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Evaluation of *Lactobacillus* Species Isolated from Swine Intestine for their Probiotic Properties in Piglets

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A B S T R A C T

Intestinal mucosal scrapings (48) were collected from twelve, 6 month old, Large White Yorkshire pigs after slaughter. Isolates (23) that were gram positive rods, were alone subjected to physiological tests (growth at pH: 4.5, 9.5; temperature; 15, 45°C; and NaCl: 2, 6.5%), four isolates exhibited no growth at all conditions studied and were rejected. Biochemical tests on the remaining nineteen isolates showed them to be catalase negative, indicating that they belong to the genus *Lactobacillus*. Six of the isolates, which were oxidase and nitrate reduction positive, were rejected. Sugar fermentation tests performed on thirteen isolates, lead to speciation of two isolates as *Lactobacillus acidophilus* and *Lactobacillus plantarum*. Both these isolates were able to thrive at pH 2 and had significantly ($P<0.05$) highest growth at pH 5. *L. plantarum* revealed significantly ($P<0.05$) higher growth than *L. acidophilus* at all incubation hours, at pH 4, 5, 8 and 9. Both isolates showed, significantly ($P<0.05$) highest growth at bile concentration of 0.05%. At all bile concentrations and incubation hours studied, except 0.05 % bile at 10 hours incubation, *L. plantarum* revealed significantly ($P<0.05$) higher growth compared to *L. acidophilus*. Both the organisms revealed antimicrobial activity towards *E. coli* and *Enterobacter sp*., antimicrobial activity of *L. acidophilus* was significantly ($P<0.05$) higher compared to *L. plantarum* against *E. coli*. Freeze drying did not affect the viability of both organisms. Twenty four Large White Yorkshire piglets 42 days of age, with an average weight of 9.4 kg, were distributed randomly into four treatment groups; T1 without probiotic supplementation; T2 supplemented with *L. acidophilus* (1.0 x10^6 CFU/g of feed); T3supplemented with *L. plantarum* (1.0 x10^6 CFU/g of feed) and T4 supplemented with (*L.plantarum*1.0 x10^3 CFU/g and*L.acidophilus*1.0 x10^3CFU/g of feed) and reared for 90 days. Average daily gain was significantly ($P<0.1$) highest (0.551 ± 0.148 kg) with significantly ($P<0.05$) lowest feed conversion ratio (2.373 ± 0.719) in T3 piglets. *L. plantarum*or *L. acidophilus* supplementation alone or in combination caused increased excretion of *Lactobacilli* and decreased excretion of *E. coli* in faeces, it had no impact on serum triglycerides but significantly ($P<0.05$) reduced total and LDL cholesterol and significantly ($P<0.05$) increased HDL cholesterol. *Lactobacillus* supplementation (T2, T3 and T4) caused significantly higher ($P<0.05$) villi width and crypt length and villi height was significantly higher ($P<0.05$) in T3 and T4.

Introduction

In India, out of the 6.27 million tons of meat produced, pork accounts to only 5.31 per cent (FAO, 2012). Generation after generation of pork producers in India follow local resource based production system, which, though self-sustainable has not lead to any marked increase in productivity. If productivity has to be increased more focus has to be given on feeding strategies. Antibiotics have been supplemented in piglet diets to address digestive issues, reduce the incidence of diarrhoea and increase their growth performance (Wang *et al.,* 2011). However, in recent years, there has been an increased interest to eliminate the use of antibiotics as growth promoters. European Union, Japan has already banned the use of antibiotics as growth promoters. Therefore, interest in alternatives to antibiotics as feed additives has grown. Probiotics are live microorganisms, used as feed additive. On its ingestion it promotes growth, improves feed efficiency, prevents diarrhoea and regulates the immune system in pigs. They produce no drug resistance or drug residues (Idoui, 2014). Micro-organisms used as probiotics should be non-pathogenic, and must possess ability to withstand low pH and high concentrations of bile acids. They should also be able to tolerate the manufacturing, transportation, storage and application processes, maintaining their viability and desirable characteristics (FAO, 2016). The effectiveness of probiotic in pigs, shows wide variation. The variation can be ascribed to several factors, the microorganism used as probiotic, age of the pigs, the dose of probiotics used, diet composition, feed form and interaction with other dietary feed additives. It is with this background a study was carried out with the objective to determine the impact of supplementing probiotic microorganisms (*Lactobacillus* species) isolated from swine intestine in piglets.

Materials and Methods

This study required no clearance from Ethical committee as major component of the study was *in vitro* and the feeding trial in piglets were carried out using surplus piglets reared for pork in Swine section of Post Graduate Research Institute of Animal Sciences, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India. However the protocol of the experiment, which was a part of MVSc, dissertation of the first author, was reviewed by the programme of research work approval committee, Tamil Nadu Veterinary and Animal Sciences University, Tamil Nadu, India.

Isolation of probiotic microorganisms

Intestinal mucosal scrapings (caecum and colon in duplicate) were collected from twelve Large White Yorkshire pigs (6months of age) immediately after slaughter. The pigs prior to slaughter were maintained on swine finisher ration (ME 3170Kcal/kg and crude protein 17%), at Post graduate Research Institute of Animal Sciences, Tamil Nadu Veterinary and Animal Sciences University, India.

After slaughter, the caecum and colon were dissected, cleared of digesta to expose the mucosa and washed with 1 per cent phosphate buffer saline solution. The superficial mucosal epithelial tissue was then scraped with sterile Bard-Parker blade. The scraped contents were swabbed with help of sterile swab and placed in a tightly closed container within an ice box. The samples were immediately transferred into refrigerator (4°C). The swabs respectively from caecum and colon mucosal scrapings were directly inoculated separately in MRS (de Man Rogosa Sharpe) broth which was then incubated at 37°C with 5 per cent CO_2 for 48 hours. After growth in MRS (de Man Rogosa

Sharpe) broth, the cultures were then pour plated on MRS agar (de Man Rogosa Sharpe Agar) and incubated at 37°C with 5 per cent CO_2 for 48 hrs. Well isolated colonies with typical characteristics *viz*., pure white, small (2-3 mm diameter), with entire margins were picked from each plate and were again inoculated into the MRS broth and labelled. Thus, the prepared de Man Rogosa Sharpe broth served as source material of respective isolates for carrying out morphological, physiological and biochemical tests.

The isolates were subjected (in triplicate in two runs) to morphological identification (Kandler and Weiss, 1986), physiological tests viz., growth at pH: 4.5, 9.5; temperature; 15, 45°C; and NaCl: 2, 6.5% (Cowan and Steel, 2003) and biochemical tests viz., catalase, nitrate reduction, oxidase, voges-Proskauer and production of ammonia from arginine (Cowan and Steel, 2003) to identify Lactobacilli. Further, sugar (glucose, lactose, arabinose, fructose, esculin, galactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose) fermentation (Lan *et al.,* 2003) to determine the species of the identified *Lactobacilli.*

In vitro assay to evaluate probiotic efficacy

To evaluate probiotic efficacy, pH and bile tolerance test (Todorov *et al.,* 2011) of the identified species was carried out in triplicate. Overnight cultures were inoculated (1%, v/v) in MRS broth whose pH was adjusted to 2, 2.5, 3, 4, 5, 8 and 9 using 1N NaOH or HCl or bile acid (Ox bile) was added at 0.05%, 0.1%, 0.15%, 0.2% and 0.3% (w/v).

The cultures were incubated at 37° C with 5% CO_2 for 10 hours. Growth was indicated by culture turbidity which was measured as optical density using colorimeter at 650 nm at two hour intervals.

Antimicrobial activity (Erdogrul and Erbilr, 2006) were performed on identified isolates. Identified *Lactobacillus* species were grown in MRS broth for 24 hours at 37°C under anaerobic conditions. Cells were removed by centrifugation (5000 g for 15 min at 4°C).

The pH of the supernatant was adjusted to 6.0 with 1 N NaOH and supernatant was filtered through 0.45 µm pore size membrane (millipore). Portions of 35 ml of Mueller-Hington agar were autoclaved and cooled to about 48°C and then 100µl of overnight cultures of *E. coli* and *Enterobactor sp.* containing approximately 2 x 10^7 cells per ml were added.

The inoculated medium was then poured into plates and wells of 6 mm in diameter were cut. Aliquots of supernatants from different *Lactobacillus* isolates were dispensed into wells and plates were incubated overnight at 37°C. The diameter of clear zones of growth inhibition around each well was measured and expressed in mm. The experiment was conducted in duplicate in two runs for each of identified *Lactobacillus* species.

Testing probiotic efficacy post freeze drying

The identified Lactobacillus species was inoculated in 100ml of MRS broth; ten per cent of skim milk powder was added and incubated at 37°C with 5% CO_2 for 24 hours, after
ascertaining purity, pre freezing was done at -45°C for 12 hours in deep freezer. Freeze drying was done at -55°C for 16 hrs in freeze drier (Christ Beta 1- 8 plus). The freeze dried vials were stored at 4°C in refrigerator. The efficacy of the probiotic organisms post freezing were tested by determining their growth in MRS medium at pH 2 and bile concentration of 0.3 % (Todorov *et al.,* 2011) and antimicrobial activity (Erdogrul and Erbilr, 2006).

Feeding trial in piglets

To assess the performance of piglets supplemented with the isolated *Lactobacillus* species, twenty four Large White Yorkshire piglets 42 ± 2 days of age (12 males and 12 females) with an average weight of 9.4 ± 0.26 kg, were distributed randomly into four treatment groups (six (3 male + 3 female) piglets/treatment) *viz.*, T1 - without probiotic supplementation; T2 - supplemented with *Lactobacillus acidophilus* (1.0 x10^6 CFU/g of feed); T3 - supplemented with *Lactobacillus plantarum* (1.0 x10^6 CFU/g of feed); T 4 - supplemented with (*Lactobacillus plantarum*1.0 x10^3 CFU/g and *Lactobacillus acidophilus* 1.0 x10^3CFU/g of feed) and reared for 90 days. Each treatment group was separately housed, adopting standard management practices.

Composition of experimental ration offered to piglets of all treatment groups and its nutritive value is presented in table 1. To the common experimental ration the respective probiotic supplementationT1 - No supplementation; T2 –*L. acidophilus* (1.0 x10^6 CFU/g of feed); T3 –*L.plantarum* (1.0 x10^6 CFU/g of feed); T 4 – *L.plantarum*1.0 x10^3 CFU/g and *L. acidophilus* 1.0 x10^3CFU/g of feed] was carried out and offered to the animals of the respective groups. Records were maintained on feed offered, left over and feed wastage to document daily feed intake of the respective groups.

Feed intake of the respective group was divided by the number of animals in the group to calculate the feed intake per animal and was expressed in kg per day. Piglets were weighed in the morning before feeding at fortnightly intervals up to 90 days of age using a platform weighing balance to calculate the average daily gain of each animal. Feed conversion ratio was calculated using the following formulae.

$$\text{Feed conversion ratio} = \frac{\text{Feed consumed per day (kg)}}{\text{Average daily gain (kg)}}$$

Fresh faecal samples were taken directly from the rectum of the each of the experimental piglet with sterile cotton swab. Around 1 g of faecal sample was serially diluted in 0.85% (w/v) saline and then pour plated (1.0 ml) on *Lactobacillus* selective agar for *Lactobacilli* colony count. For *E. coli*, colony count, Eosin methylene blue agar was used.

The numbers of viable colonies on quadruplicate plates were counted after incubation at 37°C for 48 hours (Chiang *et al.*, 2015).

Serum samples of experimental animals were analysed for total cholesterol (CHOD – PAP method), HDL cholesterol (HDL-C) Williams *et al.*, (1979) and serum triglyceride (TG) (GPO-PAP method) Allain *et al.*, (1974) by using enzymatic diagnostic kit, measured colorimetrically. The LDL cholesterol was calculated (Seyed-Ali Ahamadi, *et al.*, 2008).

A piece of mid portion of ileum was collected from three piglets from each treatment group on slaughter after electrical stunning, the samples were stored in 10% formalin and subjected to histomorphometric studies. Three cross sections from each ileum sample was prepared. After staining with hematoxylin and eosin using standard paraffin embedding procedure (Uni *et al.*, 1998),a total of 10 intact well oriented crypt villus units were selected in triplicate for each cross section (3 measurements of each samples).

Villus height was measured from tip of villi to the villus crypt junction and crypt depth was defined as the depth of invagination between adjacent villi. Villi height and crypt depth ratio was calculated. The measurements were expressed in µm.

Statistical analysis

The data collected on various parameters were grouped and subjected to statistical analysis by one way ANOVA as per the procedure of statistical analysis system (SPSS, version 20.0 for windows).

Results and Discussion

Isolation of probiotic microorganisms

Morphological characterization of the 48 isolates collected from swine intestine, revealed that only 23 isolates were gram positive rods, and these isolates were alone taken up for further screening. Physiological tests (growth at pH: 4.5, 9.5; temperature; 15, 45°C; and NaCl: 2, 6.5%) performed on the twenty three isolates revealed that, four isolates exhibited no growth, at all the conditions studied. Hence, they were not selected for further screening. Biochemical tests on the remaining nineteen isolates, revealed that all the isolates were catalase negative, favouring that the isolates could belong to the genus *Lactobacillus*. However, six of the isolates were oxidase and nitrate reduction positive and this does not favour their possibility of belonging to genus *Lactobacillus*, hence, these six isolates were not included in further screening. Sugar fermentation tests performed on the thirteen isolates identified as *Lactobacilli, speciation* of only two isolates could be ascertained, one of the isolate showed characteristics for *Lactobacillus acidophilus* and the other for *Lactobacillus plantarum*.

In vitro assay to evaluate probiotic efficacy

The results of the pH and bile tolerance test of the two *Lactobacillus* species *viz.*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* is presented in tables 2 and 3 respectively.

From table 2 it could be inferred that *Lactobacillus plantarum* and *Lactobacillus acidophilus* were able to thrive at pH 2 (porcine gastric pH). Linear increase in growth was observed when pH was increased from 2 to 8. Significantly ($P<0.05$) highest growth was observed at ph 5 (4, 8, 10 incubation hours) and pH 8 (2 and 6 incubation hour). Decline in growth in both the organisms were observed at pH 9.*Lactobacillus plantarum* revealed significantly ($P<0.05$) higher growth than *Lactobacillus acidophilus* at all incubation hours, at pH 4, 5, 8 and 9. However, at pH 2, 2.5 and 3, *Lactobacillus plantarum* revealed significantly ($P<0.05$) higher growth than *Lactobacillus acidophilus* only beyond 8, 6 and 4 hours of incubation respectively.

From table 3 it could be inferred that *Lactobacillus plantarum* and *Lactobacillus acidophilus* were able to thrive at all bile concentrations and their growth increased linearly over time.

However, significantly ($P<0.05$) highest growth of both the organism were observed at bile concentration of 0.05%.At all bile concentrations, at all incubation hours studied, except for 0.05 % bile at 10 hours incubation, *Lactobacillus plantarum* revealed significantly ($P<0.05$) higher growth compared to *Lactobacillus acidophilus.*

The study revealed that the antimicrobial activity (as demonstrated by the inhibition zone produced (mm) in the agar well diffusion assay) of *Lactobacillus acidophilus* (20.00 ± 0.59) against *Escherichia coli* was significantly ($P<0.05$) higher compared to that exhibited by *Lactobacillus plantarum* (18.00± 0.58). With regard to antimicrobial activity against *Enterobacter sp* both *Lactobacillus plantarum* (18.33 ±0.33) and *Lactobacillus acidophilus* (18.00 ±0.58) showed no significant variation.

Testing probiotic efficacy post freeze drying

Freeze drying did not affect the viability of both *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Figure 1) as is evident from their growth at pH 2 and bile concentration of 0.3 % in MRS broth. Freeze drying also, did not have any adverse effect on the antimicrobial property of *Lactobacillus sp.* as evident by the inhibition zone produced (mm) in the agar well diffusion assay. The antimicrobial activity of *Lactobacillus acidophilus* (18.67 ± 0.44) against *Escherichia coli* was significantly (P<0.05) higher compared to that exhibited by *Lactobacillus plantarum.* (15.50 ^{a} ± 0.77)With regard to antimicrobial activity against *Enterobactor sp.* both *Lactobacillus plantarum* (15.00 ± 0.33) and *Lactobacillus acidophilus* (15.17 ± 1.04) showed no significant (P<0.05) variation.

Feeding trial in piglets

The effect of feeding piglets with basal diet without or with probiotic supplement on their weight gain, feed intake, feed conversion ratio, excretion of *Lactobacilli* and *Escherichia coli* in their faeces, serum lipid profile and illeal histomorphometry is presented in table 4. Probiotic supplementation lead to comparable (T2 and T4) or significantly higher (P<0.1) (T3) average daily gain in piglets and improved their feed conversion ratio (T2, T3 and T4). The piglets on probiotic supplementation (T2, T3 and T4) excreted, significantly higher (P<0.05) *Lactobacilli* and significantly lower *(*P<0.05) *E. coli* in their faeces. The serum lipid profile of piglets revealed significantly higher (P<0.05) HDL-cholesterol and significantly lower *(*P<0.05) total cholesterol and LDL-cholesterol on probiotic supplementation (T2, T3 and T4). Ileal histomorphometry on probiotic supplementation (T2, T3 and T4) revealed significantly higher (P<0.05) villi width and crypt length. Villi height was significantly higher (P<0.05) in T3 and T4.

Isolation of probiotic microorganisms

The objective of this study was to isolate probiotic organism from swine intestine, and utilize it in producing probiotic supplement for piglets. As the study was aimed in isolating a safe probiotic, focus was only on isolating *Lactobacillus*.

Hence in morphological screening isolates that were Gram positive rods alone were considered. *Lactobacilli,* are clearly Gram positive, and their cells vary from long and slender, sometimes bent rods to short rods (Kandler and Weiss, 1986). Bharadwaj *et al.,* (2012) followed a similar protocol for preliminary screening to identify *Lactobacilli* from yoghurt. They carried out morphological identification of the isolates and identified it as *Lactobacilli* microscopically by performing Gram staining.

Optimum growth temperature for *Lactobacillus* ranges between 2 to 53°C and optimal pH ranges between 5.5 to 6.2 (Kandler and Weiss, 1986). Therefore, in the present study, isolates that did not reveal growth at 15°C or 45°C, at pH of 4.5 or 9.5 and salinity of 2% or 6.5% were rejected as they did not fulfil the characterization of *Lactobacillus*. In a study on isolated strains from yoghurt, the isolates positive for *Lactobacillus* were able to grow at pH between 4.0 and 8.0, but the optimum growth was observed at pH between 5.5 and 6.5 when grown in MRS broth at 37°C. The isolated *Lactobacillus* strains also were able to tolerate 1-9 per cent w/v concentration of NaCl in the MRS broth (Chowdhury *et al.,* 2012).

In this study, it was found that all the isolates subjected to catalase test, showed negative results. Catalase is an extracellular enzyme

secreted by microorganisms that helps in degradation of hydrogen peroxide produced during carbohydrate utilization for energy production. Kandler and Weiss (1986) stated that all *Lactobacilli* are negative to catalase test *i.e.* there is no evolution of bubbles when a drop of hydrogen peroxide is added to the smear of the organism. Hence considering the catalase test alone, it could be postulated that all the nineteen isolates could be *Lactobacilli*. Six isolates out of 19 isolates were positive for oxidase test hence were rejected. Two of the rejected isolates were also positive for nitrate reduction test, further, confirming the need for their rejection. *Lactobacilli are* reported to show negative result for oxidase test, because *Lactobacilli* are unable to use oxygen as terminal electron acceptor in respiration due to absence of cytochrome C oxidase (Acharya, 2012). Variable results for nitrate reduction test have been reported by various authors. In a study on nitrate reduction to nitrite, nitric oxide and ammonia by gut bacteria under physiological conditions (Tiso and Schechter, 2015) significant nitrate reductase activity was detected both in *E. coli* and *Lactobacillus plantarum* as the oxygen tension decreased from atmospheric level towards zero. On the contrary, *B. longuminfantis*, a micro-aero tolerant anaerobe of infant gastrointestinal tract origin, showed no ability to reduce nitrate even at high concentrations. Thus, explaining the variability of results of nitrate test being positive in probiotic organisms.

Table.1 Composition of experimental ration** (as fed basis) offered to piglets of all treatment groups and its nutritive value

Ingredient	Inclusion level (%)
Maize	60.00
Soya bean meal	23.70
Fish meal	06.00
Deoiled rice bran	01.30
Oil	06.30
Salt	00.30
Monosodium phosphate	00.50
Mineral mixture*	01.50
Lysine	00.20
Methionine	00.20
Total	100.00
Nutritive value	
Metabolisable Energy (Kcal /kg)	3360
Crude Protein (%)	20

*BIS specification (2002)

Table.2 Optical density of MRS medium containing *Lactobacillus* species at various pH measured at two hourly intervals at 650 nm to evaluate *Lactobacilli* growth (Mean* ± SE)

Organism	pH	Incubation hours				
L. plantarum		**2**	**4**	**6**	**8**	**10**
	2	0.087^{I} ± 0.003	0.087^{I} ± 0.003	0.137^{I} ± 0.003	$0.173^{b\ I}$ ± 0.007	$0.193^{b\ I}$ ± 0.003
	2.5	0.080^{I} ± 0.046	0.087^{I} ± 0.005	$0.160^{b\ I}$ ± 0.092	$0.183^{b\ I}$ ± 0.106	$0.203^{b\ I}$ ± 0.118
	3	0.117^{II} ± 0.007	$0.190^{b\ I\ II}$ ± 0.012	$0.300^{b\ II}$ ± 0.016	$0.540^{b\ II}$ ± 0.012	$0.790^{b\ II}$ ± 0.006
	4	$0.127^{b\ II}$ ± 0.003	$0.257^{b\ I\ II\ III}$ ± 0.009	$0.437^{b\ IV}$ ± 0.009	$0.840^{b\ III}$ ± 0.006	$1.430^{b\ IV}$ ± 0.006
	5	$0.173^{b\ III}$ ± 0.008	$0.407^{b\ III}$ ± 0.008	$0.790^{b\ V}$ ± 0.005	$1.430^{b\ V}$ ± 0.017	$1.843^{b\ V}$ ± 0.023
	8	$0.223^{b\ IV}$ ± 0.006	$0.358^{b\ II\ III}$ ± 0.153	$0.827^{b\ VI}$ ± 0.008	$1.040^{b\ IV}$ ± 0.015	$1.427^{b\ IV}$ ± 0.014
	9	$0.160^{b\ III}$ ± 0.015	$0.273^{b\ I\ II\ III}$ ± 0.003	$0.380^{b\ III}$ ± 0.005	$0.843^{b\ III}$ ± 0.031	$1.003^{b\ III}$ ± 0.028
L. acidophilus	**2**	0.087^{I} ± 0.003	0.087^{I} ± 0.003	0.113^{I} ± 0.003	$0.113^{a\ I}$ ±0.003	$0.113^{a\ I}$ ±0.003
	2.5	0.083^{I} ± 0.048	0.087^{I} ± 0.005	$0.123^{a\ I}$ ± 0.071	$0.123^{a\ I}$ ± 0.071	$0.123^{a\ I}$ ± 0.071
	3	0.090^{I} ± 0.000	$0.147^{a\ II}$ ± 0.012	$0.237^{a\ II}$ ± 0.012	$0.463^{a\ II}$ ± 0.003	$0.613^{a\ II}$ ± 0.012
	4	$0.093^{a\ I}$ ± 0.003	$0.223^{a\ III}$ ± 0.009	$0.350^{a\ IV}$ ± 0.006	$0.643^{a\ III}$ ± 0.003	$0.073^{a\ III}$ ± 0.003
	5	$0.127^{a\ II}$ ± 0.008	$0.350^{a\ V}$ ± 0.018	$0.700^{a\ VI}$ ± 0.006	$1.123^{a\ IV}$ ± 0.009	$1.350^{a\ V}$ ± 0.029
	8	$0.140^{a\ II}$ ± 0.005	$0.277^{a\ IV}$ ± 0.008	$0.537^{a\ V}$ ± 0.012	$0.650^{a\ III}$ ± 0.017	$0.847^{a\ IV}$ ± 0.008
	9	$0.093^{a\ I}$ ± 0.006	$0.167^{a\ II}$ ± 0.006	$0.280^{a\ III}$ ± 0.005	$0.470^{a\ II}$ ± 0.005	$0.620^{a\ II}$ ± 0.015

*Mean of three observations

Means bearing different alphabetical superscripts in a column for respective pH, between organism differ significantly ($P<0.05$).

Means bearing different roman numerical superscripts in a column between pH, within incubation hours, for respective organism differ significantly ($P<0.05$).

Table.3 Optical density of MRS medium containing *Lactobacillus* species at various bile concentration measured at two hourly intervals at 650 nm to evaluate *Lactobacilli* growth (Mean* ± SE)

Organism	Bile %	Incubation hours				
		2	4	6	8	10
L. plantarum	**0.05**	0.120 $^{b\ II}$ ± 0.017	0.277 $^{b\ IV}$ ± 0.003	0.427 $^{b\ V}$ ± 0.014	0.860 $^{b\ V}$ ± 0.017	1.283 ^{V} ± 0.008
	0.1	0.087 $^{b\ I}$ ± 0.003	0.180 $^{b\ III}$ ± 0.005	0.313 $^{b\ IV}$ ± 0.003	0.637 $^{b\ IV}$ ± 0.012	0.723 $^{a\ IV}$ ± 0.003
	0.15	0.077 $^{b\ I}$ ± 0.003	0.133 $^{b\ II}$ ±0.003	0.237 $^{b\ III}$ ± 0.008	0.413 $^{b\ III}$ ± 0.008	0.510 $^{a\ III}$ ± 0.005
	0.2	0.080 $^{b\ I}$ ± 0.001	0.100 $^{b\ I}$ ± 0.000	0.187 $^{b\ II}$ ± 0.003	0.243 $^{b\ II}$ ± 0.006	0.293 $^{b\ II}$ ± 0.003
	0.3	0.070 $^{b\ I}$ ± 0.000	0.093 $^{b\ I}$ ± 0.003	0.097 $^{b\ I}$ ±0.003	0.163 $^{b\ I}$ ± 0.008	0.243 $^{b\ I}$ ± 0.020
L. acidophilus	**0.05**	0.087 $^{a\ III}$ ± 0.003	0.177 $^{a\ IV}$ ± 0.003	0.273 $^{a\ V}$ ± 0.003	0.550 $^{a\ V}$ ± 0.01	1.283 ^{V} ± 0.008
	0.1	0.066 $^{a\ II}$ ± 0.006	0.127 $^{a\ III}$ ± 0.008	0.233 $^{a\ IV}$ ± 0.008	0.590 $^{a\ IV}$ ± 0.005	0.803 $^{b\ IV}$ ± 0.008
	0.15	0.060 $^{a\ I\ II}$ ± 0.000	0.097 $^{a\ II}$ ± 0.003	0.127 $^{a\ III}$ ± 0.003	0.230 $^{a\ III}$ ± 0.005	0.543 $^{b\ III}$ ± 0.012
	0.2	0.050 ^{aI} ±0.000	0.083 $^{a\ II}$ ± 0.003	0.097 $^{a\ II}$ ± 0.003	0.113 $^{a\ II}$ ± 0.006	0.123 $^{a\ II}$ ± 0.006
	0.3	0.050 $^{a\ I}$ ± 0.000	0.067 $^{a\ I}$ ± 0.003	0.067 $^{a\ I}$ ± 0.003	0.077 $^{a\ I}$ ± 0.003	0.076 $^{a\ I}$ ± 0.003

*Mean of three observations

Means bearing different alphabetical superscripts in a column for respective bile concentrations, between organism differ significantly (P<0.05).

Means bearing different roman numerical superscripts in a column between bile concentrations, within incubation hours, for respective organism differ significantly (P<0.05).

Table.4 Overall performance of piglets fed basal diet without or with probiotic supplements (Mean ± SE)

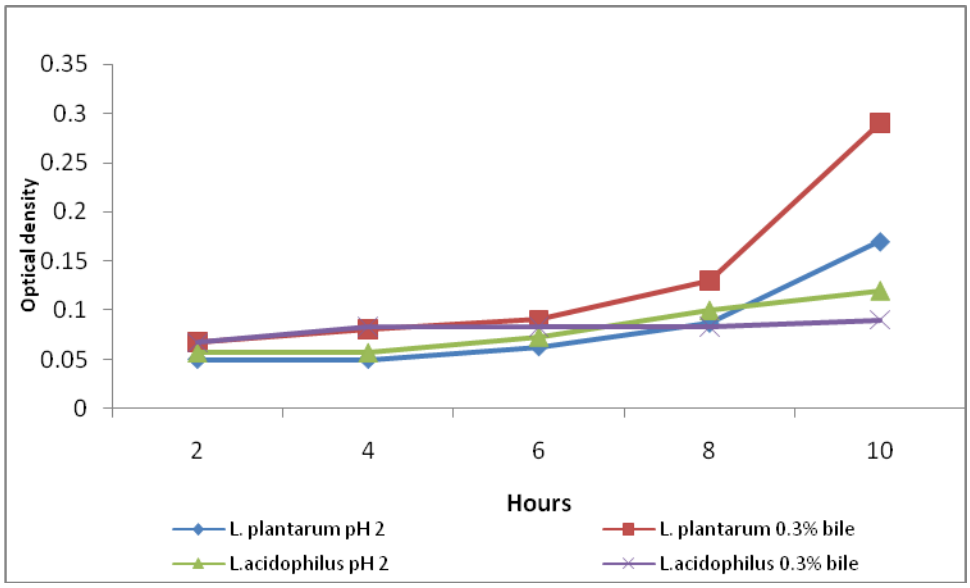
Parameters	T_1 - without probiotic supplement	T_2 - *Lactobacillus acidophilus* 1.0x10^6CFU/g of feed	T_3 - *Lactobacillus plantarum*1.0x 10^6CFU/g of feed	T_4 - *L. plantarum*1.0x 10^3CFU/g of feed *and* *L. acidophilus* 1.0x10^3CFU/g of feed
Growth parameters**				
Initial body weight (kg) on 42nd day	9.53 ± 0.46	9.37 ± 0.43	9.44 ± 0.55	9.43 ± 0.81
Final body weight (kg) on 132nd day	53.70^{ab} ± 3.43	52.65 ^{a} ± 1.39	59.31 ^{b} ± 1.59	58.21^{ab} ± 2.17
Body weight gain (kg)	44.16^{ab} ± 7.39	43.28 ^{a} ± 1.27	49.67 ^{c} ± 1.33	48.74^{bc} ± 3.71
Average daily gain (kg)	0.49 ^{ab} ± 0.03	0.48 ^{a} ± 0.14	0.55 ^{c} ± 0.14	0.54^{bc} ± 0.16
Feed intake per day (kg)	1.32 ^{d} ± 0.03	1.29 ^{c} ± 0.02	1.26 ^{a} ± 0.03	1.28 ^{b} ± 0.04
Feed conversion ratio	2.78 ^{b} ± 0.27	2.70^{ab} ± 0.09	2.37 ^{a} ± 0.11	2.39 ^{a} ± 0.01
Excretion of probiotic / pathogenic organism***				
Lactobacilli (log_{10}CFU/g faeces)	6.096 ^{a} ± 0.066	7.496 ^{b} ± 0.005	7.561 ^{b} ± 0.014	7.574 ^{b} ± 0.018
E. coli (log_{10} CFU/g faeces)	6.015 ^{b} ± 0.079	5.614 ^{a} ± 0.081	5.592 ^{a} ± 0.099	5.569 ^{a} ± 0.119
Serum lipid profile***				
Triglycerides (mg/dl)	161.36 ± 26.80	134.46 ± 12.47	134.30 ± 12.81	121.26 ± 10.91
Total cholesterol (mg/dl)	75.69^{b} ± 8.39	52.78^{a} ± 3.49	52.47 ^{a} ± 5.00	58.79 ^{a} ± 2.47
HDL-cholesterol (mg/dl)	25.59 ^{a} ± 1.88	32.43 ^{b} ± 1.57	33.72 ^{b} ± 1.62	34.52 ^{b} ± 1.05
LDL-cholesterol (mg/dl)	87.27 ^{b} ± 9.86	47.56 ^{a} ± 7.22	46.27 ^{a} ± 7.190	43.85 ^{a} ± 5.76
Ilealhistomorphometry***				
Villi height (µm)	353.51 ^{a} ± 46.32	434.36 ^{a} ± 27.31	721.15 ^{b} ± 10.94	511.52^{ab} ± 66.80
Villi width (µm)	83.79 ^{a} ± 4.489	154.61 ^{b} ± 24.39	142.62^{ab} ±10.70	117.34^{ab} ± 22.00
Crypt length (µm)	104.37 ^{a} ± 15.09	111.51^{ab} ± 7.46	129.79^{ab} ± 3.81	139.64 ^{b} ± 5.19
Villi height /crypt depth	3.65 ± 0.99	3.96 ± 0.50	5.61 ± 0.97	3.69 ± 0.57

*Mean of six observations

**Means bearing different superscripts within a row differ significantly (P<0.1).

***Means bearing different superscripts within a row differ significantly (P<0.05)

Fig.1 Optical density (650 nm) of MRS medium containing post freeze dried *Lactobacillus* species at pH 2 and bile concentration of 0.3% to evaluate *Lactobacilli* growth



Each bacterium has its own collection of enzyme that enables it to use diverse carbohydrate; this is often exploited in the identification of bacterial species. Thus carbohydrate fermentation tests are essential for speciation of bacteria (Kali *et al.*, 2015). In this study, sugar fermentation tests were carried out to identify the *Lactobacillus* species from the isolates whose genus was ascertained as *Lactobacillus*. The main task of sugar fermentation test is to investigate the ability of bacteria to ferment different types of carbohydrate (Pyar and Peh, 2014). Bacteria ferment several carbohydrates. However, not all sugars can be utilized by one organism. Depending on the ability to synthesize the enzyme catalysing the breakdown, bacteria can ferment a particular sugar. Therefore sugar fermentation tests are very useful in the identification of bacteria as the results are almost always characteristic for a species (Mudili, 2007). In total seventeen sugars, glucose, lactose, arabinose, fructose, esculin, galactose, maltose, mannitol, mannose, melibiose, rafffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose were used for conformational identification. The sugar utilization pattern was compared with those given for *Lactobacillus* species in the Bergey's manual for determinative bacteriology (Holt *et al.*, 1994). Out of the thirteen isolates screened only two isolates could be defined respectively as *Lactobacillus plantarum* and *Lactobacillus acidophilus*.

The first major hurdle the probiotic bacteria must overcome in the gastrointestinal tract is the low pH of gastric content (Casey *et al.*, 2004). From this study it was inferred that though *Lactobacillus plantarum* and *Lactobacillus acidophilus* survived between pH of 2 to 3, good growth was seen at higher pH of 5 and 8 indicating that these organisms are capable of growing at neutral pH and alkaline conditions also. The findings concur with that reported by Pyar and Peh (2014). Variation in the acid resistance was observed between *Lactobacillus plantarum* and *Lactobacillus acidophilus.* Idoui *et al.*, 2014 reported that *Lactobacillus plantarum* was resistant to pH 3 (rabbit) whereas, Lin *et al.*, (2007) observed that *Lactobacillus acidophilus* was less stable at pH 2.6 (chicken).

Bile tolerance is one of the most crucial properties for probiotic bacteria as it determines the ability of the organism to survive in the small intestine and consequently decides the capacity of the probiotic to play a functional role (Ruiz *et al.,* 2013). Both *Lactobacillus plantarum* and *Lactobacillus acidophilus* were able to tolerate bile concentration up to 0.3 %. However both the organisms showed reduced growth at higher bile concentration. As in this study, Klayraung *et al.,* (2008) also demonstrated a decrease in the viability of cells when the concentration of bile salts increased. The reason for the reduced growth with increasing level of bile salts could be due to the binding of probiotic organism with bile salts (Patel *et al.,* 2004). Similar to the results of acid tolerance in the study, bile tolerance test also revealed that *Lactobacillus plantarum* showed significantly higher growth as compared to *Lactobacillus acidophilus.* Probiotic organisms have different tendencies to utilise bile (Li, 2012).

Both *Lactobacillus plantarum* and *Lactobacillus acidophilus* showed antimicrobial activity against *E. coli* and *Enterobacter sp*. The antimicrobial activity could have been due to the effect of organic acids (Khunajakr *et al.,* 2008) or the production of bacteriocins which have high antimicrobial activity (Aween *et al.,* 2012). *Lactobacillus plantarum* is known to produce plantaricin that are active against certain pathogens (Cebeci and Gurakan, 2003). The antimicrobial agent (bacteriocin) from *Lactobacillus acidopilus* demonstrated strong antimicrobial activity against both Gram positive and Gram negative bacteria (Bharal and Shopal *et al.,* 2013).

Testing probiotic efficacy post freeze drying

Freeze-drying or lyophilization is a convenient method for preservation of bacteria. By reducing water activity to values below 0.2, it allows long-term storage and low-cost distribution at suprazero temperatures, while minimizing losses in viability and functionality (Fonseca *et al.,* 2015). In this study, freeze drying did not affect the viability of the probiotic organisms *Lactobacillus plantarum* and *Lactobacillus acidophilus.* Post freeze drying, the organisms were able to tolerate a pH of 2 and bile concentration of 0.3% and revealed antimicrobial activity.

Feeding trial in piglets

Piglets, at the time of weaning, are faced with a considerable amount of psychological stress induced by changes in feed and the environment. Probiotics can relieve weaning stress, prevent diarrhea and promote growth in piglets. These positive effects are brought about by competitive exclusion of pathogenic bacteria and colonization of beneficial bacteria in the pigs' gastro intestinal tract (Wang *et al.,* 2011). Significantly highest average daily gain in piglets supplemented with Lactobacillus*plantarum*1.0 x10^6 CFU/g of feed. Metabolites of the organism such as lactic acid could have promoted gastro intestinal peristalsis and digestion that consequently resulted in improvement of both average daily gain and feed conversion ratio.

The adhesion of probiotic *Lactobacilli* to intestinal mucus makes them colonise the gut efficiently and settle down to a stable position and avoid infectious disease condition in piglets (Li *et al.,* 2008). In addition a number of studies have identified various cell surface proteins which are stated to be involved in bacterial adhesion to intestinal mucosa, and protection against pathogen colonization (Watanabe *et al.,* 2010). Increase in *Lactobacilli* is accompanied by increase in production of short chain fatty acids that lowers the pH of intestinal contents that inhibit the growth of acid sensitive coliform

bacteria. Thus, as observed in this study, probiotic feeding could decrease the *E. coli* population and increase the *Lactobacillus* population in the intestine of weaned piglets (Chiang *et al.,* 2015).

The total cholesterol in the serum of piglets supplemented with probiotics was significantly lower than that of piglets without probiotic supplementation. Similar trend was observed for LDL – cholesterol, whereas, HDL – cholesterol was significantly higher in probiotics supplemented piglets. The mechanism by which *Lactobacilli* remove cholesterol has been studied, the mechanism that may contribute to cholesterol - lowering effects is bile salt hydrolase (BSH) enzyme activity of *Lactobacillu*s strains. Deconjugated bile salts are less efficiently reabsorbed than their conjugated counterparts, which results in the excretion of larger amounts of free bile acids in faeces. Also, free bile salts are less efficient in solubilizing and absorption of lipids in the gut. Therefore, deconjugation of bile salts could lead to a reduction in serum cholesterol either through increasing the demand for cholesterol for *de novo* synthesis of bile acids in order to replace the loss in faeces or by reducing cholesterol solubility and absorption of cholesterol in the intestinal lumen (Fazeli *et al.,* 2010). Lye *et al.,* (2010) evaluated the conversion of cholesterol to coprostanol by strains of lactobacilli such as *Lactobacillus acidophilus, L. bulgaricus* and *L. casei* ATCC 393 via fluorometric assays. The authors detected both intracellular and extracellular cholesterol reductase in all strains of probiotics examined, indicating possible intracellular and extracellular conversion of cholesterol to coprostanol.

Villi are critical component of the digestive tract and their geometry provides an indicator of absorptive capacity of the small intestine. Turnover of intestinal epithelium reflects a dynamic equilibrium between production of enterocytes in the crypts and the subsequent desquamation from the villi.

The increased villus height leads to increased surface area at the same time, promoting digestion and absorption of disaccharides and dipeptides. In addition, longer villi are correlated with activation of cell mitosis (Samanya and Yamauchy, 2002). Concurring with the findings of this study, Willing and Van Kessel (2007) have reported that crypt depth was increased in piglets inoculated with *Lactobacillus fermentum*.

In conclusion the results of the present study indicate that *Lactobacillus plantarum* and *Lactobacillus acidophilus* isolated from swine intestine were resistant to gastric pH, intestinal pH, were tolerant to bile concentrations of 0.05 to 0.3% and showed antimicrobial activity against both *E. coli and Enterobacter sp.*

They could tolerate freeze drying. Their supplementation improved ADG, FCR, serum lipid profile and ileal absorptive surface in piglets.

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