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Potential probiotic *Lactobacillus* strains for piglets from an arid coast

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Abstract - Important centres for the pork industry have become growth in arid regions in the world and pig production needs alternatives to increase the productivity. A screening of predominant *Lactobacillus* strains from healthy piglets was performed in order to select specific probiotics. The ability of 164 strains to grow at different temperatures and concentrations of NaCl was evaluated. Results showed that all of them grew at 45 °C, 75% at 50 °C and 64% resisted 680 mM of salt. Adhesion to mucus and gastric mucin was evaluated showing 45% of strains isolated from faeces were able to adhere whereas 71% of strains from mucus showed mucus binding activity. Among the 164 isolates, 27 adhesive strains were identified using comparisons with 16S rDNA and intergenic 16-23S sequences. Results indicated that *L. fermentum* and *L. reuteri* were the most common species in faeces and mucus, respectively. Ability to grow in gastrointestinal mucus was evaluated showing that 92.6% of strains were able to replicate. Additionally, bacterial strain grown in 3.5% MRS with bile salts was evaluated. These results indicated that animals inhabiting isolated arid coasts are a rich source of probiotics, which resist adverse environmental conditions and can colonise the intestinal tract of pigs.

Key words: arid coast, *Lactobacillus*, adhesion, piglets, probiotic.

INTRODUCTION

The pork industry is an important source of food in the world. Arid and semi-arid regions represent one third of the total area of the world including Australia, the southwest of Unites States and northwest of Mexico. The climatic pattern in arid zones is frequently characterised by a relatively cool dry season, followed by a relatively hot dry season, and ultimately by a scarce rainy season. Quite often, during the cool dry season, daytime temperatures peak between 35 and 45 °C and fall to 10 to 15 °C at night. Moreover, there is high rainfall variability, with annual amounts ranging between 100 and 300 millimetres (http://www.fao.org/docrep/T0122E/t0122e03.htm). In spite of arid regions present adverse climatic conditions, there are important centres for the pork industry, examples of this are Utah and Oklahoma in the United States, east of Australia and Sonora in Mexico. Actually, the northwest of Mexico experiments an important growth in this industry, mainly in Sonora state (Hernández and Maya, 2002). Baja California Sur, located in this region is the most arid state with the longest length of coast in Mexico (Troyo-Diéguez *et al*., 1994). This state is geographically isolated from the rest of the country by the Pacific Ocean and the Sea of Cortez. Here as in all arid regions of the world saline soils are common due the transport of soluble salts to the ocean does not occur because of low rainfall (Webster and Wilson, 1980).

Typical extreme conditions of arid lands, stress the animals causing susceptibility to gastrointestinal diseases when large variations in temperatures occur (Shimizu *et al*., 1978). In fact, gastrointestinal disorders caused by pathogens are the principal death cause in pigs from farms located in the arid region of Kenya (Karanja *et al.,.* 2005). Thus, the supplementation of *Lactobacillus* able to survive in arid conditions represents an alternative to maintain the health of growing pigs. At birth, piglets are exposed to a huge variety of microorganisms. Most of them come from the vagina, faeces, and skin of the mother as well as the environment (Conway, 1997). The composition of gut microbiota can be modulated by host, environmental, and bacterial factors (Thompson-Chagoyán *et al*., 2007). Recently, was reported that microbiota composition of Holstein heifers changed at elevated environmental temperatures and humidity (Tajima *et al.,* 2007). Therefore, microorganisms inhabiting arid environments should retain abilities to survive under these harsh conditions. The ability to grow at high temperatures and in presence of salts represents an important characteristic for probiotic bacteria. It has been reported that *Lactobacillus* strains adapted to heat (52 °C for 15 min) or salt (300 mM NaCl) exhibited 300 to 700 folds more tolerance to lethal temperatures used during spray

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drying technological process (Desmond *et al.,* 2001). One of the health-promoting effects attributed to *Lactobacillus* as a probiotic is its capacity to prevent microbial infections in the gastrointestinal tract (Bernet-Camard *et al*., 1997; Servin, 2004). Adherence to intestinal mucus has been associated to competitive exclusion of pathogens (Lee *et al*., 2003; Gueimonde *et al*., 2006). Two requirements have been identified as desirable properties for *Lactobacillus* to be considered as an effective probiotic microorganism (Reid, 1999), these include the ability to adhere and then to consequently colonise mucous surfaces. Mackie *et al*. (1999) suggested that during a colonisation event a bacterial population remains stable in size, with no need of periodic reintroduction of bacteria by oral doses. However, in practical terms it is well known that external factors can arise such as antibiotic treatments or a change in the nutritional regime that can disrupt the equilibrium of the normal bacterial population (Jernberg *et al*., 2005). In these cases, it is necessary to supplement the feed with probiotics to restore the balance. Therefore, the ability to replicate in mucus represents an important parameter to evaluate in potential probiotic strains. Additionally, it is recognised that resistance of potential probiotics to bile salts is a strictly necessary property (Moser and Savage, 2001). Therefore, the aim of this study was to isolate *Lactobacillus* strains harbouring probiotic potential with high temperature and high salinity resistance traits from faeces and intestinal mucus of piglets in Baja California Sur, Mexico. This selection process involved the isolation of dominant strains from healthy piglets, evaluation of their adhesiveness to mucus and mucins, ability of the strains to replicate *in vitro* in mucus, molecular identification of selected strains and growth with culture media containing bile salts. Potential probiotic strains from arid land must be available for pig industry in these areas.

MATERIALS AND METHODS

Animals. Landrace-Duroc newborn piglets from a pig farm in Baja California Sur, Mexico, were maintained with their mothers in maternity cages with grid floors during 23 days before weaned. Piglets received an intramuscular Fe injection (100 mg Fe, VITALECHON DEXTRAN) the second day after birth. Mother's milk fed piglets were given free access to commercial starter feed (17.5% crude protein, 2.5% crude fat, 5% crude fiber, 12% moisture, salts, vitamins, and minerals) and water (< 900 ppm) 2-5 days before weaning. Maternity cages were maintained at room temperature and warmed up with lamps during the night when needed. To avoid excessive stress caused by high temperatures, piglets were bathed every day at midday with saline water (> 1000 ppm). Faecal samples of 19 healthy 23-day-old preweaned piglets from different cages with weights of 10 to 12 Kg were collected in sterile falcon tubes just at the time of defecating and transported to the laboratory at 4 °C. A piglet randomly selected, was sacrificed by a humanitarian method in the laboratory and immediately, the small intestine and caecum were removed and treated as is described below.

Isolation and characterisation of bacteria. Dominant lactobacilli were isolated from the faeces and the associated mucus from the small intestine and caecum. For mucus samples, the small intestine and caecum were sectioned, opened and rinsed with ice-cold phosphate-buffer saline (PBS) (145 mM NaCl, 2.87 mM KH_2PO_4, and 6.95 mM K_2HPO_4, pH 7.2) in order to remove loosely associated intestinal material. Mucus was then released by gently scraping the small intestine and caecum with a spatula. Both faecal and mucosal samples were diluted in PBS and serial dilutions were plated on Rogosa SL agar (Difco). Plates were incubated at 37 °C for 24 h in an anaerobic jar with a Gaspack system. Counts of colony forming units (CFU) per gram of piglet faeces and for cm^2 of small intestine and caecum were reported. Colonies (3 to 6 per plate) from each faecal or mucosal piglet sample were randomly selected from the last dilutions, purified on Rogosa SL plates and grown in MRS broth (Mann, Rogosa and Sharpe, Difco). Gram staining was performed as previously described by Speck (1976). The presence of catalase activity was determined by production of oxygen from hydrogen peroxide (Whittenbury, 1964). Cell morphology was examined by microscopy. The isolates were stored at -85 °C in MRS broth containing 40% (v/v) glycerol.

Growth conditions. A primary culture from stocks stored at -85 °C were grown overnight at 37 °C in tubes containing 5 ml of MRS broth. For adhesion tests, MRS primary cultures were inoculated (1% v/v of the final volume) into LDM semi defined medium (Conway and Kjelleberg, 1989) supplemented with 2% w/v glucose and grown to an optical density at 600 nm (OD_{600}) value of 0.9-1.0. To perform chromosomal DNA extractions primary cultures were inoculated (5% v/v of the final volume) in fresh MRS broth and grown for 4 h in the same conditions.

Growth at different concentrations of sodium chloride. Growth in presence of NaCl was performed using MRS supplemented with different concentrations of salt (430, 680 or 1112 mM NaCl). Five millilitres of sterile MRS containing different concentrations of NaCl were inoculated with 50 µl of a 12 h MRS primary culture. Tubes were incubated at 37 °C and OD_{600} was determined after 24 and 48 h of incubation. Negative growth represented an OD_{600} comparable to the non-inoculated MRS medium with equivalent concentration of salt, while growth was represented by at least third of the OD_{600} observed in the MRS cultures without salt.

Growth at different temperatures. Growth at 10, 45 and 50 °C was tested in tubes containing 5 ml of MRS broth as was described above. Optical density was measured after 24 and 48 h of incubation. Negative growth represented an OD_{600} comparable to the non-inoculated MRS medium, while growth was represented by at least third of the OD_{600} observed in the MRS cultures growth at 37 °C.

Mucin and mucus preparation. Mucin type III partially purified from porcine stomach (Sigma) and hereafter referred as gastric mucin was used. Crude mucus from small intestine of a 23-days-old healthy piglet was obtained by gentle scraping and suspended in cold HEPES plus Hanks balanced salt solution (H-H buffer) (136.87 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2.2H_2O$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 0.35 mM Na_2HPO_4, 2.57 mM KH_2PO_4 and 9.98 mM HEPES, pH 7.4) as previously described (Conway *et al*., 1990). Both mucus and gastric mucin were conjugated with horseradish peroxidase (HRP) using the method of Hudson and Hay (1989) and then stored at -20 °C until used.

Mucus and mucin adhesion assay. All lactobacilli isolates were inoculated and grown in LDM medium as described above. Bacterial cells were harvested, washed and resuspended in H-H buffer until an OD_{600} of 0.9-1.0. The *Lactobacillus fermentum* 104R strain previously shown as a strain with strong adhesion properties, through the 29 kDa MAPP protein (Rojas *et al*., 2002), was used as positive control. The adhesion assay was based on methods previously described by Rojas and Conway (2001). Briefly, drops of 20 µl of bacterial suspensions were immobilised on immobilon-P Polyvinylidene difluoride membranes (PVDF, Millipore) and a 3% bovine serum albumin (BSA) solution was used as blocking agent of non-specific adhesion sites. Membranes were washed 3 times for 10 min with 10 ml H-H buffer. Blocked membrane was incubated under slow agitation for 2 h at room temperature in H-H buffer with a 1:1000 solution of labelled mucus or gastric mucin, and then washed 3 times with H-H buffer as mentioned above. To visualise the binding reaction, the membranes were rinsed briefly with 0.1 M sodium acetate buffer, pH 5.0 and then incubated in 10 ml of the same buffer containing 2.5 µl of a 30% hydrogen peroxide solution (Sigma) and 3.5 mg of 3,3′-diaminobenzidine (Sigma). Membranes were rinsed with H_20 and dried at room temperature. Adhesion assays were performed in triplicate in two independent experiments.

Molecular identification. Chromosomal DNA of 27 adhesive *Lactobacillus* strains was prepared according to Anderson and McKay (1983). Polymerase Chain Reactions (PCR) were performed with specific primers for the 16-23S intergenic region (Tannock *et al*., 1999) (16-1A: 5′-GAATCGCTAGTAATCG-3′ and 23-1B: 5′-GGGTTCCCCCATTCGGA-3′) and the 16S rDNA gene (Broda *et al*., 1999) (pA: 5′-AGAGTTTGATCCTGGCTCAG-3′ and pH*: 5′-AAGGAGGTGATCCAGCCGCA-3′). For the 16-23S intergenic region, the smallest bands (about 500 to 600 bp) were excised from a 1.5% agarose gel and then purified with the QIAquick gel extraction kit (QIAGEN). For 16S rDNA, amplified DNA fragments were directly purified using the PCR purification kit (QIAGEN) and then sent for sequencing. These sequences were edited before the alignment and approximately 500 bp for the 16-23S intergenic region and 1500 bp for the 16S genes sequences were used. The nucleotide sequences of the 27 adhesive strains were compared with reported sequences in the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST) available at the National Center for Biotechnology Information website. Sequences representing the best matches were retrieved and aligned using the clustal method (Thompson *et al*., 1994) from DNASTAR Lasergene software (Version 5). Phylogenetic relationships between strains were calculated by maximum parsimony (MP) program of PAUP 4.0b (Swofford, 2003) with a heuristic search employing general search options. Relationships were further analysed by the neighbour-joining (NJ) program of PAUP 4.0d56 (Swofford, unpublished) with the Jukes-Cantor distance measure. *Escherichia coli* ATCC 43895 was the designated outgroup in NJ analyses and *Vibrio parahaemolyticus* (accession number AY531067) in MP. Confidence limits for phylogenetic trees (not shown) were estimated from bootstrap analyses (1000 replications for neighbour-joining searches).

Growth in mucus. To test the ability of the 27 adhesive *Lactobacillus* strains to grow in mucus, a method previously described was modified (Rojas and Conway, 1996). Mucus extracts (3 ml, concentration of 4 mg/ml of protein) were sterilised in plastic Petri dishes by exposure for 5 min to UV light. Sterility of mucus was tested on Plate Count agar (Difco), Violet Red Bile agar (Difco), Potato Dextrose agar (Difco) and Lactobacilli MRS agar (Difco) according with manufacture's instructions. Sterile mucus was then aliquoted in 200 µl fractions into sterile 96-well plates, inoculated with 10 µl of 1:100000 dilutions from 12 h MRS broth cultures containing between 200 to 1000 CFU, and incubated at 37 °C for 24 h under anaerobiosis. Cultures from each well were transferred to micro tubes, and bacteria were collected by centrifugation. Pellets were washed with PBS and 50 µl of 0.01% acridine orange solution were added to each tube, which was then incubated for 30 min at room temperature. Two washes with 50 µl PBS were performed and wet mounts were prepared using 5-10 µl drops of each sample. Slides were observed in an epifluorescence microscope (Nikon Eclipse E600) with a super high-pressure mercury lamp and a B-2A filter (EX450-490, DM505 BA 515) and using objective Plan fluorDLL100x oil F3. For quantification of viable bacteria, cultures at time 0 h and 24 h from each well were diluted in H-H buffer and serial dilutions were plated on Rogosa SL agar (Difco) and incubated at 37 °C for 24 h in anaerobic conditions. Counts of CFU per gram of piglet faeces and per cm^2 of small intestine or caecum at time 0 and 24 h were reported. Additionally a Neubauer counting chamber was used to quantify by direct count the number of cells in a Nikon Eclipse E600 microscope with phase contrast.

Growth in medium added with bile salts. Ability of 27 adhesive *Lactobacillus* strains to grow in medium added with bile salts was examined as previously described with minor modifications (Pedersen *et al*., 2004). Sterile MRS broth containing 0.3, 2.0, 3.5 or 7.5% (w/v) of bile salts No. 3 (Difco) was aliquoted in 200 µl fractions into sterile 96-well plates. Two microliters of an overnight MRS broth culture were inoculated into each well and then incubated at 37 °C for 24 h under anaerobiosis. Then optical density was measured. Negative growth represented an OD_{600} comparable to the non-inoculated MRS medium with equivalent concentration of bile salts, while growth was represented by at least the third of the OD_{600} observed in the MRS cultures without bile salts. The experiment was performed by duplicate in two independent assays.

Accession numbers. 16S rDNA and intergenic 16-23S sequences reported in this paper are available at the GenBank database under the accession numbers EF113958 – EF113975 and EU547278 – EU547313.

RESULTS AND DISCUSSION

In healthy pigs, the association of beneficial bacteria as lactobacilli with the epithelial and mucosal surfaces and their presence in faeces has been previously reported (Robinson *et al*., 1981; Rojas and Conway, 1996). In the present study, bacterial population in faeces ranged between 10^7 to 10^9 CFU g^{-1} with an average value of $4.46 \times 10^8 \pm 6.3$ CFU g^{-1} (n = 19). Likewise, in intestinal mucosa, counts of 3.8×10^6 and 3.2×10^6 CFU per cm^2 of small intestine and caecum respectively were observed. Colonies from each faecal piglet sample showing different morphology were selected from plates harbouring between 30 and 100 colonies, resulting in 109 isolates. From small intestine and caecal mucus, 28 and 27 colonies were selected respectively. All strains showed a bacillary morphology, a Gram positive staining and a catalase negative reaction. *Lactobacillus* strains were found at similar levels to the previously reported (Rojas and Conway, 1996). These

TABLE 1 - Growth of lactobacilli isolates at different temperatures

Source	Number of isolated strains	Percentage of the isolated strains		
		10 °C	45 °C	50 °C
Faeces	109	0%	100%	87.2%
Small intestinal mucus	28	0%	100%	32%
Caecal mucus	27	0%	100%	70%
Total	164	0%	100%	75%

TABLE 2 - Growth of lactobacilli isolates in MRS medium added with different concentrations of sodium chloride

Source	Number of isolated strains	Percentage of the isolated strains		
		430 mM	680 mM	1112 mM
Faeces	109	97.3%	87.2%	0%
Small intestinal mucus	28	71.4%	17.9%	0%
Caecal mucus	27	66.6%	17.9%	0%
Total	164	87.8%	64%	0%

results indicate that even pigs that inhabit extreme conditions in arid coasts, maintain the levels of cultivable *Lactobacillus* strains in faeces and intestinal mucus.

It is well known that microorganisms colonising gastrointestinal tract of animals come from the mother and from the environment (Conway, 1997). Therefore to determine if *Lactobacillus* strains isolated from piglets that inhabit the arid coast of Baja California Sur, Mexico are able to resist extreme conditions of salinity and temperature, their ability to grow in the presence of NaCl or at high or low temperatures was tested as described above. From the strains isolated, 64% of *Lactobacillus* presented abilities to grow in the presence of 680 mM of NaCl. Specifically, 87.2% from faeces strains and 18% from mucus strains resisted the salt concentration. Additionally 75% of the strains isolated were able to grow at 50 °C. Of these, 87.2% from faeces and 51% from mucus presented the ability to withstand the higher temperature (Table 1 and Table 2). It is well known, that some of the technological characteristics evaluated for potential probiotic strains are the resistance to heat and osmotic shocks caused by NaCl in the medium (Prasad *et al*., 2003). In a previous report, was evaluated the ability of *L. fermentum* strains to grow at 45 °C and 680 mM NaCl. These strains isolated from chickens in a tropical weather grew at this temperature but not in presence of that concentration of salt (Reque *et al.,* 2000). These results suggest resistance to salt could be related to strains origin. Moreover, was shown that *Lactobacillus* strains previously adapted to high temperatures are able to resist lethal temperatures during their processing (Desmond *et al.,* 2001). These abilities are important considering that probiotic bacteria are exposed to high temperatures and presence of NaCl during their technological preparation as pelleted or dried feed for pigs.

To determine the probiotic potential of *Lactobacillus* isolates, the adhesion to intestinal mucus or mucin was evaluated. The adhesion assay of the 164 isolates to porcine mucus and mucin allowed visualise strains that bind mucus or gastric mucin in a qualitative manner as is shown in Fig. 1. During the dot blot assay, the membrane was maintained wet in order to avoid changes in the surface of the bacterial cells. Additionally, this permitted a direct interaction between molecules on the bacterial surface and the mucus or gastric mucin components. Results

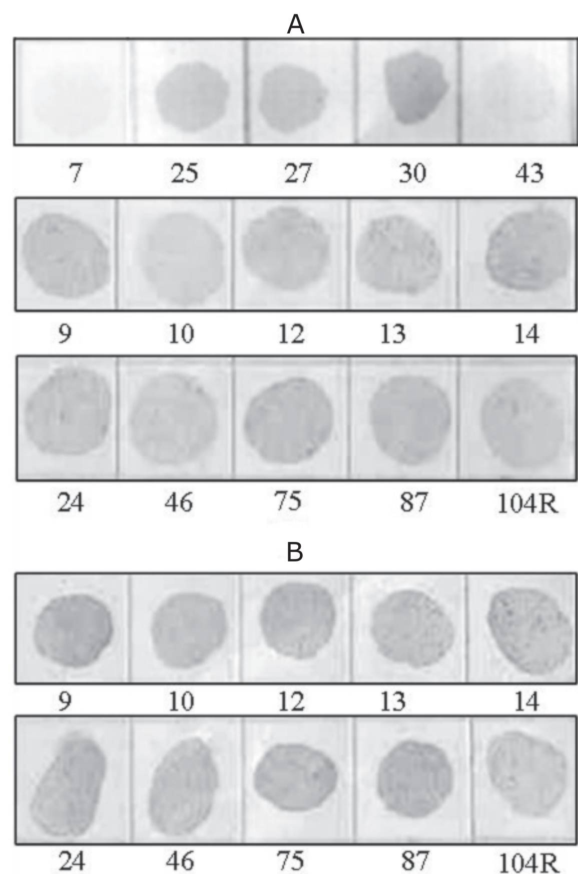

FIG. 1 - Dot-blot adhesion assay of piglets *Lactobacillus* strains using HRP-labelled (A) gastric mucin and (B) intestinal mucus. Results show no adhering and adhering isolates: *Lactobacillus* sp. BCS7, *Lactobacillus* sp. BCS43 and *Lactobacillus fermentum* BCS25, BCS27, BCS30, BCS9, BCS10, BCS12, BCS13, BCS14, BCS24, BCS46, BCS75, BCS87. 104R: *L. fermentum* 104R (positive control). For each isolate, experiments were performed in triplicate.

TABLE 3 - Qualitative adhesion of *Lactobacillus* strains isolated from faeces and mucus of piglets, as reveled by a dot-blot adhesion assay using HRP-labelled mucus and gastric mucin as probe

Source	Number of isolated strains	Adhesion (% of isolated strains)	
		Strong [a]	Weak [b]
Faeces	109	45%	55%
Small intestinal mucus	28	64%	36%
Caecal mucus	27	78%	22%
Total	164	53.7%	46.3%

[a] Adhesion similar or stronger than adhesion shown by *L. fermentum* 104R strain.
[b] Adhesion weaker than adhesion shown by *L. fermentum* 104R strain.

indicated that 88 isolates representing 53.7% of the 164 strains, presented adhesion to both mucus and gastric mucin similar to the positive control (*L. fermentum* 104R, Rojas *et al*., 2002) as is shown in Table 3. From the total of faecal strains 45% showed binding ability, whereas from intestinal and caecal mucus strains 64 and 78% presented adhesion ability respectively. Adhesion represents a prerequisite to colonise mucosal surfaces (Beachey, 1981). These results showed the highest percentage of adhesive strains in the caecum and intestine compared with faeces. Pathogens as *Escherichia coli* that inhabit the gastrointestinal tract of pigs present surface proteins that recognise in a specific manner receptors in the mucus (Fang *et al.,* 2000). Moreover these results support the previously observed by Namba *et al.* (2007) who reported that intestinal bacteria which colonise the surface of intestinal mucosa, showed a high-adhesive capability to fish intestinal mucus. Adhesive strains isolated from faeces could be released to the lumen during the renewal of mucus. Different adhesive abilities between faecal and mucosal strains could be also explained if is considered that microflora in the intestine differs from that in faeces (Marteau *et al.,* 2001). Moreover, adhesive properties are strain-dependent and differences exist even if strains were isolated from the same source (Kinoshita *et al.,* 2007).

For molecular identification 16-23S intergenic region and 16S rDNA gene sequences of 27 adhesive strains were amplified and then analysed. Sequence analysis of the smallest 500-550 bp bands corresponding to the 16-23S intergenic regions showed between 87 to 99% identity to sequences of *Lactobacillus* species reported in databases (Table 4). Moreover, analysis of 16S rDNA gene sequences showed between 98 to 100% identity to *L. fermentum* (17 strains), *L. johnsonii* (one strain), *L. vaginalis* (2 strains), *L. crispatus* (one strain) and *L. reuteri* (5 strains) spe-

TABLE 4 - Molecular identification of adhesive Lactobacillus strains based on the 16-23S intergenic region and 16S rDNA gene analysis

Source	Strain	Identity (%)[a]	
		16-23S sequence	16S rDNA sequence
Faeces	BCS9	97% to *L. fermentum*	99% to *L. fermentum*
	BCS10	96% to *L. fermentum*	99% to *L. fermentum*
	BCS12	97% to *L. fermentum*	99% to *L. fermentum*
	BCS13	98% to *L. fermentum*	99% to *L. fermentum*
	BCS14	97% to *L. fermentum*	99% to *L. fermentum*
	BCS21	98% to *L. fermentum*	99% to *L. fermentum*
	BCS24	98% to *L. fermentum*	99% to *L. fermentum*
	BCS25	98% to *L. fermentum*	98% to *L. fermentum*
	BCS27	98% to *L. fermentum*	99% to *L. fermentum*
	BCS30	97% to *L. fermentum*	99% to *L fermentum*
	BCS36	97% to *L. fermentum*	100% to *L. fermentum*
	BCS41	99% to *L. johnsonii*	100% to *L. johnsonii*
	BCS46	97% to *L. fermentum*	99% to *L. fermentum*
	BCS68	94% to *L. panis*	99% to *L. vaginalis*
	BCS75	95% to *L. fermentum*	99% to *L. fermentum*
	BCS80	98% to *L. fermentum*	99% to *L. fermentum*
	BCS81	97% to *L. fermentum*	99% to *L. fermentum*
	BCS82	98% to *L. fermentum*	99% to *L. fermentum*
	BCS87	98% to *L. fermentum*	99% to *L. fermentum*
Small intestinal mucus	BCS113	87% to *L. delbrueckii* subsp. *bulgaricus*	92 % to *L. delbrueckii* subsp. *bulgaricus*
	BCS125	98% to *L. acidophilus*	99% to *L. crispatus*
	BCS127	97% to *L. reuteri*	99% to *L. reuteri*
	BCS154	94% to *L. panis*	99% to *L. vaginalis*
Caecal mucus	BCS134	99% to *L. reuteri*	99% to *L. reuteri*
	BCS136	99% to *L. reuteri*	99% to *L. reuteri*
	BCS142	98% to *L. reuteri*	99% to *L. reuteri*
	BCS159	98% to *L. reuteri*	99% to *L. reuteri*

[a] E values of 0.0 except for the strain BCS113 who presented an E value of 2e-61 for the intergenic sequence.

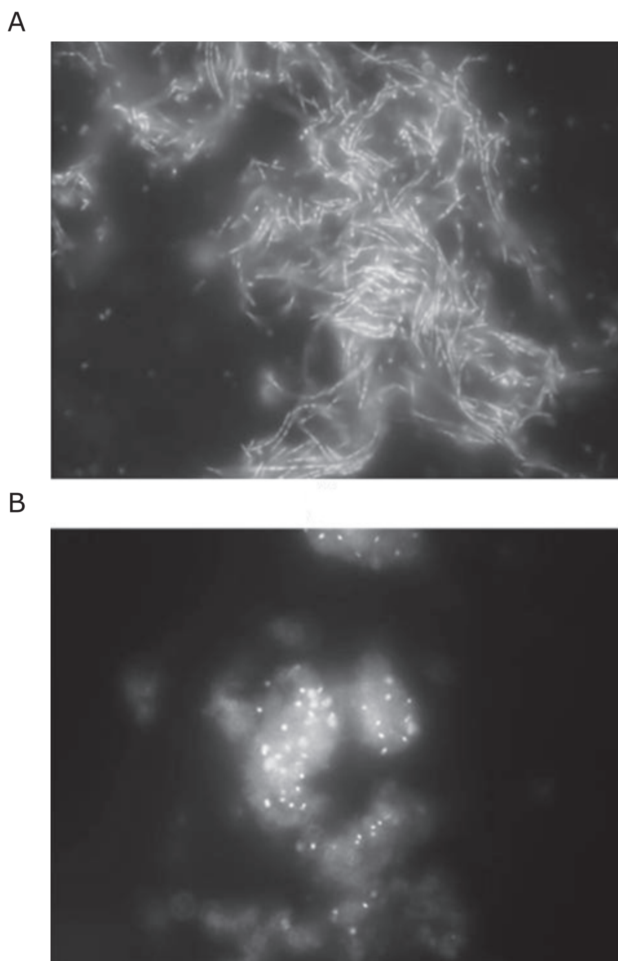

FIG. 2 - Lactobacilli in small intestinal mucus of piglets stained with acridine orange. Growth was observed by epifluorescence microscopy at 1000X magnification. A: *Lactobacillus fermentum* strain BCS87; B: negative control (non-inoculated mucus, no fluorescence was detected). For each isolate, experiments were performed in triplicate.

cies (Table 4). Except strain BCS113 that showed 92% identity to 16S rDNA of *L. delbrueckii* subsp. *bulgaricus*. *Lactobacillus fermentum* was predominant in faecal adhesive isolates whereas *L. reuteri* was the principal in mucus of caecum. In small intestinal mucus there was not predominant specie. These observations agree with previously reported by De Angelis *et al*. (2006) and Lin *et al*. (2007) who found both species in faeces and mucus of pigs. This result confirms the relevance of these species in the intestinal tract of pigs (Lin *et al*., 2007) even in arid coasts. Moreover, *L. fermentum* and *L. reuteri* species have been reported as good candidates as probiotics (De Angelis *et al*., 2007; Zoumpopoulou *et al*., 2008). Another species identified as *L. johnsonii*, *L. delbrueckii* subsp. *bulgaricus*, *L. vaginalis* and *L. crispatus* have been reported by their probiotic potential in humans and animals (Matijasic *et al*., 2006; Chen *et al*., 2007; Ohashi *et al*., 2007).

Adhesive strains were examined for their ability to grow in porcine intestinal mucus. Sterility of mucus was confirmed on specific media, and only one CFU per millilitre of mucus on plate count agar was found. The method described in this study discriminated in a qualitative and quantitative manner between bacteria able or unable to develop (Fig. 2). Adhesive bacterial strains (25 among the 27 selected) reached a high bacterial population visible through epifluorescent microscopy (Fig. 2). Quantification of CFU was carried out by classical microbiological methods resulting in the increase of 2 to 4 orders of magnitude after 24 h. Strains BCS113 and BCS154 did not grow in mucus under these conditions. Based on these observations was confirmed that lactobacilli inhabit, small intestine, colon and do not depend entirely on the host diet for growth but can utilise components from the mucus. It was already demonstrated that commensal intestinal bacteria as *Escherichia coli* MG1655, grows in mice mucus using it as the sole carbon source and energy (Chang *et al*., 2004). This strain showed a high expression of genes involved in amino-sugar catabolism and in the tricarboxylic acid cycle. Moreover, these results are in accordance with those of Rojas and Conway (1996) who demonstrated that *Lactobacillus* strains are able to utilise the small intestinal mucus for growth. Ability to develop in high number in intestinal mucus could represent an advantage for potential coloniser bacteria but also for potential probiotic *Lactobacillus*.

Finally, it is well known that in the intestinal tract, indigenous microbiota is exposed to bile salts, which are products of cholesterol metabolism in the liver and play an important role in the digestive process due to their amphipathic nature (Taranto *et al*., 2006). In the present study growth of selected strains in presence of bile salts was tested. Results showed that all strains increased their optical density levels at 24 h (1-1.5 at 600 nm) in medium with 3.5% of bile salts and no differences were observed between strains isolated from faeces or mucus. Only the strain BCS142, which was isolated from caecal mucus, was able to grow in MRS containing 7.5% of bile salts. Based on these results, one can propose that strains are good candidates to survive and colonise the intestinal tract.

Accordingly, of the 88 *Lactobacillus* strains with adhesive characteristics reported in this study, it is proposed that at least 27 of them could be used *in vivo* trials to maintain a good level of *Lactobacillus* in the gastrointestinal tract of piglets during the postweaning periods in arid coasts.

In conclusion, the isolated *Lactobacillus* strains showed to be able to survive in dry parched coastal environments with temperatures that range from 10 to 40 °C (Domínguez-Cadena *et al*., 2003) and concentration of NaCl in saline soils that range from 85 to 170 mM (Murillo-Amador and Troyo-Dieguez, 2000). These characteristics, suggest strains could resist technological processing as pelleted or drying animal feed. Moreover, the 27 selected strains showed to be able to survive and colonise *in vitro* the intestinal tract of pigs. Although little is known about characteristic of pathogens which infect piglets in this environment, we can suggest the isolates with probiotic potential are good candidates to be used in pigs that inhabit arid lands. These strains can prevent gastrointestinal disorders and avoid the unbalanced intestinal microflora typical of weaned piglets. Moreover *L. fermentum* and *L. reuteri* were the main species in faeces and in mucus respectively confirming the relevance of these species in the intestinal tract of pigs.

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