DPC6005</td>
<td>NG</td>
<td>0/3</td>
</tr>
<tr>
<td>323</td>
<td><em>L. salivarius</em> DPC6005</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>314</td>
<td><em>P. pentosaceus</em> DPC6006</td>
<td>5/5</td>
<td>3/9</td>
</tr>
<tr>
<td>315</td>
<td><em>P. pentosaceus</em> DPC6006</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>316</td>
<td><em>P. pentosaceus</em> DPC6006</td>
<td>0/5</td>
<td>ND</td>
</tr>
<tr>
<td>317</td>
<td><em>P. pentosaceus</em> DPC6006</td>
<td>5/5</td>
<td>0/10</td>
</tr>
<tr>
<td>402</td>
<td><em>L. pentosus</em> DPC6004</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>403</td>
<td><em>L. pentosus</em> DPC6004</td>
<td>0/4</td>
<td>1/9</td>
</tr>
<tr>
<td>404</td>
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<td>0/5</td>
<td>ND</td>
</tr>
<tr>
<td>405</td>
<td><em>L. pentosus</em> DPC6004</td>
<td>0/5</td>
<td>0/10</td>
</tr>
<tr>
<td>408</td>
<td><em>L. murinus</em> DPC6002</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>409</td>
<td><em>L. murinus</em> DPC6002</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>410</td>
<td><em>L. murinus</em> DPC6002</td>
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<td>7/7</td>
</tr>
<tr>
<td>420</td>
<td><em>L. murinus</em> DPC6003</td>
<td>1/1</td>
<td>ND</td>
</tr>
<tr>
<td>421</td>
<td><em>L. murinus</em> DPC6003</td>
<td>5/5</td>
<td>ND</td>
</tr>
<tr>
<td>422</td>
<td><em>L. murinus</em> DPC6003</td>
<td>5/5</td>
<td>10/10</td>
</tr>
<tr>
<td>423</td>
<td><em>L. murinus</em> DPC6003</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>414</td>
<td>Culture mix</td>
<td>2/5 (DPC6002)</td>
<td>ND</td>
</tr>
<tr>
<td>415</td>
<td>Culture mix</td>
<td>3/5 (DPC6003)</td>
<td>1/10 (DPC6004)</td>
</tr>
<tr>
<td>416</td>
<td>Culture mix</td>
<td>2/5 (DPC6003)</td>
<td>9/10 (DPC6006)</td>
</tr>
<tr>
<td>417</td>
<td>Culture mix</td>
<td>1/5 (DPC6003)</td>
<td>8/10 (DPC6003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4/5 (DPC6004)</td>
<td>2/10 (DPC6002)</td>
</tr>
</tbody>
</table>

* ND, not determined in these animals.
* NG, no growth of selected isolates in MRS broth.
* a For pigs receiving the culture mix, the strain identified is given in parentheses.

bacillus mix fed at 10^10 CFU per day accounted for no higher than 2.5% of the total *Lactobacillus* population (30) and a *L. shirota* strain fed at the same rate was recovered at only 10^6 CFU/g of feces (28). However, it is not always possible to compare intestinal survival of administered culture(s) in feeding trials, as investigators often do not enumerate the strains fed (4, 38, 39). In the present study, it was perhaps surprising that *L. murinus*, a species usually associated with mice and rats (18), performed so well in the porcine gut. However, *L. murinus* was recovered relatively frequently from pig cecal samples in an initial screening in our laboratory (3), indicating that it is a common inhabitant of the pig gut and so may possess properties that enable it to compete successfully in that environment. We also observed strain variation in terms of survival and persistence in the porcine gut, as demonstrated by others (20, 30, 31). This indicates that there may be advantages to feeding a culture mix, where if one strain is unsuccessful, others may compensate. Indeed, we found that individual strains within a mix performed differently in individual animals, and in general, the culture combination resulted in high fecal counts and good strain persistence. Interestingly, this is supported by the work of Pedersen et al. (30), who found that when a culture mix of four *Lactobacillus* strains was fed, fecal counts were maintained at 10^6 to 10^7 CFU/g for 5 to 7 days postadministration, whereas an *L. acidophilus* strain alone persisted for only 3 days.
points (Fig. 6). As a result, no significant effects were observed when examining the impact of culture administration on fecal Enterobacteriaceae during the entire culture administration period (day 3 to 22). However, at day 15, mean fecal Enterobacteriaceae counts were significantly lower \( (P < 0.05) \) in animals fed \( L. \) murinus DPC6003 \( (1.6 \times 10^5 \text{ CFU/g}) \) than in either the \( L. \) murinus DPC6002 \( (2 \times 10^7 \text{ CFU/g}) \) or the culture mix \( (8.7 \times 10^6 \text{ CFU/g}) \) groups. Apart from these statistically significant effects, some nonsignificant trends were observed when mean preadministration \( (\text{day } -5) \) counts were compared with those obtained after 21 days of culture administration \( (\text{day } 22) \). For example, in pigs fed strains DPC6005, DPC6006, DPC6004, and the culture mix, Enterobacteriaceae counts decreased \( \sim 10\)-

\[ \text{to } 50\text{-fold from } 7 \times 10^6 \text{ to } 1.3 \times 10^6 \text{ CFU/g to } 5 \times 10^5 \text{ to } 4 \times 10^5 \text{ CFU/g (Table 2). This represents mean reductions of 98, 87, 97, and 97\%, respectively, when the mean preadministration counts within each treatment group are taken as 100\% (Table 2). However, mean fecal counts also decreased by 83\% in the control group (Table 2). Ratcliffe et al. (33) observed similar Enterobacteriaceae-reducing effects in pigs fed acidified milk and concluded that this was due, at least in part, to its low pH and/or lactic acid content. In our study, in all cases, the Enterobacteriaceae reduction persisted through to day 5 post-administration, when numbers declined even further (Table 2). Mean counts in the \( L. \) murinus DPC6003 group also decreased to \( \sim 10^5 \text{ CFU/g at this time point (Table 2), perhaps reflecting} \]

![Image](https://example.com/image.png)

**FIG. 4.** (A) Predominant strains detected in the feces at day 5 postadministration and in the cecal contents at day 9 postadministration (as determined by RAPD PCR fingerprinting of a representative number of isolates) in pigs fed \( \sim 10^{10} \text{ CFU of a combination of the five porcine cultures/day. The combination contained } L. \) salivarius DPC6005 Rif\(^\text{r} \), \( L. \) pentosus DPC6004 Rif\(^\text{r} \), \( L. \) murinus DPC6002 Rif\(^\text{r} \), and \( P. \) pentosaceus DPC6006 Rif\(^\text{r} \). (B and C) RAPD fingerprints (generated with R2 primer) of representative fecal (B) or cecal (C) isolates obtained from selected pigs fed this 5-strain combination on day 5 or 9 postadministration (lanes 6 to 15, respectively) compared with RAPD fingerprints of each of the strains administered in the mixture (lanes 1 to 5). Lane 16 contains a 100-bp ladder.
the fact that Enterobacteriaceae counts in this treatment group were significantly lower ($P < 0.05$) than in some other groups at day 15, as outlined above. In addition, mean Enterobacteriaceae in the cecal contents of the two animals per treatment sacrificed did not differ substantially between treatment groups (Fig. 7). However, animals fed $L. \text{ murinus}$ DPC6003 and $L. \text{ salivarius}$ DPC6005 had $\sim$10-fold-lower counts than those fed $L. \text{ pentosus}$ DPC6004, $L. \text{ murinus}$ DPC6002, or the culture mix ($\sim 2 \times 10^6$ versus $\sim 1 \times 10^7$ CFU/g), although there was no correlation between lower Enterobacteriaceae counts and low cecal pH (Fig. 7).

Interestingly, although $L. \text{ murinus}$ DPC6002 and DPC6003 were excreted at the highest level in the feces, they did not result in Enterobacteriaceae reductions (although $L. \text{ murinus}$ DPC6003 may have had some inconsistent effects). This correlates with in vitro findings for Salmonella inhibition, with $L. \text{ salivarius}$ DPC6005, $P. \text{ pentosaceus}$ DPC6006, and $L. \text{ pentosus}$ DPC6004 showing greater inhibitory activity than $L. \text{ murinus}$ DPC6002 and DPC6003 (3). However, due to the variations observed in counts between individual animals and at different time points and the fact that a reduction was also observed in the control group, it is difficult to draw clear conclusions on the effects of culture administration on intestinal Enterobacteriaceae.

Administration of lactic acid bacteria has been shown to reduce intestinal coliform and Enterobacteriaceae counts in the majority of previous studies (4, 22, 24, 28, 38, 39), although some have seen no effects (7, 13). The trend towards positive effects observed in ours and other studies, although not significant, should not be ignored, as individual variations in the responses of different animals are to be expected due to the complexity of the intestine (36). Furthermore, it has been suggested that hygienic conditions in scientific institutes may be too favorable to investigate effects on pathogenic bacteria without deliberate challenge (36). Future experiments with larger treatment groups and deliberate infection with Salmonella should provide further information on the possible pathogen-lowering ability of the potentially probiotic cultures investigated in the present study.

While others have found increases in total lactobacilli as a result of administration of Lactobacillus strains (20, 28, 38), no significant differences in total fecal Lactobacillus numbers were observed between any of the treatment groups in the present study (Fig. 8). However, nonsignificant $\sim$10-fold increases (from $\sim 10^6$ to $\sim 10^9$ CFU/g) were observed in pigs fed $P. \text{ pentosaceus}$ DPC6006, $L. \text{ pentosus}$ DPC6004, and $L. \text{ murinus}$ DPC6002 and DPC6003 when preadministration (day $-5$) fecal counts were compared with counts after 21 days of culture.

### TABLE 2. Effect of administration of porcine cultures on mean fecal Enterobacteriaceae counts in pigs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day $-5$ count* (baseline)</th>
<th>Day 22 count* (after 21 days of culture administration)</th>
<th>% Reduction after 21 days of culture administration$^a$</th>
<th>Day 5 postadministration count*</th>
<th>% Reduction up to 5 days postadministration$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>7.39 ± 0.36</td>
<td>6.63 ± 0.8</td>
<td>83</td>
<td>5.45 ± 1.5</td>
<td>99</td>
</tr>
<tr>
<td>$L. \text{ salivarius}$ DPC6005</td>
<td>7.42 ± 0.77</td>
<td>5.67 ± 1.22</td>
<td>98</td>
<td>5.41 ± 0.86</td>
<td>99</td>
</tr>
<tr>
<td>$P. \text{ pentosaceus}$ DPC6006</td>
<td>6.87 ± 1.6</td>
<td>6.0 ± 0.52</td>
<td>87</td>
<td>4.64 ± 0.36</td>
<td>99.6</td>
</tr>
<tr>
<td>$L. \text{ pentosus}$ DPC6004</td>
<td>8.1 ± 0.73</td>
<td>6.58 ± 0.53</td>
<td>97</td>
<td>6.06 ± 1.15</td>
<td>99</td>
</tr>
<tr>
<td>$L. \text{ murinus}$ DPC6002</td>
<td>6.24 ± 1.21</td>
<td>6.59 ± 0.53</td>
<td>0$^d$</td>
<td>6.51 ± 1.53</td>
<td>23</td>
</tr>
<tr>
<td>$L. \text{ murinus}$ DPC6003</td>
<td>6.56 ± 0.57</td>
<td>6.69 ± 0.63</td>
<td>0$^d$</td>
<td>5.78 ± 0.64</td>
<td>84</td>
</tr>
<tr>
<td>Culture mix</td>
<td>7.22 ± 0.31</td>
<td>5.74 ± 0.96</td>
<td>97</td>
<td>5.47 ± 0.65</td>
<td>98</td>
</tr>
</tbody>
</table>

$^a$ Mean values of results for four pigs in log CFU/gram of feces ± SD, except for $L. \text{ murinus}$ DPC6002 treatment, which is the mean value of the results for three pigs ± SD.

$^b$ Calculated as ($N_a - N_b/N_b$) × 100, where $N_a$ is the mean day $-5$ count and $N_b$ is the mean day 22 (both expressed as CFU/gram of feces).

$^c$ Calculated as ($N_a - N_b/N_b$) × 100, where $N_a$ is the mean day $-5$ count and $N_b$ is the mean day 5 postadministration count (both expressed as CFU/gram of feces).

$^d$ Counts increased in these treatment groups.

FIG. 5. Counts on MRS-RIF at day 9 postadministration in the cecal contents of individual pigs administered porcine cultures, as indicated.
administration (day 22) (Fig. 8). A similar nonsignificant increase was also seen in the control group, which contradicts the findings of Ratcliffe et al. (33), who observed a decrease in lactobacilli in pigs fed acidified milk. Interestingly, although L. salivarius DPC6005 produces a bacteriocin with anti-Lactobacillus activity in vitro (data not shown), this strain did not reduce total fecal Lactobacillus populations. However, as we have limited knowledge on its spectrum of activity, it is difficult to say whether this bacteriocin inhibits any of the Lactobacillus species present in the porcine gut. Mean Lactobacillus counts in the cecum show little correlation with fecal counts and were 10-fold higher in pigs fed L. murinus DPC6003 and L. salivarius DPC6005 (2.8 × 10^8 to 3.9 × 10^8 CFU/g) than in pigs fed control milk or L. pentosus DPC6004 (3.2 × 10^7 to 4 × 10^7 CFU/g) (Fig. 7).

We have demonstrated that porcine intestinal isolates, selected on the basis of in vitro probiotic criteria, can be effectively delivered to the porcine GIT by oral administration either individually or as a strain combination. All five strains investigated survived gastrointestinal transit in pigs, and some may have the ability to reduce undesirable microorganisms in the gut. However, it was evident that certain cultures survived at higher numbers in the porcine gut, persisted for longer in the cecum after culture administration had ceased, and were
more efficacious in reducing pathogenic indicator species. This strain variation highlights the advantages of using combination probiotics. Based on the results of this study, the most promising strains or strain combinations will be selected for administration to larger numbers of animals in future trials. We conclude that, although further characterization of their in vivo efficacy is necessary, the present findings provide a strong basis to explore the potential of these porcine-derived isolates as probiotic feed additives for pigs.

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