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Indigenous Porcine Gastro-intestinal Tract as Veritable Source of Probiotic Isolates

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Piglets weaning age is usually fast tracked to between 3-4 weeks for economic reasons. This burdens the immature gastro-intestinal tract of the weaned piglets with associated post weaning diarrhoeal syndromes (PWDs). In the European Union, the use of antibiotics as growth promoter in animal feed has been banned since 2006, so, in an attempt to finding alternative to the management of PWDs, this research was designed to isolate and characterize probiotic from indigenous South African Windsnyer pig breed. Four isolates namely: *Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC 13956 were characterized based on their probiotic attributes. The isolates resisted bile salt up to 1.2%v/v and survive at low pH of 3 with OD_{590} values ranging from 0.45 to 1.26 after 12 h incubation. From the adherence and survival assay, the average viable cell count after 6h incubation is 5.15×10^8 cfu/mL, with the highest viable count of 9.4×10^8 cfu/mL observed in *Lactobacillus salivarius* ZJ614 after 6 h of incubation. These results revealed probiotics that could be used in pig husbandry, efforts are on-going to further characterise these probiotics, develop a porcine gastro-intestinal model and ultimately establish an *in vivo* protocol.

Key words: post weaning diarrhoeal syndromes, antibiotics, probiotics, Gastro intestinal tracts, bile salt, viable cell, porcine.

Piglets weaning age is habitually fast tracked to between 3-4 weeks for economic motives. This burdens the immature gastro-intestinal tract of the weaned piglets with accompanying post weaning diarrhoeal syndromes (PWDs). The weaning progression increases the stress on piglets, and thus their vulnerability to viral and bacterial infections. Weaning causes stress to pigs due to the concurrent effect of several physiological, psychological and ecological factors i.e. introduction of adult feed, separations from sows, new environment, forced living together with other pigs in large groups etc.[1]. Nonetheless, the microbial factor plays a critical role in post-weaning diarrhoea[2]. Diarrhoea or scours in piglets is very common at the post-weaning period, it is one of the foremost causes of mortality in piglets; often associated with poor sanitation, inappropriate husbandry, traumatic environment and inappropriate feeding factors. Potential etiological agents of diarrhoea include *Escherichia coli* i.e. enterotoxigenic *E. coli* (“!), enterohaemorrhagic *E.coli* (EHEC)or verocytotoxin *E.coli* (VTEC) and shiga toxin producing *E.coli* (STPEC) *Rotavirus*, transmissible gastroenteritis (TGE), salmonellosis, *Campylobacter* and *Brachyspira hyodysenteriae*[3–5].

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Gastrointestinal disorders in pigs are a great challenge to intensive pig farming. They cause substantial economic losses related to mortality, stunted growth and prolonged time for reaching slaughter weight[6]. Therefore, gastrointestinal diseases of growing pigs are economically important for pig production worldwide and enteric bacterial infections are often treated with antimicrobials[7].

A study conducted in Denmark showed that prescriptions for weaner pigs accounted for more than one third of the total antimicrobial consumption in pigs and that gastrointestinal disease were the most common indications for prescriptions in this age group[2]. In-feed antibiotics have played a major role in managing PWDs; but the use of antibiotics as growth promoters in animal feed has been banned in most parts of the world, because of the accompanying spreading of antibiotic resistance genes to humans. Antimicrobial resistance in pathogenic bacteria is a global threat and therefore increasing attention is being paid to the prudent use of antibiotics in food-producing animals[6] thus, the need to identify alternatives to antibiotics.

Probiotics are considered alternatives in this regard, they maintain the balance in intestinal microbiota, enhance the digestion of feed, reduce the activity of carcinogenic enzymes, lower serum cholesterol, stimulate immune response, prevent digestive disorders, etc. Probiotics are most effective when administered at weaning. This research was designed to isolate and characterise potential probiotic from indigenous pigs; these pigs have proven records of tolerance to diseases, high weaning rates and low incidences of PWDs.

METHODS

Raising of Piglet

Piglets used in this study were of the South African indigenous Windsnyer breed and were all raised on the Agricultural Research Council research farm, and approval of the research institute animal ethical committee was sought and obtained before the commencement of this research. Piglets and sows were kept in pens of 6.25 m^2. A farrowing crate (2.4 m by 0.9 m) was installed in the centre of each pen to prevent the sow from falling onto or trampling on the piglets. Each pen was furnished with a 260 Watt infrared element. Ammonia and methane gasses emanating from the faeces were extracted through roof canopies. At the rear of the pen is a flush channel, covered with steel grid, was disinfected on a routine basis. Twelve sows with their new-borns were selected at random and kept in separate pens for the first ten days after birth. Sows received their normal feed and pre-farrowing treatments as part of a routine procedure. Piglets were fed in a creep feeder located at the back of the pens. Water was supplied with nipples. As from day 11, the piglets and sows were divided into eight groups of six to eight piglets per group. Eight suckling piglets (1per group) were selected prior to weaning, and were all sacrificed humanely at weaning for the isolation of probiotics.

Isolation of Potential Probiotics

Piglets with body weight of between 10.0 - 12.0 kg were selected, one from each of the eight groups (one piglet representative of each group). After slaughtering, a sample (5 cm in length) was cut from the ileum of each piglet. The ileum sample was cut open aseptically on the side and the mucus layer scraped from the surface with a sterile glass slide as aseptically as possible. One ml of mucus sample was dispensed into 9 ml sterile saline solution, serially diluted and plated on MRS agar (selective medium for the cultivation of lactic acid bacteria). Colonies were counted after 24 h anaerobic incubation at 37^0C to determine the colony forming unit (cfu) count of lactic acid bacteria present in each ileum sample collected from the eight piglets. Bacterial colonies on MRS plates were picked randomly and streaked out for pure culture determination. Selected colonies were stored in MRS broth overlaid with sterile glycerol solution (40% v/v, final concentration) at -70^0C. Gram staining was performed on these colonies; slides were viewed under a phase contrast Microscope. The catalase test was then done on Gram-Positive strains. Lactic acid bacterial are Gram-positive and Catalase-negative. Eighteen pure strains met these criteria, and were selected and stored as potential probiotic bacteria in MRS broth overlaid with sterile glycerol solution at -70°C. The 18 isolates were later screened for various probiotic bacteria properties which include:

Growth at Low (acidic) pH

The method of Maré (2005)[8] was used to determine the growth of selected potential

probiotic bacteria at different pH. A range of MRS broth was adjusted from pH 2.0 to pH 4.0, with 1M HCl and autoclaved at 121^{0}C for 15 min. Accuracy of the adjusted pH was monitored using a calibrated pH meter. The wells of the Microtitre plates were filled with 180 µl of the pH adjusted media and inoculated with 20 µl of an overnight culture. One hundred and eighty microliter of the pH adjusted media and 20 µl of the broth without inoculum served as controls. Growth in the test cultures were monitored at one hour interval for 5 h by measuring the absorbance at 590_{nm} using a Microtitre plate reader. The 0 h reading was taken just before the reaction mixture plate was incubated at 37^{0}C.

Resistance to Bile salts assay

This assay was performed according to the method described by Chateau and co-workers with little modifications[9]. The bile tolerance of the LAB isolates was tested using sterile flat-bottom 96-well Microtitre plates. MRS broth was supplemented with Oxgall to 0.3%, 0.6%, 0.8%, 1.0% and 1.2% (w/v), respectively and autoclaved at 121^{0}C for 15 min. The wells of the Microtitre plates were filled with 180 µl of the bile-containing media inoculated with 20 µl of an overnight potential probiotic culture. One hundred and eighty microliter of the bile-containing media and 20 µl of the broth without inoculum served as controls. Growth in the test cultures was monitored at one hour interval for 5 h by measuring the absorbance at 590_{nm} using a Microtitre plate reader. The 0 h reading was taken just before the reaction mixture Microtitre plate is incubated at 37^{0}C.

Antimicrobial activity against pathogens

The ability to produce antimicrobial compounds was tested by screening for activity against a panel of indicator pathogenic strains. Fresh 18 h LAB MRS Culture supernatants were collected by centrifugation (15,000g, 15 min, 4^{0}C). The cell free supernatants were then adjusted to between pH 6.5 to 7.0 with sterile 1M NaOH and then stored at -80^{0}C. The antimicrobial activity was tested using well diffusion assays. In the well diffusion assays, 1×10^5 CFU/ml of the pathogenic strains were incorporated into soft agar (1%, v/v) plates of MRS. LAB supernatant samples (1 ml) were pipetted into holes drilled into the agar and MRS broth adjusted at pH 6.5 served as control. Plates were incubated for 12 to 18 h at 37^{0}C and examined for zones of growth inhibition. Antimicrobial activity was recorded as growth free inhibition zones around the well. All tests for each strain were performed in triplicate. A zone of at least 10 mm in diameter was regarded as positive for the production of an antimicrobial compound.

Adhesion and survival in ileum model assay

The method of Brink et al.,[10] was used, porcine ileum was aseptically dissected and kept on ice for a maximum time of 9 h Isolates (LAB) were inoculated (2% v/v) into 250 ml MRS broth and incubated at 37^{0}C to OD 1.2 at 600_{nm}. A section of ileum was added to one set of the cultures and incubated for 6 h at 37^{0}C on a rotary water shaker. Sample of the culture was drawn every 2 h, serially diluted and plated on MRS agar. Colonies were counted after 14 h incubation at 37^{0}C. The ileum sections were aseptically removed from the flasks and the mucus layer scraped off with a sterile glass slide. Preparations of the mucus samples on microscope slides were treated with LIVE/DEAD® BacLight™ Bacterial Viability Kit, and left for 10 min in the dark at room temperature and viewed on an Epi-flourescent microscope (Nikon Eclipse 50i) [11]. This method determines both the adherence and survival of the cells.

Data processing and statistical analysis

SPSS version 12.0.1 for Windows (SPSSInc., Chicago, IL, USA) was used for statistic analysis of the data. P-Values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Isolation and identification of potential probiotics

Eighteen pure cultures of lactic acid bacteria (LAB) were isolated with only four showing potential of novel probiotic. Combinations of both the analytical profile index, using API CHL50 identification kits (API® bioMerieux, SA) and molecular (16S ribosomal RNA) methods were used in identification of the four probiotics. Preliminary confirmation was done with the API CHL 50 identification kits and then confirmed with partial sequencing of the 16S rRNA gene of the four potential probiotics; the four identified and characterized bacteria are: *Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC 13956.

LABs are established early in piglet

intestine, and although succession occurs throughout lifetime of the pigs, they may remain as one of the predominant elements of the bacterial community[12]. Other lactobacilli that have been isolated and identified previously from porcine ileum include, among others, *Lactobacillus agilis,* and even *Lactobacillus delbrueckii* which primarily in habit food stuffs[13]. In this work, we isolated and identified four obligate hetero fermentative LAB species: *Lactobacillus reuteri* ZJ625, *Lactobacillus reuteri* VB4, *Lactobacillus salivarius* ZJ614 and *Streptococcus salivarius* NBRC 13956 respectively. Similar results were reported by [14, 15] in intestinal tract of healthy adult pigs. Those results indicated that isolated species are maintained in the intestine throughout the whole life of pigs [12, 16].

Growth at Low pH

The results for growth in acidic pH for the four isolates revealed that they were able to survive at low pH of 3 with OD_{590nm} values ranging from 0.45 to 1.26 after 5 h incubation (Table 1). The highest OD_{590nm} value of 1.2 at pH 3 was recorded for *Lactobacillus reuteri* ZJ625 after 5 h of incubation. *Streptococcus salivarius* NBRC 13956 was the weakest of all the four isolates tested in their ability to grow at low pH, after incubation for 5 h, the isolate could only grow to absorbance of 0.61 at OD_{590nm}. Overall, there is an appreciable growth in all the four isolate at pH 3, which indicate their ability to survive in acidic environment.

Probiotic microorganisms need to resist the adverse factors in the gastrointestinal tract when they pass through it, like the stomach acidity and bile salts, excreted in duodenum. In general, variable results have been documented in respect to the resistance of low pH of the LAB[17]. The *Lactobacillus genus* has optimal growth in pH 6.0. It is characterized by its capacity to produce lactic acid mainly, which creates environments with pH up to 4.0, where they are able to remain viable for variable periods, depending on the activity of their H^+-ATPase[18]. Some authors have reported that the conjugated salts, mainly the glycodeoxicolic acid, are lethal for this bacterial genus and the mortality rate increases as pH diminishes. One of the baseline properties for probiotics is the ability to survive in the upper GI tract[19,20]. The gastric pH of a suckling piglet can reach values below three, and one to two weeks after weaning it can be as low as 1.6–3.9[21]. Studies have shown that the pH values recorded in the stomach one centimeter distal from the pylorus were between 5.1 and 6.5 in suckling piglets, between 4.2 and 6.1 at the moment of weaning, between 3.2 and 5.8 one week post weaning, and between 2.4 and 6.0 two weeks post weaning[21]. The relatively high pH (remained above 2.5) in the stomach of a suckling and recently weaned piglets can be explained by several factors. The stomach of young piglets has not yet developed the capacity to secrete hydrochloric acid [22]. The sow milk does not stimulate this secretion and has considerable buffering capacity. Lastly, lactic acid production from LAB in the upper alimentary tract of piglets partially suppresses hydrochloric acid production [22]. This relatively high pH in the stomach, at weaning age may explain the isolation of only few (in this case, four) lactic acid bacteria from the just weaned porcine ileum.

Resistance to Bile salts assay

The entire four identified LAB were able to resist bile salt up to 1.2%v/v (Fig. 1), though with a longer lag phase of between 2-4h. The best bile salt resistance activity was observed in *Lactobacillus reuteri* VB4 and *Lactobacillus reuteri* ZJ625. The two isolates were able to tolerate

Table 1. Effects of low pH on the growth and survival of the ileum isolates

Time	Average of triplicate Absorbance values at 590nm for pH3			
(h)	*Lactobacillus reuteri* VB4	*Lactobacillus reuteri* ZJ625	*Lactobacillus salivarius* ZJ614	*Streptococcus salivarius* NBRC 13956
0.00	0.450	0.450	0.450	0.450
1.00	0.485	0.489	0.533	0.452
2.00	0.677	0.743	0.551	0.457
3.00	0.762	0.974	0.558	0.557
4.00	0.889	1.135	0.755	0.581
5.00	1.004	1.275	0.873	0.61

Table 2. The antibacterial sensitivity patterns of Potential probiotics and reference probiotics against the test reference pathogenic bacterial strains

Test reference bacterial strains	Probiotic Zones of inhibition in (±mm)*					
	Lactobacillus reuteri VB4	*Lactobacillus reuteri* ZJ625	*Lactobacillus salivarius* ZJ614	*Streptococcus salivarius* NBRC 13956	*Lactobacillus plantarum* ATCC 8014	*Lactobacillus casei*ATCC 393
Pseudomonas aeruginosa ATCC 19429	19.00	12.5	25.00	17.00	15.00	15.00
Escherichia coli ATCC 35218	15.00	15.00	22.00	11.00	15.00	20.00
*Staphylococcus aureus*ATCC 33591	0.00	0.00	12.00	0.00	13.00	17.00
*Bacillus cereus*ATCC 10876	0.00	0.00	14.00	0.00	10.00	15.00
*Salmonella typhimurium*ATCC 49416	20.00	16.50	17.00	15.00	12.00	15.00
*Serratia marcescens*ATCC 14041	15.00	12.00	12.00	10.00	0.00	15.00
*Vibrio paraheamolyticus*ATCC 17802	21.50	20.00	24.00	0.00	30.00	10.00
*Proteus vulgaris*ATCC 6380	15.00	20.00	30.00	20.00	22.00	29.00
*Enterococcus faecalis*ATCC 49532	20.50	17.00	21.00	22.00	18.00	22.00
Shigella flexineriiATCC12022	14.00	16.50	22.00	10.00	18.00	20.00

Key: ATCC = American typedculture collection; (mm)** = Mean of three replicates; 0.00 = No activity; NBRC = Biological Resource Center

and grow in 1.2% v/v bile salt to absorbance of approximately 0.6 from 0.4 after 4 h incubation at $OD_{590nm.}$

After passage through the stomach, the bile-rich environment of the small intestine can also be damaging to bacteria. It has been found that biliary salts hydrolases produced by some *Lactobacillus strains*, are involved in the resistance[23-25]. One of the requirements of an ideal probiotic for inclusion in animal diet includes resistance to bile salts[26]. The concentration and residence time of bile varies in the different section of the GIT. Suckling piglets have less bile acid and thus also lessactive hydrolysis compared to weaned piglets [27].The enzyme cholesterol 7 α- and 27-hydroxylases that determine the rate at which bile acid is formed is present at lower concentrations during the first 21 days of the suckling period, but increases in piglets between 21 and 49 days old[28]. Piglets consume high-fat milk, and have no problems with digestion of the fat. Neither the relatively lower bile acids nor the lower enzyme activity that regulates the bile acid synthesis affects the ability of piglets to produce sufficient bile acids to digest and absorb milk fat[28].

Antimicrobial activity against pathogens

Table 2 present the results of the abilities of the potential probiotics to inhibit the growth of enteric bacteria that have been implicated in post weaning diahorreal syndrome (PWDs) in piglets. The production of antimicrobials and antagonistic abilities of the four isolates were compared to those of two standards LAB (*Lactobacillus plantarum* ATCC 8014 and *Lactobacillus casei* ATCC 393). Zones of inhibition ranged from 10–30 mm for the entire LAB isolates. The least activity with an inhibition zone diameter (IZD) of 10 mm was observed in *Streptococcus salivarius* NBRC 13956 against *Serratia marcescens* ATCC 14041 and *Shigella flexinerii* ATCC12022 and the highest antibacterial activity was observed in *Lactobacillus salivarius* ZJ614 against pathogenic strain of *Proteus vulgaris* ATCC 6380 with zone of inhibition of 30 mm diameter. The MRS culture supernatants of the four LAB strains showed antagonistic effect on the growth of enteric pathogens tested. Probiotic bacteria produce organic acids, hydrogen peroxide and bacteriocins as antimicrobial substances that suppress the multiplication of pathogenic bacteria[29]. The most

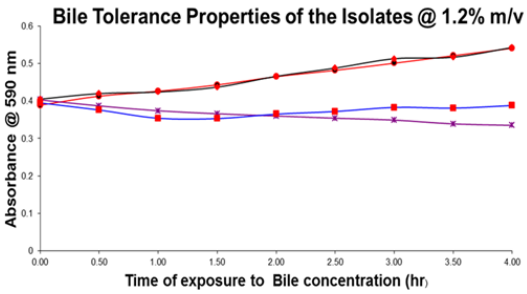
Table 3. The Adherence to the ileum model and viability studies of the isolates showing the viable counts from 0 - 6 h of incubation

Time (h)	*Streptococcus salivarius* NBRC 13956(cfu/ml)	*Lactobacillusreuteri* ZJ625 (cfu/ml)	*Lactobacillus salivarius* ZJ614 (cfu/ml)	*Lactobacillus reuteri* VB4 (cfu/ml)
0.00	1.90×10^7	1.60×10^7	1.63×10^7	1.79×10^7
6.00	5.15×10^8	5.75×10^8	9.4×10^8	7.03×10^8

widely produced anti-microbial substances are organic acids, especially lactic and acetic acids, and consumption of certain probiotic strains reduces the intestinal pH and inhibits the growth of enteric pathogens[30]. Probiotic bacteria can maintain immune homeostasis and treat gastrointestinal diseases; including infectious diarrhoea in children, recurrent *Clostridium difficile*- induced infection, and some inflammatory bowel diseases by inhibiting the growth of enteric pathogens[31]. Candela *et al.*[32] reported that *Lactobacillus acidophilus* Bar13 could protect enterocytes against acute inflammatory response by inhibiting enteric pathogens *S. typhimurium* and *E. coli* H10407.

Adhesion and survival in ileum model assay

From the adherence and survival assay, the lowest LAB viable cell count after 6 h of incubation is 5.15×10^8 cfu/mL observed in *Streptococcus salivarius* NBRC 13956, and the highest viable count of 9.4×10^8 cfu/mL observed in *Lactobacillus saliviarus* ZJ614 after 6 h of incubation. Adhesion is considered an important property of probiotic LAB, i.e. ability to colonise



= *Lactobacillus reuteri VB4*
◊ = *Lactobacillus reuteri ZJ625*
□= *Lactobacillus salivarius* ZJ614
x = *Streptococcus salivarius* NBRC 13956

Fig. 1. The ability of the potential probiotics to resist bile salt exposure at 1.2% m/v bile salt

the gastrointestinal tract which is essential for the exertion of a beneficial effect in the host. The adhesion ability to intestinal mucosa is one of the most crucial selection criteria forprobiotics[33-35] reported that Immunostimulatory activity of *Lactobacillus reuteri* DC421 and *Lactobacillus plantarum* 2035 strains occurs in association with their adhesive capacity. *Lactobacillus* species have been known to prevent gastrointestinal infections by competing with pathogens to adhere to the gastro-intestinal tract[35]. Preising *et al.* [34] reported that Bifidobacteria strains showing higher adhesion to IECs also hada higher anti-inflammatory capacity[36]. Kotzamanidis *et al* reported that *L. plantarum* strains had high adhesion to HT-29 cells and strongly antagonised the adhesion and invasion of entero invasive *E. coli* to the HT-29 cells[35]. The cell surface protein plays an important role in the adhesive property of *Lactobacillus*. It has been confirmed that the adhesion promoting factors of *Lactobacillus* strains were protein aceous components[37-38]. MacKenzie and co-workers demonstrated that proteins involved in mechanisms of the adherence of lactobacilli to the host, although present inmany *L. reuteri* isolates, show a high genetic heterogeneity among strains[39]. May be the difference in the adhesive properties among the four LAB isolates tested in this study was ascribed to the cell surface protein of the strains. The protective activity of the four LAB isolates against pathogens adhering and invading intestinal epithelial cells will be further studied.

CONCLUSIONS

Use of antibiotics as growth promoter in animal feed has been banned in the European Union since 2006 because of development and spread of antibiotic resistant bacteria. Need for research into alternatives to antibiotics is becoming

urgent in South Africa if niche export markets are to be identified and developed. New export market for the pork industry would be identified and antibiotic free meat products could be delivered both locally and internationally. A complete new income sector for the pork industry could be established. Our results were able to identify potential probiotics that could be used in pig husbandry, efforts are on-going to further characterise these probiotics and ultimately to establish an *in vitro* protocol.

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