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PCR for Screening Potential Probiotic Lactobacilli for Piglets

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1. Introduction

1.1 Screening of potential probiotic lactobacilli

To continuously select probiotic bacteria, is needed to look for new strategies to make easy this task. In this chapter the characterization and identification by PCR of presumptive adhering lactobacilli to piglet gastrointestinal tract components is described and compared with previous reports. *Lactobacillus* is one of the major bacterial groups in the gastrointestinal tract of humans and animals (Smith, 1965; Dubos, 1965). Moreover, there is accumulating scientific evidence which strongly suggest that lactobacilli are associated with health (Bibel, 1988; Sanders, 2011). Consequently lactobacilli are frequently used as probiotics. This term refers to preparations of living microbes that can be added to the diet to improve health in humans and in farm animals (Fuller, 1989; Guilliland et al., 2001). The number of reports of health-promoting effects attributed to *Lactobacillus* strains has been increased in recent years where antagonistic activities against enteropathogens and modulation of immune system are well documented (Collado, 2006). The worldwide impact of advances in the scientific knowledge in this area is being enormous. For instance, diarrheal diseases affect millions of people throughout the world, having the greatest impact among children in developing countries (Guerrant et al., 1990; Guarino et al., 2011; Mondal et al., 2011). *Lactobacillus* have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* (Ashenafi 2005; Harris et al., 1989), *Escherichia coli*, *Salmonella* spp. (Chateau & Castellanos, 1993; Hudault et al., 1997), and others (Coconnier et al., 1997). When lactobacilli could be commonly used to prevent or alleviate some of the infections by enteropathogens, e. g. *E. coli*, *Salmonella*, *Shigella*, *Campylobacter*, etc. it could be an achievement for human beings. From an economical point of view, lactobacilli could reduce the risk for major economic losses due to decreased performance and health in the farm industry. For example, pig rising has become more industrialized and intestinal disturbances, e. g. diarrhea, affect significantly the piglet health and decrease intestinal performance (Goswami et al., 2011; Oostindjer et al., 2010).

Antibiotics have been used successfully against these infections, however there is an increasing concern consuming meat containing antibiotic residues as well as the potential hazards from spreading of resistance factors. Lactobacilli *Lactobacillus* is an alternative to maintain the health of growing pigs, mainly were environmental conditions are not controlled (Chiduwa et al., 2008). Under these conditions are a large number of pig farms worldwide. These conditions stress the animals, causing susceptibility to gastrointestinal diseases. It is well known that lactobacilli is a habitant of the intestinal tract of pigs and has been found as dominant microbiota. However confinement in small yards, large variations in temperatures, diet and other conditions, stress the animals, causing susceptibility to gastrointestinal diseases (Shimizu & Shimizu, 1978). Lactobacilli should retain special features to survive under these harsh conditions. 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 (Jonsson & Conway, 1992). Composition of gut microbiota can be modulated by host, environmental, and bacterial factors (Thompson-Chagoyán et al., 2007). The colonization potential of lactobacilli has been investigated using small intestinal mucus extracts from 35 day old pigs. Numbers of lactobacilli in different portions of the small intestine of 35 days old pigs were enumerated. Mucus isolated from the small intestine of pigs was investigated for its capacity to support the growth of lactobacilli and results confirmed that *Lactobacillus* spp inhabit the mucus layer of the small intestine and can grow and adhere to ileal mucus (Rojas & Conway, 1996). The survivability and colonization of probiotics in the digestive tract are considered critical to ensure optimal functionality and expression of health promoting physiological functions. Muralidhara (Muralidhara, 1977) reported that viable counts of lactobacilli in tissue homogenates from the duodenum and upper jejunum of 3 weeks old pigs were 5.5-6.21 $\log_{10}$ per g mucosa. In addition, when segments of the small intestine of piglets, from the duodenum to the ileum were examined, it was found that lactobacilli increased from 6.4 to 8.2 $\log_{10}$ per g of mucosa (McAllister et al., 1979). From the total numbers of identified strict anaerobic organisms associated with the cecal mucosa, anaerobic lactobacilli were much lower (4.0-5.7 $\log_{10}$) per cm^2 than the numbers of obligated anaerobes. Although differences in the counts of the different groups of organisms have been quite large for the various reports, *Lactobacillus* appears to be dominant group in cecal and colonic content.

Screening for functional and probiotic attributes in lactobacilli new isolates is commonly performed, following these assays: Gram stain, acid and bile salt tolerance, cell surface hydrophobicity, adhesion to mucus and mucin, autoaggregation, Caco-2 cell-binding as well as antibacterial activity against *E. coli, L. monocytogenes, S. typhi*, etc. and antioxidative activities (Jacobsen et al., 1999; Macías-Rodríguez et al., 2008; Kaushik et al., 2009). Recently a screening of predominant *Lactobacillus* strains from healthy piglets has been performed in order to select specific probiotics for arid land piglets. 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 (Macías-Rodríguez et al., 2009). Likewise probiotics are increasingly used as nutraceuticals, functional foods or prophylactics and considering that probiotics strains have shown to be population-specific due to variation in gut microbiota, food habits and specific host-microbial interactions (Kaushik et al., 2009), screening of new indigenous probiotic strains in different region of the world is necessary.

1.2 Colonization by lactobacilli

Colonization studies of lactobacilli to the gastrointestinal tract first were concentrated on the attachment to the non secretory epithelium from the stomach. Cell morphology by electron microscopy, viable counts and biochemical test have been very important tools to identify lactobacilli attached to the keratinized squamous epithelium of the stomach of mice (N. Suegara et al., 1975; Moser & Savage, 2001; Savage, 1992; Tannock & Savage, 1974; Conway & Adams, 1989) and pig (Fuller et al., 1978; Pedersen & Tannock, 1989; Tannock et al., 1987; Henriksson et al., 1991). Later other reports on colonization by lactobacilli to other regions in the intestinal tract were found. Colonization of lactic acid bacteria isolated from rats and humans in the gastrointestinal tract of gnotobiotic rats has been studied by performing viable counts of the contents and tissue homogenates from the different regions of the intestinal tract. It was observed that lactobacilli seem to be retained, and to multiply on the mucosal surfaces along the intestinal tract (Kawai et al., 1982). In other report lactobacilli were ingested by human volunteers and samples of jejunal fluid at varying intervals were cultured for lactobacilli (Robins-Browne & Levine, 1981). It was shown that lactobacilli entered the small intestine and persisted there for 3-6 h after which time, levels returned to the base-line (Dixon, 1960). Studies on the possible interaction of lactobacilli with mammalian extracellular proteins have been performed. It was shown that specific collagen binding is common among lactobacilli of various origins (Aleljung et al., 1991).

Attention has been focused on interactions of lactobacilli with the mucosa of the intestinal tract. The gastrointestinal tract is covered by a protective mucus layer consisting of glycolipids and a complex mixture of large and highly glycosylated proteins called mucins as the main components. Mucus layer represents the first barrier of contact between bacteria contained in the lumen and the epithelial cell layer of the host (Tassell et al., 2011). Ability of commensal bacteria to adhere mucus is an important characteristic that is evaluated in probiotic bacteria (Ma et al., 2005). Adherence of lactobacilli to the intestinal epithelium and mucus is associated with stimulation of the immune system and inhibition of adhesion of pathogens (Herías et al., 1999). Caco-2 and HT-29 cells and a subpopulation of mucus secreting HT29-MTX cells have been used to study the adhesion of human isolated *L. acidophilus* BG2F04 strain. These studies showed scanning electron micrographs where mucus secreting HT29-MTX monolayer covered by the dense mucus gel produced by these typical goblet cells, bound to lactobacilli. In addition they proposed a model for the adherence of this *Lactobacillus* strain to human intestinal cells (Coconnier et al., 1997). Other workers used human colon mucosa in an *in vitro* assay, to test the capacity of five *Lactobacillus* strains to colonize; a dense population of lactobacilli was observed covering the whole mucosal surface of the colon tissue (Sarem-Daamerdji et al., 1995). Other contributions for understanding the interactions between gastrointestinal mucosa and lactobacilli have been reported. The diversity of *Lactobacillus spp* on healthy and diseased human intestinal mucosa biopsies has be studied (Molin, 1993). These workers assessed the potential of the *Lactobacillus* isolates for treating intestinal disorders, suggesting that there are no general differences in the type of dominating *Lactobacillus* microbiota between mucosa from different regions of the intestine. In another report, different *Lactobacillus* strains in fermented oatmeal soup were administered to healthy human volunteers. Biopsies were taken from both the upper jejunum region and the rectum before one and eleven days after administration. Results showed significantly increased counts of lactobacilli on the jejunum mucosa and high levels of all those strains that remained eleven days after

administration (Johansson et al., 1993). Colonization experiments in mice, also showed that the number of lactobacilli detected in samples collected from various regions of the gastrointestinal tract, two weeks after inoculation, were not statistically significant different, no matter which strain had been used to colonize mice. In addition, it was concluded that bile salt hydrolase production was not an essential attribute for lactobacilli to colonize the murine gastrointestinal tract. Furthermore, the growth rate of mice that consumed a nutritionally balanced diet were not affected by the presence of bile salt hydrolase producing or not lactobacilli in the gastrointestinal tract (Bateup et al., 1995). The capacity of different lactobacillus strains to grow in and adhere to small intestinal mucus as well as the characteristics of binding was studied. It was shown that six *Lactobacillus* strains isolated from porcine small intestinal mucosa, one isolated from faeces, one isolated from stomach and one more isolated from human feces, all grew equally well in intestinal mucus extract. Growth was monitored by enumerating the colony forming units. During growth in mucus, a visible precipitation was developed because lactobacilli formed clusters surrounded by mucus. In this study it was observed that when lactobacilli were grown in mucus, the ability to adhere to mucus was reduced from 35% to 10% of the adhesion. This could occur because adhesin(s) on the surface of the bacteria were being blocked by receptors or receptors-like components in the mucus (Rojas & Conway 1996). Adhesion assays of *Lactobacillus fermentum* 104R (Actually identified as *L. reuteri* 104R) indicated that this strain adhered to mucus when it was grown in synthetic media. Adhesion data were analyzed by Scatchard plot and it was noted that the binding of lactobacilli to mucus is not mediated by a single adhesin-receptor interaction. The quantitative interpretation of the binding data for this system was not possible to perform because the complexity of the system. These results correlate with other report suggesting that lactobacillus species adhere to intestinal cells via mechanisms which involve different combination of factors on the bacterial cell surface (Greene & Klaenhammer, 1994). Adhesion promoting compound(s) from *L. reuteri* 104R were found in the spent culture medium on the late stationary phase of growth. The spent culture fluid was used to inhibit adhesion to mucus of whole *L. reuteri* 104R strain, revealing that proteinaceous compound(s) were involved in the binding (Rojas & Conway, 1996).

1.3 *Lactobacillus* adhesins

Bacteria can have many types of surfaces, including sheaths, S-layers, capsules and walls. In the laboratory certain surface types are usually expressed. For example, *E. coli* K12 contains only core polysaccharide plus lipid A in its lipopolisaccharide that was why this strain is restricted to a laboratory habitat since it cannot withstand the rigors of a natural environment. This strain possesses only an outer membrane as its surface component surfaces components, but a related strain, K-30, is enclosed in a capsule. Frequently, it is the natural environment and their intrinsic stress that elicit expression of the surface attributes of a bacterium (Costerton, 1988; Brown et al., 1988). A bacterium in its native habitat will often possess a wall overlaid by a multiplicity of superficial layers. After several subcultures in laboratory medium these layers are not longer required and are lost (Costerton, 1988). This surface character could makes difficult the correlation of laboratory studies on adhesins of the bacteria with the *In vivo* state. Intestinal mucus extract from the small intestine of pig was used for lactobacilli growth and for studying the production and expression of the mucus and mucin adhesion promoting proteins.

Cell wall of Gram positive bacteria is composed primarily of peptidoglycan, which often contains peptide interbridge and large amounts of teichoic acids (polymers of glycerol or ribitol joined by phosphate groups). Amino acids and sugars are attached to the glycerol and ribitol groups. These molecules are important for maintaining the structure of the wall. Capsules, slims S-layers, sheaths or even pili (fimbriae) can occur as superficial layers above the cell wall. They can occur singly or in combination. Distinction among them is based primarily on their structural attributes (Beveridge, 1989; Beveridge & Graham, 1991). The term adhesin has been used to denote functions that are involved in one or more of the three following activities: 1) they may promote attachment and then initiate colonization of surface habitats, 2) They may be responsible for the organization of microbial communities and assemblages, and 3) they may be instrumental in promoting cell to cell contact as a phase preceding the transfer of genetic information between cells. The term adhesion has been used to describe the relatively stable, irreversible attachment of bacteria to surfaces, and the term receptor has been used for both known and putative entities on surfaces to which adhesins bind to effect specific adhesion (Jones & Isaacson, 1984). While there is a considerable amount of information published about proteinaceous bacterial adhesins and their receptors on pathogenic bacteria (Jones & Isaacson, 1984; Klemm, 1994; Bonazzi & Cossart, 2011), there are fewer studies on the mechanisms of adhesion of lactobacilli to gastrointestinal mucosa. Adhesion of *L. acidophilus* to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer protein (S-layer) protein was reported (Schneitz & Lounatma, 1993), and the adhesion to collagen by *L. reuteri* NCIB 11951 was shown to be mediated by a 29 KDa protein (Aleljung et al., 1994) and to *L. crrispatus* JCM 5810 was mediated by a 120 KDa S-layer protein (Toba et al., 1995). Another interesting finding was a 32 KDa protein, an aggregation promoting factor on *L. plantarum* strain 4B2 which increased the frequency of conjugation (Reniero et al., 1992; Reniero et al., 1993). The ability of probiotic bacteria to aggregate should be considered a desirable characteristic because they potentially inhibit adherence of pathogenic bacteria to intestinal mucosa either by direct coaggregation with the pathogens to facilitate clearance, by forming a barrier via self-aggregation or coaggregation with commensal organisms on the intestinal mucosa. Surface proteins from lactobacilli have been reported to be affected by freeze drying (Ray & Johnson, 1986; Brennan et al., 1986) and by the composition of the culture media (Pavlova et al., 1993; Cook et al., 1988).

Purification and characterization of proteins from lactobacilli which promote the adhesion to mucus have been well studied. The purification of a mucus and mucin adhesion promoting protein (MAPP) from the surface of *L. reuteri* 104R was performed by using LiCl (1M). A variety of different agents to extract proteins have been used. EDTA (0.1M), urea (8M) or $MgCl_2$ have been used to effectively release surface associated material from bacterial cells of various genera. Solutions of detergents such as sodium lauryl sarcosinate, triton X 100 (1% v/v final concentration), sonication and sodium dodecyl sulphate (SDS, 1%, w/v) have been shown to be effective in extracting proteins from *L. reuteri* strain 100-23 (Boot et al., 1993; Chagnaud et al., 1992). Guanidine hydrochloride (4M, GHCl) was used to extract regular arrays from the cell walls of different strains (Masuda & Kawata, 1983) and an S-layer protein from *L. acidophilus* ATCC 4356 (Boot et al., 1993). GHCl (2M) was used to extract a collagen bindig S-layer protein from *L. crispatus* JCM 5810 (Toba et al., 1995) while LiCl (1M) for 20 h at 20°C after treatment with lysozyme (2 mg per ml) for 1 h, was used to extract another collagen binding protein from *L. reuteri* NCIB 11951 (Aleljung et al., 1994).

The MAPP protein from *L. reuteri* 104R was extracted from the surface, by treating the cells after 14-16 h growth in a semi-defined medium (LDM), with LiCl (1M) for 1 h with gently mixing at 4°C. However, when other lactobacilli strains isolated also from intestinal tract which presented binding to mucus and mucin were treated as above did not show the characteristic band of the MAPP adhesin as it was visualized by western blot with the horse radish peroxidase labeled mucin (Rojas et al., 2002). The adhesion of *L. reuteri* JCM1081 to HT-29 cells mediated by a cell surface protein was reported. Results showed a 29-kDa surface protein which displays significant peptide sequence similarity to the Lr0793 protein from *L. reuteri* ATCC55730 (71.1% identity), whereas the protein Lr0793 is homologous to the ABC transporter component CnBP, which previously has been described as a collagen binding protein. The 29-kDa surface protein of *L. reuteri* JCM1081 probably is classified as a member of the ABC transporter family, as well as CnBP from *L. reuteri* NCIB11951 and MapA from *L. reuteri* 104R (Wang et al., 2008). The mucus-binding properties of a large collection of *L. reuteri* strains isolated from a range of vertebrate hosts and the correlation of the adherence of a subset of strains to the presence and expression of MUB was performed by immunodetection, microscopic immunolocalization of MUB on the bacteria, characterization of cell-surface extracts and spent media by gel electrophoresis, Western blotting and mass spectrometry, quantification of *mub* gene expression by qRT-PCR, cell aggregation and cell-surface MUB quantification. Results revealed that the particular MUB investigated is highly specific to a very small set of closely related strains of *L. reuteri.* This was observed despite the fact that 17 proteins with a putative MucBP domain were found in the available genomes of *L. reuteri.* strains 100-23, DSM 20016T, MM2-3, MM4-1, ATCC 55730 and CF48-3A, nine of which were present in the rodent isolate 100-23 (Mackenzie et al., 2010).

1.4 Adhering probiotic *Lactobacillus*

Two requirements have been identified as desirable properties for *Lactobacillus* to be considered as an effective probiotic microorganism, these include the ability to adhere (Reid, 1999), and then to consequently colonize mucous surfaces. Mucus layer is the first physical barrier to host-cell stimulation by bacteria in the gut. Adhesion to mucus is therefore the first step required for probiotic organisms to interact with host cells and elicit any particular response. Adherence to intestinal mucus has been associated to competitive exclusion of pathogens (Gueimonde et al., 2006; Lee et al., 2008) considering it as a critical event for colonization not only for lactobacilli but also for pathogenic bacteria (Beachey, 1981; Soto & Hultgren, 1999). In the gastrointestinal tract, mucus is the outermost luminal layer, and is the first intestinal component of surface that microorganisms are likely to contact before they reach epithelial cells. Mackie (Mackie et al., 1999) suggested that during a colonization event, bacterial population remains stable in size, with no need of periodic reintroduction of bacteria by oral doses. This implies that colonizing bacteria multiply in a given intestinal niche at a rate that equals or exceeds their rate of washout or elimination from the intestinal site. 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 recognized that resistance of potential probiotic to bile salts is a testable and is a necessary property (Moser & Savage, 2001).

The mechanisms used by lactobacilli to recognize and adhere to gastrointestinal components, until now is not completely understood. Protein and carbohydrate play an important role in mediating the adhesion to mucosal and or epithelial host surfaces. Some cell-surface biomolecules as exopolysaccharides and proteins have been recognized by their ability to bind gastrointestinal components (Vélez et al., 2007; Rojas et al., 2002; Sun et al., 2007). The best characterized are proteins present in the surface of lactobacilli that can be attached covalently or not to the cell wall (Vélez et al., 2007). Recently, proteins that adhere to mucus or mucins have been described and characterized. Adhering protein molecules characterized from *Lactobacillus* are Mucus-binding protein (Mub) of *L. reuteri* 1063 (Roos & Jonsson, 2002), the lectine-like mannose-specific adhesin (Msa) of *L. plantarum* WCFS1 (Pretzer et al., 2005), the mucus adhesion promoting protein (MAPP or MapA) from *L. reuteri* 104R reported by its ability to bind porcine mucus and mucin (Rojas et al., 2002) and Caco-2 cells (Miyoshi et al., 2006) and the Mub of *L. acidophilus* NCFM (Buck et al., 2005). Moreover, two proteins EF-Tu (Elongation Factor-Tu) and GroEL (a class of heat shock protein) of *L. johnsonii* La1 NCC533 showed abilities to adhere to mucins at specific conditions of pH (Granato et al., 2004; Bergonzelli et al., 2006). Recently a piglet mucus adhesion protein was completely characterized from the potential probiotic *L. fermentum* strain BCS87 (Macías-Rodríguez et al., 2009).

1.5 Genes codifying for *Lactobacillus* adhesins

Genetic research on *Lactobacillus* is underway in many laboratories around the world. Research has centered on 1) characterization and construction of vectors based on endogenos *Lactobacillus* plasmids which are capables to replicateof replicate and express molecules in specific lactobacilli strains, 2) molecular cloning of genes and operons from lactobacilli encoding important metabolic pathways, proteinases and adhesins 3) methods for introduction of genes *In vivo* and *In vitro* through conjugation, transfection and transformation (Chassy, 1987), and more recently 4) the global analysis of proteins and genes using the new tools of proteomic and genomic and the data base information of diferent species of *Lactobacillus* which are in public data bases. The development of cloning systems of *Lactobacillus* have increased in the last years. Methods for the introduction and stable maintenance of DNA into *Lactobacillus* are routine now and can be applied to almost any *Lactobacillus* species. Both broad host-range and narrow host range multi-copy plasmid vectors based on a variety of replicons have become available for the introduction and expression of homologous and heterologous genes (Pouwels & Leer, 1993). The sequenced genomes of lactobacilli are increasing and their availability might lead to the identification of the adhesin domain containing proteins in other species of *Lactobacillus* and in the specific functions of this surface proteins. Genes codifying for above adhesins are well known. The cloning and sequencing of the *L. reuteri* 104R gene encoding the adhesion promoting protein (MAPP) that binds to porcine gastrointestinal mucus was also studied. The sequence revealed one open reading frame consisting of 744 nucleotides corresponding to 244 aminoacids with deduced pI of 10.57, net charge at pH 7 of 16.23 and a molecular mass of 26.4 KDa. No putative promoter was found, however a start codon (ATG) appeared 6 bases downstream from the beginning of the sequence. The open reading frame ended with stop

codons in all three reading frames (TGA A TAA T TAA). Computer search of the nucleotide and aminoacid sequences, showed that this adhesin is related to proteins encoding adherence factors from several pathogenic bacteria, as well as amino acid transporter

binding protein precursors (Rojas, 1996; E. Satoh et al., 2000). Expression by real time PCR of the genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* by *L. plantarum* 423 grown in the presence of bile, pancreatin and at low pH, was reported. It was found that under normal physiological concentration of bile and pancreatin, expression of the *Mub* gene was affected, the *MapA* gene was over expressed and the *EF-Tu* gene remained stable, suggesting that whilst the expression of certain mucus genes may be affected by bile and pancreatin, others mucus genes are switched on, enabling the strain to adapt to physiological conditions and adhere to the gastrointestinal tract (Ramiah et al., 2007). To confirm the *MapA* results will be interesting to search in *L. plantarum* genome the compete sequence of this gene and find the adhering function in the reported specie or strain. By searching bacterial genome sequences and the UniProt protein data base for potential mucus binding proteins based on the sequence of the Mub domains of *L. reuteri* and *L. plantarum*. Boaekhorst et al, 2006. found that MUB domain is variable in size and sequence, making it difficult to determine precise domain boundaries. However the high variability in the number of MUB domain in putative mucus-binding proteins suggested that the MUB domain is often duplicated or deleted in evolution and appears to be only present in lactic acid bacteria, with the highest abundance in lactobacilli of the gastrointestinal tract, fulfilling an important function in host-microbe interactions (Boekhorst et al., 2006). Characterization of 32 Mmubp and *32-mmubp* gene from the potential probiotic strain previously isolated from piglet *L. fermentum* BCS87 was reported (Macías-Rodríguez et al., 2008). In the adhesion of this wild type strain to mucus and mucin, two proteins were identified, one of them, the 32Mmubp was characterized and the gene that codes for it was reported. Results indicate that the gene encoding this adhesin is conserved for *L. fermentum.* Other results suggested that 32Mmubp is released to the medium, but it could be anchored to cell wall by electrostatic interactions with acidic groups. It was indicated that Mmubp protein is a member of an ABC transporter system and is part of the OpuAC family. Based on homology and sequence domain search and in a phylogenetic tree with sequences of a seed group of the OpuAC family were shown conserved sequences between prokaryotic proteins of substrate-binding region on ABC type glycine/betaine transport systems. Some members of the corresponding taxa having similar ecological niches to those occupied by lactobacilli (gastrointestinal and respiratory tracts), i.e. *Helicobacter pylori* and *Mycobacterium tuberculosis,* did not group together suggesting that adhesion mechanisms is not a phylogenetic associated trait (Macías-Rodríguez et al., 2009). Recently was discovered only in the genome of the probiotic *Lactobacillus rhamnosus* GG, two different pilus fiber in the *spaCBA* and *spaFED* gene clusters. Moreover the expression and localization of intact SpaCBA pili on the cell surface of this strain were confirmed by immunoblotting and immunogold-labeled electron microscopy using antiserum specific for the Spa pilin. SpaCBA pilus-mediated binding of *L. rhamnosus* GG cells to human intestinal mucus was revealed (Kankainen et al., 2009). More recently pilin subunits SpaA, SpaB, SpaD, SpaE and SpaF encoded by genes in the *spaCBA* and *spaFED* genes clusters were cloned in *E. coli*. Recombinant, overproduced proteins were purified and assessment of the adherence to human intestinal mucus was performed. Results suggested that SpaC and

SpaB may be involved in SpaCBA pilus-mediated adherence to intestinal mucus. It was established that the SpaF minor pilin is the only mucus binding component in the putative SpaFED pilus fiber (von Ossowski et al., 2010). Aggregation promoting factors (Apf) are secreted proteins that have been associated with a diverse number of functional roles in lactobacilli, including self aggregation, coaggregation with other commensal or pathogenic bacteria, maintenance of cell shape and the bridging of conjugal pairs. Genes encoding Apf´s have been characterized for several *Lactobacillus* species, including *L. crispatus, L. johnsonii, L. gasseri, L. paracasei* and *L. coryniformis*. Investigation of the functional role of the putative *apf* gene (LBA0493) in *L. acidophilus* NCFM by mutational analysis was performed. It was observed that survival rates mutant strain NCK2033 decreased when stationary phase cells were exposed to simulated small intestinal and gastric juices. Furthermore, NCK2033 in the stationary phase showed a reduction of *In vitro* adherence to Caco-2 intestinal epithelial cells, mucin glycoproteins and fibronectin. It was suggested that the Apf-like proteins may contributes to the survival of *L. acidophilus* during transit through the digestive tract and, potentially, participate in the interactions with the host intestinal mucosa (Goh & Klaenhammer, 2010). The ability to tolerate the toxic levels of bile salts accumulated therein is the essential requirement to survive in the gut and it is generally included among the criteria used for selection of the potential probiotic strains and their application as functional ingredients in foods and nutraceuticals. Expression of bile salt hydrolase and surface proteins were targeted to look at their expression profile in two putative probiotic *L. plantarum* Lp9 and Lp91, (compared with standard strain CSCC5276) by quantitative real time PCR (RT – qPCR). Expression ratio for *bsh, mub, mapA* and *EF-Tu* genes under *In vitro* simulated gut conditions was tested for significance by qBase-Plus software. Amongst the three probiotic strains used in that study, Lp91 showed the highest level of *bsh* gene expression when the medium was supplemented with 0.01% mucin along with 1% of both bile and pancreatin in all the three strains. Results suggested that the expression of *mub* is a characteristic of not only the specie but could also be strain specific. The highest level of expression of *mapA* gene was recorded when normal gut conditions (Mucin, 0.01% and 0.3% each of bile and pancreatin, 0.3% supplemented in MRS at pH 6.5) were used. The relative expression of EF-Tu gene was significantly up-regulated in Lp9 in presence of mucin along at 0.01 and 0.05%, respectively at pH 7.0. It was concluded that the efficacy of both Lp9 and Lp91 with regards to expression of *mub, mapA* and *EF-Tu* was found to be either superior or comparable to that of standard probiotic strain (Duary et al., 2011). To confirm the *MapA* results in this last report it is important to find if the *L. plantarum* genome contains this gene to probe then its functionality.

1.6 Methods for screening mucus or mucin adhering bacteria

Mucus provides protective functions in the gastrointestinal tract and plays an important role in the adhesion of microorganisms to host surfaces. Mucin glycoprotein forms a framework to which microbial population can adhere, including probiotic *Lactobacillus* strains. Numerous factors have been shown to influence binding of lactobacilli to mucus *in vitro*. Experimental methods should be reviewed and compared to get a better understanding of the bacteria-mucosa interaction. The mechanism of this interaction could help to determine the degree of probiotic functionality imparted by adhesion (Tassell et al., 2011). Different methods to measure adhesion to mucus have been reported. Mucus contains about 80% of carbohydrates which occur as oligosaccharides and most of the glycans are present in

clusters flanked by naked regions of the protein core (Clamp & Sheehan, 1978). Since mucins from different sources could be substituted with different oligosaccharides, properties such as the linear charge density could vary considerably. Porcine and rat mucin differ markedly in glycosylation and charge density (Malmsten et al., 1992). This characteristic of mucin, need to be considered when performing experiment to test the interaction between bacteria and mucus or mucin. A common method used to test *E. coli* adhesion to mucus extract prepared from the large and small intestine of mice involved immobilizing the mucus extracts on polystyrene. Radioactively labeled bacterial suspensions were added to the immobilized mucus compound and after a short inoculation time, the unbound cells were removed and adhesive cells were enumerated by measuring the amount of radioactivity (Laux et al., 1984; Laux, 1986). This method was adapted for studying *E. coli* adhesion to ileal mucus extracts from pigs (Conway et al., 1990; Blomberg & Conway, 1989). It has also been used to study the adhesion of *L. reuteri* 104R to small intestinal mucus extracts from pig (Rojas & Conway, 1996). This method still is used with some modifications (Mackenzie et al., 2010), however, it was not suitable for studying adhesion to mucin since it bound poorly to the polystyrene. In a control experiment where horse radish peroxidase labeled mucus and mucin were used, it was shown that mucin adhered to polystyrene a less extent than mucus. These results are consistent with other finding where rat and pig mucin layers on hydrophobic surfaces were studied. It was found by ellipsometry and surface force measurements, by using mica and silica surfaces, that the adsorption equilibrium of rat gastric mucin was reached after 5 hours, however for pig gastric mucin equilibrium it was not reached. It was demonstrated that for such layers, as the repulsive forces become weaker the slower the surfaces are brought together (Malmsten et al., 1992). Dot blot assay, a qualitative *In vitro* assay to detect the binding of bacterial cell surface components to mucus extracts was developed whereby extracts containing bacterial components and fractionated proteins were immobilized in a solid phase matrix and then blotted with enzymatically labelled mucus (Rojas and Conway, 2001). Results were compared to those obtained using the inhibition assay. In addition, whole cells of *Lactobacillus* and *E. coli* were tested in the dot blot assay and results compared with a modification of the method of Laux and coworkers (Conway et al., 1990). The results obtained using the dot blot assay provided further information about the binding of *Lactobacillus* and *E. coli* to gastrointestinal mucus, not only because adhesion promoting compounds could be detected in fractionated extracts but also because porcine gastric mucin as well as small intestinal mucus could be used for blotting (Rojas & Conway, 2001). Other methods have used to study adhesion to mucosa. Cultured cells have been suggested to be the best available models to study intestinal attachment of bacteria and viruses (Coconnier et al., 1997). Particularly, mucus secreting cells could be the best to study *Lactobacillus*-mucus and mucin interactions. Unfortunately this method has the same limitations as the mucus immobilization method of Laux et al. (Conway et al., 1990) for studing adhesins in soluble extracts.

1.7 Genetic tools to study the expression of genes encoding adhesins

The number of genetic tools that have been developed has increased tremendously during the last 20 years. Genetic analysis is made possible for several lactobacilli strains of known probiotic action, such as *L. plantarum* WCFS1, *L. acidophilus* NCFM, *L. johonsonii* NCC533, *L. salivarius* UCC118, *L. reuteri* ATCC 55730 and *L. rhamnosus* GG. Mutant studies are of the

utmost importance in the unraveling of modes of action of lactobacilli as they can often directly relate genotype to phenotype. Nevertheless the number of currently identified genetic loci hypothesized to encode features supporting probiotic action confirmed by mutant analysis is still limited (Lebeer et al., 2008). Although the availability of genome sequences will certainly advance the field, they need to be complemented with functional studies. Methods that start to be applied for differential gene expression analysis of lactobacilli under relevant conditions are genome-wide comparisons of RNA profiles using microarrays, comparison of protein profiles with two dimensional (2D) difference gel electrophoresis, *In vivo* expression technology (IVET) using a promoter probe library and differential-display PCR (DD-PCR) (Lebeer et al., 2008).

2. Materials and methods for screening probiotic potential lactobacilli

2.1 Animals

Newborn piglets (*Landrace-Duroc)* from a pig farm 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.

2.2 Sampling

Faecal samples of 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. Piglets randomly selected, were sacrificed by a humanitarian method in the laboratory and immediately the small intestine and cecum were removed and sectioned with a sterile dissection kit. These pieces were opened and rinsed with sterile 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 cecum with a spatula and used to isolate lactic acid bacteria.

2.3 Isolation of bacteria

Isolation and characterization of bacteria was previously performed as reported before (Rojas & Conway, 1996; Macías-Rodríguez et al., 2008) . Briefly, lactic acid bacteria from faeces and from associated small intestine and cecum mucus of healthy preweaned piglets were isolated. 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 and for cm^2 were reported. Colonies 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). Aliquots of each strain were kept in 1.5 ml tubes with 50% of glycerol at -85° C. Fresh cultures were used to perform the adhesion assay.

Source	Strain	Accession numbers (16-23S/ 16Sr DNA)	% identity
			Based on 16S rDNA sequence
Faeces	BCS9	EF113967/ EF113958	*99% to Lactobacillus fermentum*
Faeces	BCS10	EF113968/ EF113959	*99% to Lactobacillus fermentum*
Faeces	BCS12	EF113969/ EF113960	*99% to Lactobacillus fermentum*
Faeces	BCS13	EF113970/ EF113961	*99% to Lactobacillus fermentum*
Faeces	BCS14	EF113971/ EF113962	*99% to Lactobacillus fermentum*
Faeces	BCS21	EU547278/ EU547296	*99% to Lactobacillus fermentum*
Faeces	BCS24	E113972/ EF113963	*99 % to Lactobacillus fermentum*
Faeces	BCS25	EU547279/ EU547297	*99% to Lactobacillus fermentum*
Faeces	BCS27	EU547280/ EU547298	*99% to Lactobacillus fermentum*
Faeces	BCS30	EU547281/ EU547299	*99% to Lactobacillus fermentum*
Faeces	BCS36	EU547282/ EU547300	*100 %Lactobacillus fermentum*
Faeces	BCS41	EU547283/ EU547301	*100% Lactobacillus johnsonii;*
Faeces	BCS46	EF113973/ EF113964	99% to *Lactobacillus fermentum*
Faeces	BCS68	EU547284/ EU547302	99% to *Lactobacillus vaginalis*
Faeces	BCS75	EF113974/ EF113965	99% to *Lactobacillus fermentum*
Faeces	BCS80	EU547285/ EU547303	99% to *Lactobacillus fermentum*
Faeces	BCS81	EU547286/ EU547304	99% to *Lactobacillus fermentum*
Faeces	BCS82	EU547287/ EU547305	99% to *Lactobacillus fermentum*
Faeces	BCS87	EF113975/ EF113966	99% to *Lactobacillus fermentum*
SI mucus*	BCS113	EU547288/ EU547306	92 % to *Lactobacillus delbrueckii subsp. bulgaricus*
SI mucus*	BCS125	EU547289/ EU547307	99% to *Lactobacillus crispatus*
SI mucus*	BCS127	EU547290/ EU547308	99% to *Lactobacillus reuteri*
SI mucus*	BCS154	EU547294/ EU547312	99% to *Lactobacillus vaginalis*
C mucus**	BCS134	EU547291/ EU547309	99% to *Lactobacillus reuteri*
C mucus**	BCS136	EU547292/ EU547310	99% to *Lactobacillus reuteri*
C mucus**	BCS142	EU547293/ EU547311	99% to *Lactobacillus reuteri*
C mucus**	BCS159	EU547294/ EU547313	99% to *Lactobacillus reuteri*

*Intestinal tract mucus, ** Cecum mucus

Table 1. Strains isolated from faeces and mucus of healthy piglets used in this study (Macías-Rodríguez et al., 2008).

2.4 Oligonucleotide design and synthesis

Oligonucleotides used for PCR amplifications were designed with the Primer Select tool of the Laser gene software (Version 5) and synthesized at the Instituto de Biotecnología, UNAM (Mexico). All are listed in Table 2.

Oligonucleotide name	Orientation	Sequence
MAP1F	Forward	5' ATGCCTGCAGGAATCACAA 3′
MAP1R	Reverse	5' AGTAATATCTGCACCGAAGTA 3'
MEF7	Forward	5´ ATTTACGCCCTGGCCCTGGAAAAG-3´
MER9	Reverse	5´ AGAGGGTGTATTTGTTGCCATTGG-3´
MAP2F	Forward	5′ TCTTATGCGACCCACAGTTTG 3′
MAP2R	Reverse	5′ CTAAGAGCCCCGTCGTTC 3′

Table 2. Oligonucleotides used for PCR amplifications

2.5 PCR amplification of the *32-Mmubp* gene

Amplification of the *32-Mmubp* gene of *L. fermentum* previously reported by (Macías-Rodríguez et al., 2009) was performed using as template the chromosomal DNA of *Lactobacillus* strains previously characterized as potential probiotic by traditional methods (Table 1). A combination of gene specific oligonucleotides for an internal fragment MEF7 and MER9 was used to perform the amplification. The PCR solution contained a final concentration of 1× Taq polymerase buffer, 3mmol l^{-1} $MgCl_2$, 0.4mmol l^{-1} for each dNTP, 120 pmol of each primer, 250 ng chromosomal DNA and 1 U of Taq DNA polymerase in a total volume of 25 µl. Amplification reaction was performed in a thermocycler (Perkin-Elmer mod. GeneAmp 2400) with the following temperature program: 1 cycle at 94°C for 5 min; 30 cycles consisted in a denaturation step at 94° C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72° C for 1 min. A final extension was performed at 72° C for 5 min. PCR products were then analyzed in a 1.5% agarose gel.

2.6 PCR amplification of the *mapp* or *mapA* gene

Amplification of the gene *mapp* or *mapA* (Genebank accession number AJ293860) previously described (Rojas 1996, Satoh et al., 2000 and Miyoshi et al., 2006) was performed using as template the same chromosomal DNA of *Lactobacillus* strains used for amplification of the 32*Mmubp* gene. A combination of gene specific oligonucleotides for an internal fragment of the open reading frame MAP1F and MAP1R (Table 2) was used. The PCR solution contained a final concentration of 1× Taq polymerase buffer, 3mmol $MgCl_2$, 0.4mmol for each dNTP, 60 pmol of each primer, 300 ng chromosomal DNA and 1 U of Taq DNA polymerase in a total volume of 25 µl. Amplification reaction was performed in a thermocycler (Perkin-Elmer mod. GeneAmp 2400) with the following temperature program: 1 cycle at 94°C for 5 min; 28 cycles consisted in a denaturation step at 94° C for 1 min, an annealing step at 49°C for 1 min and an extension step at 72° C for 2 min. A final extension was performed at 72° C for 5 min. PCR products were analyzed in a 1.5% agarose gel.

2.7 PCR amplification of the operon containing the *MapA* gene

Polymerase Chain Reactions was performed with primers MAP2F and MAP2R (Table 2) for an internal fragment of the operon containing the *mapA* gene (Genebank LOCUS AJ293860) using as template the chromosomal DNA of *Lactobacillus* strains *map*A positive. The PCR solution contained a final concentration of 1× Taq polymerase buffer, 2mmol $MgSO_4$, 0.4mmol for each dNTP, 100 pmol of each primer, 300 ng chromosomal DNA and 2 U of Platinum *Taq* DNA Polymerase (Invitrogene), in a total volume of 25 µl. Amplification reaction was performed in a thermocycler (Perkin-Elmer mod. GeneAmp 2400) with the following temperature program: 1 cycle at 94°C for 5 min; 30 cycles consisted in a denaturation step at 94° C for 1 min, an annealing step at 62°C for 1 min and an extension step at 68° C for 2 min. A final extension was performed at 68° C for 5 min. PCR products were analyzed in a 1.0% agarose gel.

3. Results and discussion

The association of lactobacilli with the epithelial and mucosal surfaces and their presence in faeces in pigs has been well studied (Rojas & Conway 1996; Macías-Rodríguez et al., 2008). It was shown that *Lactobacillus* population in faeces ranged between 10^7 and 10^9 CFU gr^{-1}. Likewise, in intestinal mucosa, counts of $3.8x10^6$ and $3.2 x10^6$ CFU per cm^2 of small intestine and cecum respectively were reported. Cultivable *Lactobacillus* strains has been found in similar amounts in faeces and intestinal mucus of pigs that inhabit different environmental conditions, cool countries (Rojas and Conway, 1996) and warm arid coasts (Macías-Rodríguez et al., 2008). It was found too that *L. fermentum* and *L. reuteri* are the major strains which colonize the gastrointestinal tract of pigs. Therefore the screening of *Lactobacillus* with probiotic potential for piglets with the ability to interact with the host should be addressed to this species. It has been reported that species of *Lactobacillus* which colonize humans, differ in number and specie from one region to other in the world. Likewise *In vivo* trials have been shown that probiotic effect of one strain in one region of the world could produce confused results in other. This finding supports the idea to look for a new generation of specific probiotics for animals and humans inhabiting specific region in the world.

Traditionally the screening of *Lactobacillus* with probiotic potential involve the isolation and purification of many colonies of lactic acid bacteria, confirmation that correspond to presumptive lactobacilli (grown in selective medium, Gram stain, catalasa reaction, etc), selection according to adhesion profile, growth in mucus, bile salt resistance, growth in broad range of temperature and salt concentration, bacteriocin production, growth and adhesion inhibition of enteropathogens, molecular identification, etc. Previously, more than 150 strains were isolated from mucus and feaces of piglets. Results showed that 64% of presumptive *Lactobacillus* presented abilities to grow in the presence of 680 mM of NaCl. Additionally 75% of the isolates were able to grow at 50 °C. 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. The adhesion assay of the 164 isolates to porcine mucus and mucin allowed visualize strains that bind mucus or gastric mucin in a qualitative manner. Results 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. reuteri* 104R, (Rojas et al., 2002). From the total of faecal strains 45% showed binding ability, whereas from intestinal and cecal mucus strains, 64 and 78%

presented adhesion ability respectively. These results showed the highest percentage of adhesive strains in the cecum and intestine compared with faeces. 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 microbiota in the intestine differs from that in faeces (Marteau, 2002). 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 the most common amplified sequences by PCR are the 16-23S intergenic region and 16S rDNA gene. The 27 strains used in this work were identified by these methods. Analysis of 16S rDNA gene sequences showed that 17 strains belong to *L. fermentum* specie (between 98 to 100% identity), one strain *to L. johnsonii*, 2 strains to *L. vaginalis*, one strain to *L. crispatus* and 5 strains *to L. reuteri* species (Table 1). Except strain BCS113 that showed 92% identity to 16S rDNA of *L. delbrueckii* subsp. *bulgaricus*. These results showed that *L. fermentum* was predominant in faecal adhesive isolates whereas *L. reuteri* was the principal in mucus of cecum. In small intestinal mucus there was not predominant specie. These observations agree with previously reported by Lin *et al*. (Lin et al., 2007) and (De Angelis et al., 2006) who found both species in faeces and mucus of pigs. This result confirmed the relevance of these species in the intestinal tract of pigs. 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 (Chen et al., 2007; Matijasic et al., 2006; Ohashi et al., 2007).

To understand the relevance of surface proteins in the adhesion of *Lactobacillus* to mucus and mucin, the purification and characterization of the adhesins should be performed. In previous reports proteins have been obtained by treatment with chaotropic agents as LiCl. From the spent, centrifuged growth medium and from soluble cytoplasmic extracts. A western blot assay using labelled mucus and mucin has been usually performed to show the protein bands with their relative molecular weight (MW) and in order to characterize them, N-terminal and internal peptide sequences has been determined. The MAPP adhesin of *L. reuteri* and the *Mmubp of L. fermentum* have been characterized in that manner (Rojas et al., 2002; Macías-Rodríguez et al., 2008). Recently the mucus-binding proteins (MUBs) have been revealed as one of the effectors molecules involved in mechanisms of the adherence of lactobacilli to the host; *mub*, or *mub*-like, genes were found in all of the six genomes of *L. reuteri* that are available but the MUB was only detectable on the cell surface of two highly related isolates when using antibodies that were raised against the protein (Mackenzie et al., 2010).

The complete process to get new strains of probiotic potential lactobacilli has been long and complex. Above a review of the different methods and results used was exposed and the results of a proposal are described.

The strains listed in Table 1 were selected because they were the predominant cultivable lactic acid bacteria in a selective medium (Rogosa agar, DIFCO); attached strongly to mucus and mucin when tested by the Dot Blot adhesion assay; grew in mucus, in presence of bile salt and in a broad range of temperatures. Likewise the molecular identification confirmed that *L. fermentum* and *L. reuteri* were the main isolates with probiotic potential for piglets (Macías-Rodríguez et al., 2008). In addition the genes *mapp* or *mapA* of *L. reuteri* and *mmub* of

L. fermentum, which codified for mucus adhesins have been well characterized. Here these genes are described and the results of this proposal are discussed.

3.1 Amplification and sequencing of the *32-Mmubp* encoding gene (*32-mmub*)

Primers MEF7 and MER9 were previously deduced from the complete nucleotide sequence of *32-mmub* gene. The gene presented an ORF (open reading frame) of 903 bp encoding a predicted primary protein of 300 amino acids. This protein presented a signal peptide of 28 amino acids. Cleavage site between residues 28 and 29 were detected with the Signal P 3.0 prediction software. The prediction of transmembrane helices showed that the first 1 to 7 amino acids are predicted to be inside of the cell whereas residues 7 to 29 could be in the membrane and finally the region encompassing amino acids 30 to 300 could be outside. The mature protein consists of 272 residues with a molecular mass of 29,974 Da, an isoelectric point of 9.78 and a positive net charge of 21.22 at pH 7.0. This adhesin protein showed high identity only to *L. fermentum* (BAG27284). A search of homology (BLAST) with the genome of *L. fermentum* IFO 3956 recently published (Morita et al., 2008) showed that 32-Mmubp in *L. fermentum* BCS87 is part of an ABC transporter system and belongs to the PBPb superfamily. It showed to be conserved between prokaryotic protein sequences of substrate binding domains on the ABC-type glycine/betaine transport systems of the OpuAc familiy (PF04069). This family is part of a high-affinity multicomponent binding proteins-dependent transport system involved in bacterial osmoregulation and members of this family are often integral membrane proteins or predicted to be attached to the membrane by a lipid anchor. Some members of the corresponding taxa having similar ecological niches to those occupied by lactobacilli (gastrointestinal and respiratory tracts), i.e. *Helicobacter pylori* and *Mycobacterium tuberculosis*, do not group together suggesting that adhesion mechanisms is not a phylogenetic associated trait.

To confirm that 32-Mmubp of *L. fermentum* BCS87 is specific for this especie, a PCR using the MEF7 and MER9 oligonucleotides was performed. Chromosomal DNA of the 26 adhering strains of Table 1 was used as template to amplify an internal product of *32-mmub* gene. PCR products of the same size (550 bp) were observed in *L. fermentum* strain BCS87 and in all strains which belong to the same specie (Figure 1). Moreover a weak band was also observed in species *L. johnsonii* BCS41, *L. vaginalis* strains BCS68 and BCS154, *L. delbrueckii* subsp. *bulgaricus* BCS113, *L. crispatus* BCS125 and *L. reuteri* strains BCS127, BCS134, BCS136, BCS142 and BCS159 (Figure 1) suggesting *32-mmub* gene is conserved in piglets adhesive *L. fermentum*.

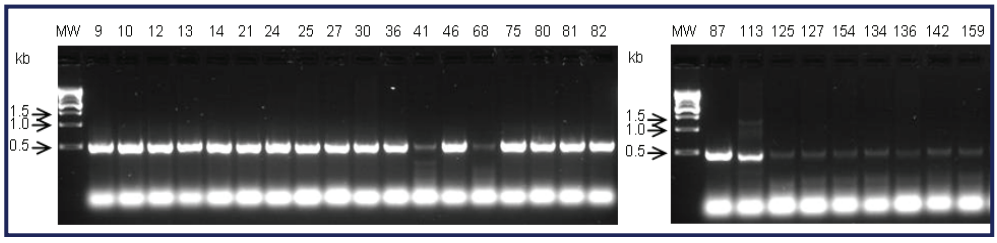

Fig. 1. Amplification of internal fragment of the *32-Mmubp* gene in adhesive strains of *L. fermentum* isolated from piglets intestinal tract. Lane MW, Molecular weight. Numbers 9 to 159 represents the identification code for each *Lactobacillus* strains from Table 1.

3.2 Amplification and sequencing of the *mapp* or *mapA* gene

A mucus adhesion promoting protein (MAPP) from *L. reuteri* 104R was reported (Rojas et al., 2002; Rojas, 1996). The gene encoding this MAPP adhesin (*mapp* gene) was found by using a PCR strategy were peptide derived oligonucletides were carefully devised and PCR reactions performed using chromosomal DNA of *L. reuteri* 104R as template. A PCR product was cloning and sequencing. Southern blotting of digested chromosomal DNA with selected enzyme mixtures was performed by using a 189 bp PCR product as a probe. Then a subgenomic DNA library of the hybridized fragment approximately of 4600 bp was running out. DNA fragments in this region were ligated in the pGEM3 vector and cloned in *E. coli*. Hybridization with the same probe showed a 4500 bp fragment containing the *mapp* gene. A subcloning and sequencing strategy (Figure 2) was used to determine the nucleotide sequence of the *mapp* gene. Nucleotide sequence analysis and search of the nucleotide and deduced aminoacid sequenced were searched in different data bases (NCBI). The complete gene *mapp* was sequenced. The sequence revealed one open reading frame which consists of 744 nucleotides corresponding to a protein of 244 aminoacids with a deduced pI of 10.57

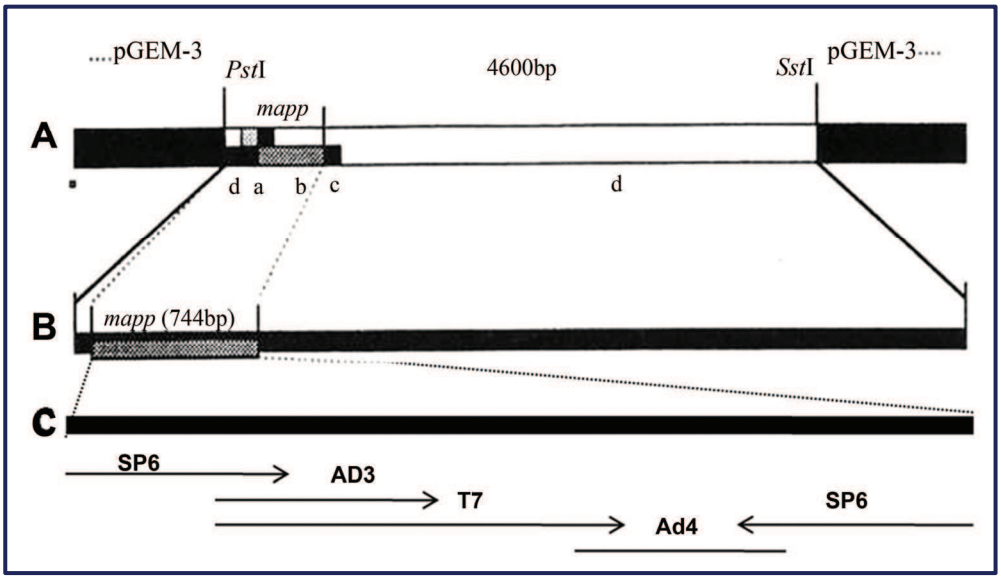

Fig. 2. Schematic drawing of the subcloning and sequencing strategy to determine the nucleotide sequence of the *mapp* gene. A) The stippled box represents the pGEM-3 vector used to clone the chromosomal DNA fragment (4500 bp) from *L. reuteri* 104R and to subclone the fragments a, b, c and d. (inside boxes): a)189 bp, PCR fragment b)610 *Bgl*II-*Bgl*II fragment, c)146 bp *Bgl* II-*Bgl* II fragment and d) vector plus fragment without the two *Bgl* II fragments. B) The largest box represent the 4500bp fragment and the inside box represent the *mapp* gene. Universal primers are indicated, arrows indicate nucleotides determined and the heads of the arrow indicate the transcription direction. C) The box represents the 744bp open reading frame of the *mapp* gene. Universal and sequence specific primers are indicates, arrows indicate nucleotides determined and the heads of the arrow indicate the transcription direction.

and a molecular mass of 26380.90 Da. No putative promoter was found, however, a start codon (ATG) was noted 6 bases downstream of the beginning of the sequence and 30 bases upstream of the first N terminal aminoacid derived codon. The open reading frame ends with stop codons in all three reading frames (TGA A TAA T TAA) (Rojas, 1996).

The *mapp* gene described in Rojas, 2006, was later reported in Gene Bank as *MapA* and as part of one operon whose expression is controlled by a mechanism of transcription attenuation involved cysteine, with accession number AJ 293860 (Satoh et al., 2000). The relation between MapA and adhesion of *L. reuteri* to human intestinal (Caco 2) cells was reported. Quantitative analysis of adhesion of *L. reuteri* strains to Caco 2 cells showed that various strains bind also intestinal epithelial cells. In addition purified MapA bound to Caco 2 cells and this binding inhibited the adhesion of *L. reuteri* in a concentration dependent manner. Additionally it was concluded that multiple receptor-like molecules are involved in the MapA binding to Caco 2 cells (Miyoshi et al., 2006).

To confirm that *MapA* gene is specific for adhesive *L. reuteri* strains, a PCR using the MAPF1 and MAPR1 oligonucleotides was performed. Chromosomal DNA of the 26 adhering strains of Table 1 was also used as template to amplify the *MapA* gene. PCR products of the same size were observed only in the *L. reuteri* strains tested (Figure 3) but not in other species. This result strongly suggests that *MapA* gene is conserved in piglet adhesive *L. reutri* strains.

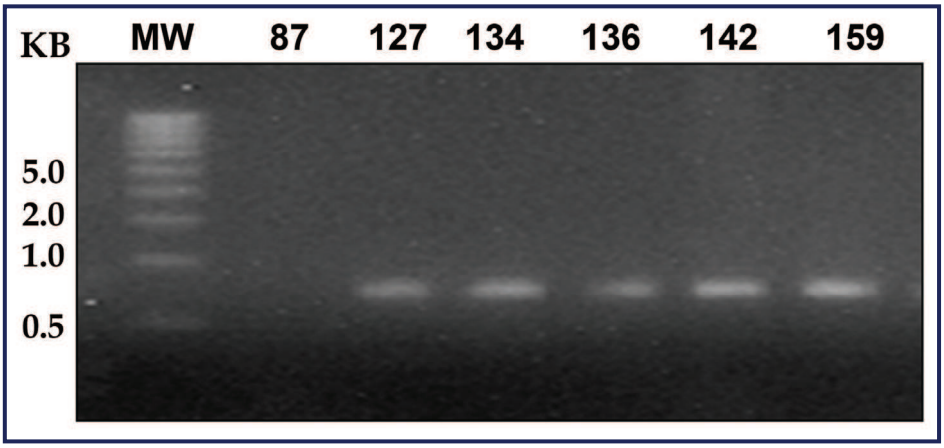

Fig. 3. Amplification of the gene *MapA* in adhesive *L. reuteri* strains isolated from piglets intestinal tract. KB Kilobases. MW; Molecular weight. Names on the lanes represent the identification code for each *Lactobacillus* strains from Table 1.

Expression of the mucus adhesion genes *Mub* and *MapA,* adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* by *L. plantarum* 423 was reported. Growth in the presence of bile, pancreatin and at low pH, was studied by real-time PCR. It was found that *Mub, MapA* and *EF-Tu* were up-regulated in the presence of mucus, proportional to increasing concentrations. Expression of *Mub* and *MapA* remained unchanged at pH 4.0, whilst expression of *EF-Tu* and *plaA* were up-regulated. Expression of *MapA* was down-regulated in the presence of 1.0 g/l l-cysteine HCl, confirming that the gene is regulated by transcription attenuation that involves cysteine (Ramiah et al., 2007). However the gene and

operon *MapA* were not found in *L. plantarum* by a nucleotide data base search in blastn suite (NCBI). However results in this work suggested that functional MapA gene is specific for at least adhesive *L. reuteri* strains.

Mucus-binding proteins (MUBs) are molecules involved in mechanisms of the adherence of lactobacilli to the host (Roos & Jonsson, 2002). It was suggested that MUB domain is an LAB –specific functional unit that performs its task in various domain contexts and could fulfils an important role in host-microbe interactions in the gastrointestinal tract (Boekhorst et al., 2006). Recently was reported that in spite that *mub*, or *mub*-like, genes are found in all of the six genomes of *L. reuteri* and further demonstrated that MUB and MUB-like proteins are present in many *L. reuteri* isolates, MUB was only detectable on the cell surface of two highly related isolates when using antibodies that were raised against the protein. There was considerable variation in quantitative mucus adhesion *in vitro* among *L. reuteri* strains, showing a high genetic heterogeneity among strains (Mackenzie et al., 2010). Different results were observed for the *MapA* gene which was present in all the adhesive *L. reuteri* strains used to amplify this gene.

Recently was reported a well-defined degradation product with antimicrobial activity obtained from the mucus adhesion-promoting protein (MapA) termed AP48-MapA from *L. reuteri* strain. The peptide was purified and characterized. This finding gave a new perspective on how some probiotic bacteria may successfully compete in this environment and thereby contribute to a healthy microbiota (Bøhle et al., 2010). This finding correlate with a report where trypsin digestion of the MapA protein resulted in peptides that bound to mucin suggesting that MapA protein could be involved in colonization of the intestinal mucosa of piglet, since the adhesive capacity could be retained in the intestinal mieleu (Rojas et al., 2002).

To find if *L. reuteri* strains which contain the *MapA* gene present the same operon as strain 104R, amplification was run out (Figure 4).

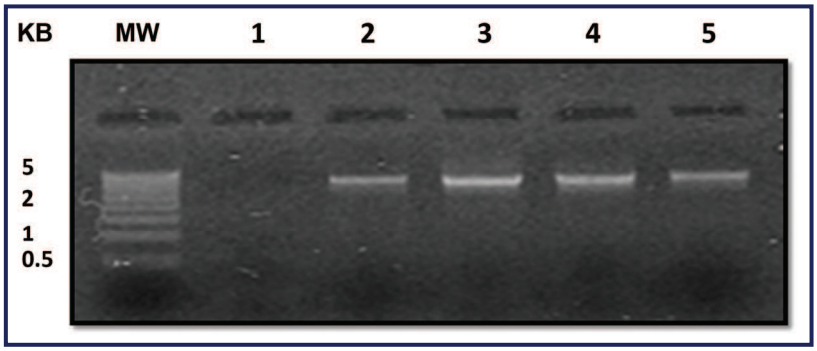

Fig. 4. Amplification of the *MapA* operon (3.9Kb) from different adhesive *L. reuteri* strains isolated from piglets intestinal tract. Lane MW) 500-5000 bp ladder lane 1) Control strain, *L. fermentum* BCS87 lane 2) *L. reuteri* BCS136, lane 3) *L. reuteri* BCS127, lane 4) *L. reuteri* BCS159 and lane 5) *L. reuteri* BCS142

These results together with the review of adhesins from *L. fermentum* and *L. reuteri* and their genes indicate that *Mmubp* and *MapA* genes are conserved in these species, at least in

adhesive strains isolated from intestinal tract of piglets. In Addition these strains are considered the main *Lactobacillus* species which colonize the intestinal tract of piglets. Therefore the traditional methods for screening new probiotic strains for piglets could be reduced as described.

Take faeces and intestinal tract mucus samples from healthy piglets and make a viable count in a selective medium (Rogosa Agar, DIFCO). Incubate at 36°C in anaerobic conditions for 24-48 h and select colonies from the plates with the more diluted samples to grow and purify the DNA. Perform a PCR reaction using the specific primers for the *Mmub* and *MapA* genes. Strains which amplify a fragment with the size mentioned above should be *L. fermentum* for the *Mmub* gene and *L. reuteri* for the *MapA* gene.

4. Conclusion

Bacteria cultivated in the laboratory for long time could mutate and lost probiotic attributes, therefore it is important to look for an easy strategy to routinely screening for probiotics. Screening for new probiotic *Lactobacillus fermentum* and *Lactobacillus reuteri*, which are the dominant microbiota in healthy piglets and present the ability to adhere the intestinal tract mucus is described in this chapter. The main advantage of this method is the expend time.

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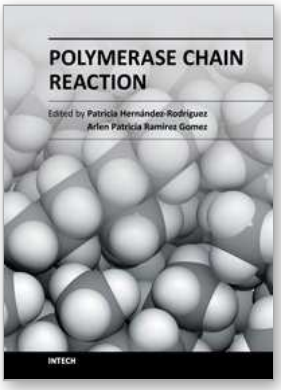

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