Selection of porcine intestinal isolates as probiotics for pathogen reduction in pigs

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Abstract: Putative lactobacilli were isolated from the porcine small intestine and assessed in vitro for potential probiotic traits. Viability of all isolates tested was unaffected at porcine bile concentrations of 0.3 % (w/v), with some tolerating up to 3.5 % (w/v) bile. Randomly amplified polymorphic DNA (RAPD) and/or pulsed field gel electrophoresis (PFGE) were used to genetically fingerprint the porcine isolates. Some of these cultures demonstrated antagonistic activity against pathogens Escherichia coli O157:H45 and Salmonella typhimurium DT104 when assayed on agar plates and in co-culture. For example, no Salmonella were detected after 8 h of associative culture with one intestinal isolate, L. salivarius B-24. However, when co-cultured under constant pH conditions, no inhibition was observed, indicating that acid may be one of the mechanisms involved in the antimicrobial activity of this strain. Assessment of these strains is ongoing with a view to the development of probiotic feed additives which could potentially reduce pathogen carriage in pigs.

Keywords: probiotic, Lactobacillus, Salmonella, Escherichia coli, antimicrobial

Introduction: Pigs have been identified as a principle reservoir of enteropathogens, such as Salmonella (Davies et al., 2000). Probiotic microorganisms aimed at restoration and maintenance of a healthy gut microflora offer potential as a means of controlling such enteric pathogen carriage in farm animals. Protective cultures have principally been applied to poultry; for example, an FDA-approved defined mix of 29 bacterial strains significantly reduces Salmonella carriage in chickens (Nisbet, 1998). Inoculation with probiotic bacteria can also reduce faecal shedding of E. coli O157:H7 in both cattle and lambs and Salmonella colonisation in pigs (Nisbet, 1998). Although the exact mode of action by which microbial cultures reduce intestinal carriage of pathogens is unknown, suggested mechanisms include; competitive exclusion and the production of inhibitory compounds, such as organic acids, hydrogen peroxide and bacteriocins.
The use of high levels of viable microorganisms selected in vitro on the basis of these criteria may improve probiotic efficacy in vivo. The objective of this study was to isolate and characterise probiotic bacteria with the potential to reduce enteric pathogen carriage in pigs.

**Materials and Methods:** **Source and maintenance of cultures** Washed porcine duodenum samples obtained at slaughter were homogenised and both the washings and homogenate were diluted 10-fold in maximum recovery diluent and pour-plated on *Lactobacillus* selective (LBS) agar. Following anaerobic incubation at 37 °C for 5 days, selected colonies identified as Gram positive, catalase negative rods were stored at -80 °C in MRS containing 40 % glycerol and thereafter, were routinely cultured in MRS broth anaerobically at 37 °C. The isolate used in co-culture experiments was identified as *L. salivarius* ssp. *salivarius/salicinius* by SDS-PAGE analysis of total cell protein (Pot et al., 1993). *S. typhimurium* DT104 isolated from a pig carcass and *E. coli* 0157:H45, both grown at 37 °C, in brain heart infusion broth and M17 supplemented with 0.5 % (w/v) glucose, respectively were used as indicators to assess antimicrobial activity.

**Assessment of bile tolerance** Overnight MRS broth cultures of each of the porcine isolates were streaked on MRS agar containing 0, 0.3, 0.5, 0.8, 1.0, 1.3, 1.5, 1.8, 3.0 or 3.5 % (w/v) porcine bile. Plates were examined for growth after 2 d of anaerobic incubation at 37 °C.

**Genetic fingerprinting by RAPD and PFGE** For RAPD analysis genomic DNA was isolated from each culture, PCR was performed using a primer of arbitrary sequence and products were analysed on agarose gels (Gardiner et al., 1998). For PFGE analysis high molecular weight genomic DNA was isolated, digested with *Apa I* or *Sma I* and products separated using a CHEF (DR III) PFGE system.

**Determination of antimicrobial activity** Aliquots (50 μl) of cultures of each of the porcine isolates or the corresponding cell-free supernatants were added to wells in plates seeded (0.05-0.5 %) with the indicator organisms and the plates were examined for zones of inhibition after overnight incubation at 37 °C. One selected porcine isolate was also examined in co-culture with *S. typhimurium* DT104 or *E. coli* 0157:H45. In some experiments the growth medium was maintained constant at pH 6.8 by the automatic addition of 4 N NaOH. One pathogenic microorganism together with *L. salivarius* B-24 were inoculated (1 %) into 150 ml of MRS broth and incubated at 37 °C. Pure MRS broth cultures of each strain were used as controls. *Lactobacilli*, salmonellae and *E. coli* were enumerated at intervals by spread-plating on LBS, xylose lysine deoxycholate (XLD) and violet red bile (VRB) agars, respectively.
Results and Discussion: This study initially set out to generate a bank of bacterial isolates of porcine origin for potential use as probiotics in pigs. Forty four isolates from the porcine small intestine of 2 pigs were identified as putative lactobacilli (Gram positive, catalase negative rods). While RAPD differentiated only 11 strains within a group of 19 isolates from one animal, PFGE proved more discriminatory and distinguished each of the 19 isolates as individual strains (data not shown). Bile tolerance of 14 of the porcine strains was assessed in vitro in order to evaluate their potential to survive small intestinal transit in pigs. Viability was unaffected at porcine bile concentrations considered physiologically relevant (0.3 % w/v) and 12 isolates tolerated concentrations as high as 3.5 % (w/v).

Figure 1. Co-culture of L. salivarius B-24 with S. typhimurium DT104 in MRS broth at 37 °C (A) without pH control and (B) maintained at a constant pH of 6.8. Viable cells of S. typhimurium in pure ( ) and mixed ( ) cultures and of L. salivarius B-24 in pure ( ) and mixed (×) cultures. Results are means of duplicate experiments with SD indicated by vertical bars.

Preliminary plate assays set up to assess the antimicrobial activity of the porcine isolates, revealed that certain strains exhibited antagonistic activity against indicator strains of E. coli and S. typhimurium. Further characterisation of one isolate identified as L. salivarius showed that E. coli O157:H45 numbers were reduced 1000-fold (to 10^3 cfu/ml) over a 24 h period as a result of co-culture with this strain, while a 100-fold increase in E. coli levels was observed in the control culture without lactobacilli added (data not shown). L. salivarius B-24 also inhibited the growth of S. typhimurium DT104 with no Salmonella detected after only 8 h of associative culture (Fig. 1A). At the end of this 8 h period the pH of the associative culture had decreased to 4.0, while the pH of the S. typhimurium control culture was 5.9, suggesting that the inhibitory effect may be due to acid
production by *L. salivarius* B-24 or that acidic conditions augmented the antimicrobial activity of other inhibitory factors. Further co-culture experiments conducted at constant pH (6.8) confirmed this, as no salmonella inhibition was observed (Fig. 1B).

**Conclusions:** A bank of putative lactobacilli of porcine intestinal origin was generated and isolates were identified which exhibited antimicrobial activity against pathogens *in vitro*, possibly through the production of acid. Further characterisation is required *in vivo* in order to assess the effectiveness of these isolates as probiotic feed additives for the control of enteric pathogen carriage in pigs.

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**References:**