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# Protein-carbohydrate interactions between *Lactobacillus salivarius* and pig mucins<sup>1</sup>

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**ABSTRACT:** Adherence to the gastrointestinal tract is a key element desirable for many of the proposed beneficial health effects of probiotic bacteria. The aims of this study were to determine the amounts of adhesion of 3 *Lactobacillus salivarius* strains (Lb6, Lb9, and Lb10) to porcine small intestinal mucins and to determine whether adhesion is a function of lectin-like activities. Dot and Western blot assays were performed to investigate bacterial adhesion. Several carbohydrates and glycoproteins were evaluated to determine whether they interfered with adhesion of the *Lactobacillus* strains to intestinal mucins and to determine whether they had lectin-like activities. The Lb9 and Lb10 strains had greater association with piglet mucins than did those from 22- to 24-wk-old finishing pigs ( $P = 0.021$  and  $0.037$ , respectively), whereas the Lb6 strain adhered to both ( $P = 0.138$ ). Western blot assays showed that bacterial adhesion detected piglet mucosa from the

duodenum, jejunum, and ileum. In finishing pigs, the adhesion was variable throughout the gastrointestinal tract. Galactose and mannose diminished the interaction of the Lb9 and Lb10 strains in intestinal mucosa ( $P = 0.028$  and  $0.026$ , respectively), whereas pig gastric mucin reduced the adhesion of the Lb6 strain ( $P = 0.013$ ). Adhesion of the Lb9 and Lb10 strains to intestinal mucosa was less after protease treatment ( $P = 0.023$  and  $0.018$ , respectively), which indicates that proteins are needed for the Lb9 and Lb10 strains to recognize mucin. The Lb6 strain also demonstrated diminished adhesion after periodate treatment ( $P = 0.038$ ). From these results, we suggest that the nature of the bacterial lectin-like substance is a surface protein that loosely binds to the bacterial cell surface. All the tested strains adhered to specific targets in the small intestinal mucosa of piglets, and the bacteria had lectin-like proteins involved in this adhesion.

**Key words:** adhesion, carbohydrate, *Lactobacillus salivarius*, piglet mucin

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## INTRODUCTION

Colonization of different portions of the intestinal tract (IT) by probiotic microorganisms is the first defensive barrier against the invasion of pathogenic microorganisms, and this colonization is considered to be an important characteristic for health benefits to the host (Lee et al., 2003). Intestinal epithelial cells are covered by a relatively thick (400  $\mu\text{m}$ ) mucus layer consisting of mucin, which is a 2-MDa gel-forming glyco-

protein, and a large number of smaller glycoproteins, proteins, glycolipids, and lipids (Piel et al., 2004). This mucus layer has been implicated in interactions with bacteria in several ways. These include involvement of the mucus layer as an initial site for bacterial adhesion, a protective barrier against bacterial colonization, a source of nutrients for bacterial growth, and a matrix for bacterial replication and colonization (Laux et al., 2005). Bacterial enteropathogens must traverse the intestinal mucus layer to approach and adhere to epithelial cells (Talay, 2005).

The molecular species involved in the adherence of *Lactobacillus* to intestinal mucosa and epithelial cells is not fully understood (Roos and Jonsson, 2002). It is thought that the attachment of probiotic bacteria to mucus and epithelial cells can be mediated by adhesins or lectins (specific carbohydrate-binding proteins or glycoproteins) located on bacterial surfaces. These adhesins could recognize the oligosaccharide moieties

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of glycoproteins or glycolipids placed either in the mucus layer or in the surface membrane of epithelial cells (Gao and Meng, 2004). The aims of this study were to determine whether 3 *Lactobacillus salivarius* strains isolated in our laboratory from the small intestine of healthy piglets would adhere to the intestinal mucosa of weaned piglets and to determine whether this adhesion was mediated by lectin-like activities.

## MATERIALS AND METHODS

The protocols used in this experiment complied with the guidelines of the Centro de Investigación en Alimentación y Desarrollo A.C. concerning animal experimentation and the care of experimental animals. All reagents used were obtained from a commercial company (Sigma, St. Louis, MO) unless otherwise indicated.

### *Bacterial Strains and Culture Conditions*

Three *L. salivarius* strains (Lb6, Lb9, and Lb10) were isolated from the small intestine of 10-d-old crossbred pigs (Landrace × Large White). Bacteria were previously identified by sequencing a 492-bp fragment from the 16S rRNA gene (Iñiguez-Palomares et al., 2007). Strains were stored in de Man, Rogosa, and Sharpe (MRS) broth at  $-20^{\circ}\text{C}$  in 30% (vol/vol) glycerol (Difco-Becton Dickinson & Company, Sparks, MD). These strains were previously characterized as potential probiotics according to Gusils et al. (2002), and they displayed a tolerance to gastric juice and bile salts. They also inhibited *Escherichia coli* K88 in vitro (Iñiguez-Palomares et al., 2007). For subsequent experiments, individual strains were inoculated in a 10-mL MRS broth (Difco-Becton Dickinson & Company) with 0.5 g of Cys·HCl/L and incubated for 18 to 24 h at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

### *Mucin Preparation*

Pig intestinal mucin glycoproteins were isolated from each portion of the small intestine (duodenum, jejunum, and ileum) from 10-d-old healthy piglets ( $n = 6$ ) and 22- to 24-wk-old finishing pigs ( $n = 6$ ) by scraping. After sedation with 5 mL of a 5% (wt/vol) solution of veterinary ketamine (Richmond Vet Pharma, BA, Buenos Aires, Argentina), piglets were euthanized with an overdose of sodium pentobarbital (Penta-Hypnol, Agrovot Market S.A., Lima, Peru) in 6.5% (wt/vol) solution. The abdominal cavity was opened and the entire gastrointestinal tract was immediately removed. The small intestine was isolated, and the length was determined. Samples of duodenum (15 cm proximal to the pyloric junction), jejunum (55 cm proximal to the pyloric junction), and ileum (15 cm distal to the ileocecal junction) were obtained from each piglet and flushed with PBS-A (10 mM phosphate, pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, and 10 mM EDTA. Intestinal sections

were opened longitudinally and rinsed with PBS-A. Mucus was gently removed with a sterile glass slide and suspended in 4 vol of ice-cold PBS-A. The suspension was shaken for 1 h at  $4^{\circ}\text{C}$  and centrifuged for 30 min at  $2,600 \times g$  at  $4^{\circ}\text{C}$  to remove particulate matter. Mucus was isolated from the supernatant by dual precipitation with ice-cold ethanol. After centrifugation at  $2,600 \times g$  at  $4^{\circ}\text{C}$  for 30 min, the pellet was dissolved in water (Milli-Q water, Millipore Corp., Billerica, MA), lyophilized, and then weighed to determine the amount of mucus obtained (Lee et al., 2004). Intestinal segments of finished pigs were provided by a pork processing plant (federally inspected plant) in Hermosillo, Sonora, México. This plant complies with the hazard analysis and critical control point system. Mucin samples were obtained at slaughter following the same procedure described for piglet mucins.

### *Adhesion Assay of L. salivarius*

Dot blot and Western blot assays were conducted to detect lectin-mediated interactions between *Lactobacillus* strains and pig and piglet mucins. Overnight cultures of bacterial strains were grown in MRS broth with Cys·HCl (0.5 g/L). After harvesting, bacterial cells were washed twice in 0.02 MPBS at pH 7.2 and the suspensions were adjusted to 9 log cfu by optical densities equal to 1.0 at 450 nm (modified from Gao and Meng, 2004). Bacterial cells were then labeled with sulfo-N-hydroxysuccinimide (NHS)-biotin (Pierce Biotechnology Inc., Rockford, IL) as described previously by Mukai et al. (2002). Briefly, intact bacterial cells were washed 3 times with PBS and resuspended (approximately  $1 \times 10^9$  cells) in 1 mL of the same buffer. Sulfo-NHS-biotin was added (4  $\mu\text{g}$  of a 25 mg/mL solution), and cells were incubated at room temperature for 2 min. Unbound sulfo-NHS-biotin was removed by washing 4 times with PBS. Labeled bacteria were stored at  $-80^{\circ}\text{C}$  for no more than 2 d before use. Viability of bacterial cells was evaluated before the adhesion assay.

Twofold serial dilutions containing between 2.0 mg/mL and 6.0 ng/mL of protein were prepared from piglet and pig mucins for use in the blotting assays. Aliquots of 15  $\mu\text{L}$  were applied to nitrocellulose. The Gal $\alpha$ (1–3)Gal-BSA and Gal $\beta$ (1–4)GlcNAc-BSA (Calbiochem, San Diego, CA) neoglycoconjugates were included as positive controls. Untreated BSA was used as a negative control. Membranes spotted with glycoproteins or neoglycoconjugates were blocked for 2 h in PBS (0.02 M PBS and 0.05% Tween 20, pH 7.2) containing 2% (wt/vol) BSA. After washing with PBS, membranes were overlaid with biotinylated bacteria to a final concentration of approximately 9 log in PBS. After overnight incubation at  $4^{\circ}\text{C}$ , membranes were washed to remove unbound bacteria and were developed with avidin-peroxidase to detect bacterial adhesion (Ruhl et al., 1996; Rojas et al., 2002).

In preliminary experiments, we observed no differences among duodenum, jejunum, and ileum mucins in

bacterial adhesion (data not shown). Therefore, mucin obtained from the entire small intestine was used for the dot blot assays. Additionally, bacteria adhered at all probed intestinal mucin concentrations (from 2.0 mg/mL to 6.0 ng/mL), but the 5.0  $\mu\text{g/mL}$  dilution showed differences in dot color intensity for each strain. For further analysis, 5.0  $\mu\text{g/mL}$  of mucin was used. Dot intensity was measured with a densitometer and software (GS-700 Imaging Densitometer & Molecular Analyst Software v.2, Bio-Rad Labs Inc., Hercules, CA).

Western blot assays were performed as described by Towbin et al. (1979). Mucins from each portion of the small intestine from both piglets and finishing pigs (50.0  $\mu\text{g}$ ) were separated by 10% SDS-PAGE under nonreducing conditions (Laemmli, 1970) and transferred to nitrocellulose membranes at 0.06 V for 1 h (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad Labs Inc.). The interaction between biotinylated bacteria and mucin proteins was performed as described previously.

### ***Inhibition of Lectin-Mediated Adhesion of *L. salivarius****

To test the inhibition of lectin-mediated adhesion of *L. salivarius* strains to piglet mucin by different carbohydrates, glycoproteins, or neoglycoconjugates, 15.0- $\mu\text{L}$  samples of 5.0  $\mu\text{g/mL}$  mucin were spotted onto nitrocellulose and incubated with 9 log of biotin-labeled bacteria strains as a positive control. The *L. salivarius* strains were mixed with different concentrations (6.0 ng to 2.0 mg) of the following carbohydrates: D(+)-mannose, D(+)-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-lactose, and sucrose. Glycoproteins (asialofetuin, ovalbumin, and pig gastric mucin) and the neoglycoconjugates Gal $\alpha$ (1–3)Gal-BSA and Gal(31–4)GlcNAc-BSA (Calbiochem) were also tested. These compounds were preincubated for 1 h at 37°C before being overlaid on nitrocellulose membranes as described by Gao and Meng (2004).

### ***Enzymatic and Chemical Treatment of Bacteria***

To investigate the bacterial components involved in the adherence of strains to intestinal mucins, bacteria were exposed to chemical and enzymatic treatments before adhesion. Bacterial cells were adjusted to an absorbance of 1.0. For enzymatic treatments, trypsin (2.0 mg/mL) and 0.4% (wt/vol) protease (endoprotease from *Aspergillus oryzae*) were evaluated. Sodium periodate (20.0 mM) was used as a chemical treatment as described by Tuomola et al. (2000). Viable bacterial suspensions were incubated for 1 h at 37°C with each of the treatments. After centrifugation at  $2,600 \times g$  at 4°C, the pellets were washed 5 times with a PBS buffer with pH 7.2. The final bacterial cell density was adjusted with the same buffer solution as before and then labeled with biotin as described by Mukai et al. (2002).

Dot blot assays were conducted with immobilized piglet mucin at 5.0  $\mu\text{g/mL}$ .

### ***Statistical Analysis***

All experimental data were analyzed as completely random designs. Statistical comparisons between different adhesion responses, carbohydrate inhibition of lectin-mediated adhesion, and the effect of enzymatic or chemical treatments in bacterial adhesion were performed by repeated measures ANOVA (NCSS, Keyville, VA). The Tukey-Kramer multiple comparisons test was used to determine differences ( $P < 0.05$ ) between treatments means.

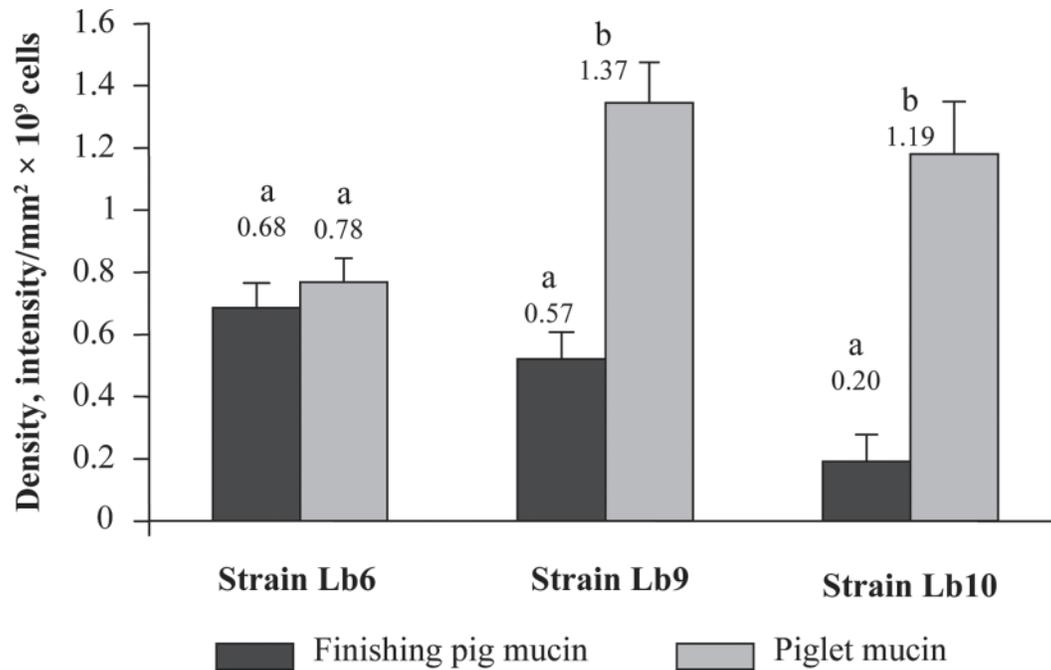
## **RESULTS AND DISCUSSION**

Figure 1 shows the adhesion of the Lb6, Lb9, and Lb10 strains to piglet and finishing pig mucin. The Lb9 and Lb10 strains showed greater interaction with piglet mucins ( $P < 0.05$ ) than did the Lb6 strain. These results indicated that piglet mucins contain specific moieties recognized with greatest affinity by molecules located at the surface of the Lb9 and Lb10 strains. In other bacterial species, a small number of AA substitutions confer different but related binding specificities to lectin-like adhesins, which recognize glycan moieties expressed in mucins and on epithelial cells located in the intestine of piglets (Willemsen and de Graaf, 1992; Verdonck et al., 2004).

In contrast to the piglet mucins, finishing pig mucins were recognized with less affinity by the Lb9 and Lb10 strains. These results indicate that the intestinal receptors for these *Lactobacillus* bacteria may vary with the age of the animal. Glycans in intestinal mucins can also be a niche for bacterial adhesion (Dai et al., 2000). Mucins and epithelial cells work as receptors for bacterial adhesion in piglet pathogens such as *E. coli* K88 and 987P (Grange et al., 2002). The presence of these pathogens has been found to be age dependent, and intestinal adhesion usually decreases with age (Conway et al., 1990; Grange et al., 2002). In probiotic bacteria, some strains, such as *Bifidobacterium*, diminish their presence with the age of the host, whereas others remain in the intestine for the entire lifetime of the host (Fontaine et al., 1994; Lee et al., 2004; Sun et al., 2007).

Kirjavainen et al. (1998) examined the correlation between age and adhesion of *L. salivarius* to newborn and 2-mo-old infant mucins and epithelial cells. However, no correlation was observed when Ouwehand et al. (1999) studied the adhesion of several probiotic *Lactobacillus* strains to human intestines. The data indicated that each strain has its own adhesive characteristics and that extrapolation from related strains is not possible. This may explain why the Lb6 strain showed similar adhesion in both piglet and finished pig mucins (Figure 1).

Specific strains recognize specific receptors in the intestinal mucins of piglets. The Lb6, Lb9, and Lb10



**Figure 1.** Adhesion of *Lactobacillus salivarius* strains (Lb6, Lb9, Lb10) to whole small intestinal mucins (5 µg/mL) from finishing pigs and piglets by dot blot assay. Values with different letters (a, b) differ ( $P < 0.05$ ).

strains bind to different glycoproteins of the 3 intestinal sections (duodenum, jejunum, and ileum; Figure 2). This is in contrast with *E. coli* K88, which can adhere better to duodenal than ileal mucins (Grange et al., 1998; Ramos-Clamont et al., 2007).

Western blot assays showed that the biotin-labeled Lb9 and Lb10 strains interacted with more protein bands from piglet mucins than from finishing pig mucins. The Lb6 strain interacted with a similar number of proteins at both ages. We found that specific strains recognize specific glycoproteins in the intestine (Figure 2b and 2d) because binding patterns from each segment of intestine were different. Bacterial adhesion in finishing pigs varied according to the tested portions of the small intestine. However, we always observed adherence to proteins from the duodenal, jejunal, and ileal sections from piglet small intestines (Figure 2).

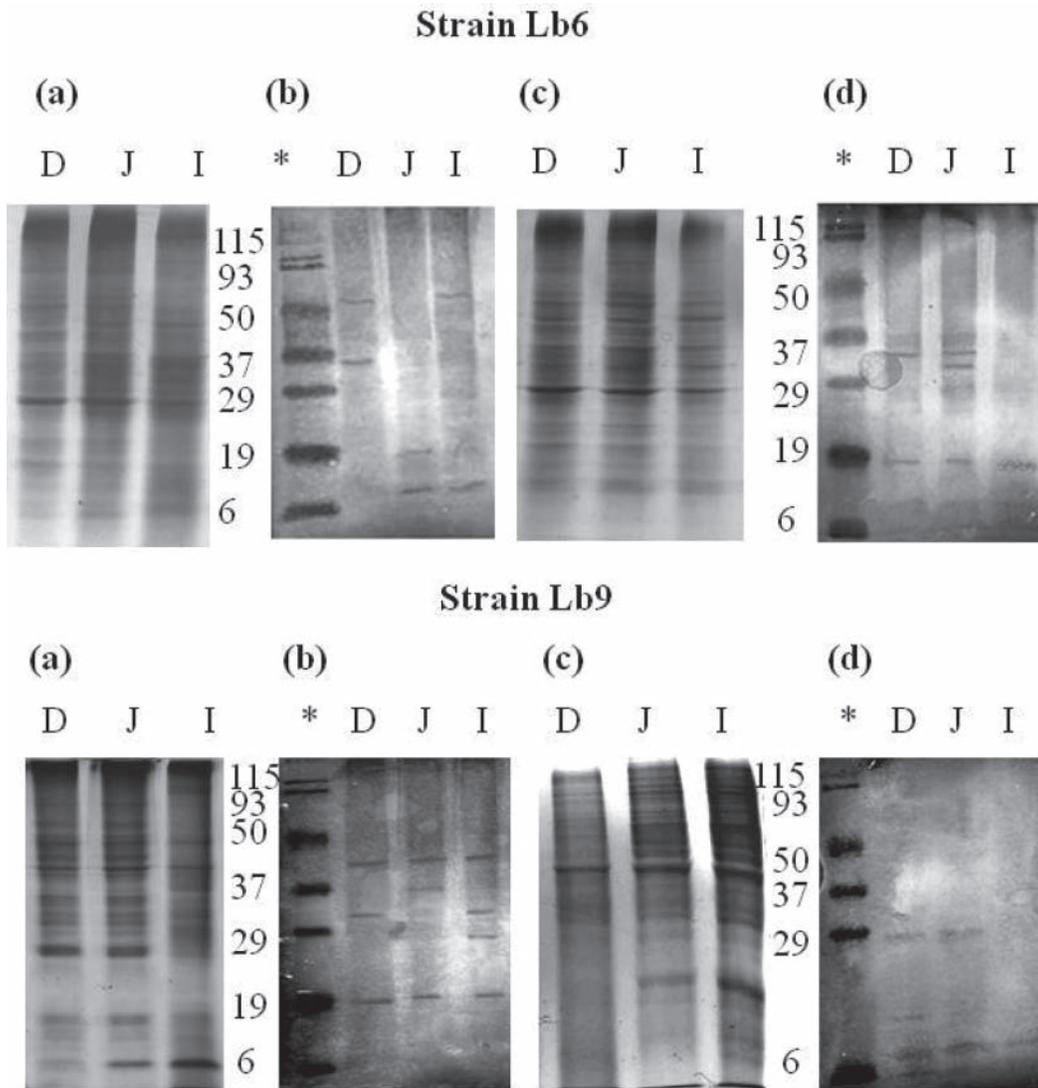
Fang et al. (2000) isolated 26- and 41-kDa glycoprotein receptors for *E. coli* K88 from piglet mucus in the small intestine. The Lb9 and Lb10 strains also interacted with proteins of similar molecular mass. In contrast, the Lb6 strain did not show interactions with proteins of this molecular mass (Figure 2). In previous work, receptors for K88 seemed to contain D-glucosamine- or D-galactosamine-like residues (Metcalf et al., 1991). Other studies have demonstrated that K88 possesses multiple receptors for mucus and brush borders, and that these receptors can vary in monosaccharide distribution, chain length, and sialic acid content, leading to variations in migration during electrophoresis (Billey et al., 1998; Jeyasingham et al., 1999).

In finishing pig mucus, the adhesion of *Lactobacillus* strains was poorly observed in the duodenum and jejunum and was absent from the ileum (Figure 2).

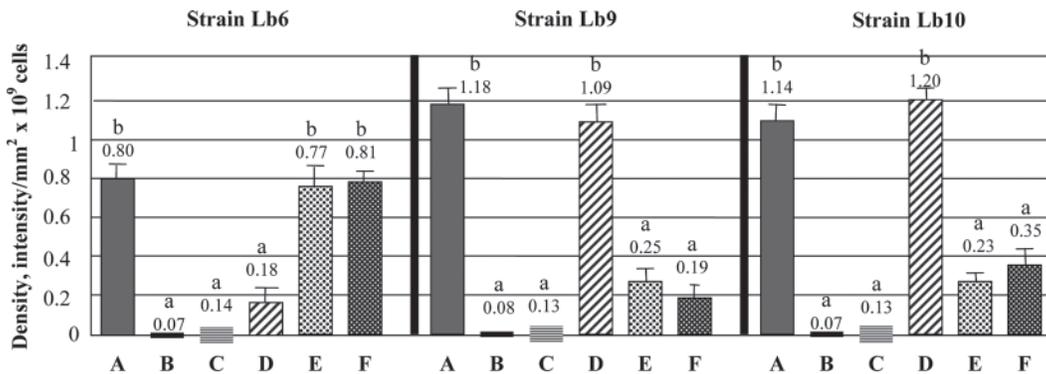
It is possible that the receptors change or disappear with age or intestinal turnover, leading to diminished bacterial binding (Conway et al., 1990; Grange et al., 2002). Roos and Jonsson (2002) found that a surface protein with a molecular mass between 15 and 45 kDa from *Lactobacillus reuteri* 1063 could adhere to finishing pig mucus, in addition to mucus components with a very high molecular mass that could not migrate in the acrylamide gel. Our strains recognized proteins from finishing pig mucus with molecular masses between 10 and 40 kDa and did not recognize proteins with a high molecular mass. In piglet mucus, bacteria were found to bind to proteins between 10 and 50 kDa (Figure 2).

Competitive studies showed that adhesion to intestinal mucins was inhibited for the Lb6, Lb9, and Lb10 strains by neoglycoproteins in concentrations of 1.25 µg/mL ( $P = 0.013, 0.028, \text{ and } 0.026$ , respectively; Figure 3). These strains could adhere strongly to Gal $\alpha$ (1–3)Gal-BSA and Gal $\beta$ (1–4)GlcNAc-BSA, which are also receptors for K88 (Grange et al., 2002). The bacteria possibly compete for sites in the small intestine of piglets and colonize it, preventing the adhesion of the pathogenic bacterium. Sun et al. (2007) found that *Lactobacillus plantarum* Lp6 has a mannose-specific adhesin that competes for binding sites with *Salmonella* Typhimurium and some *E. coli* strains in the gut.

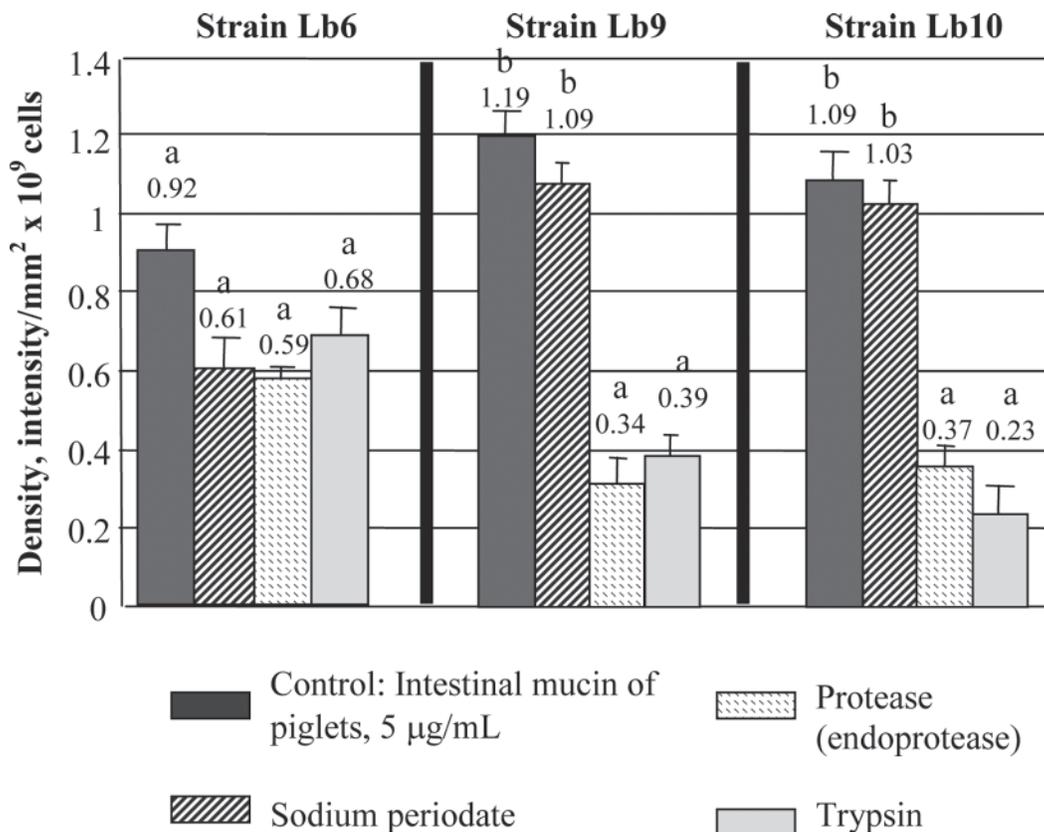
It was also observed that the Lb9 and Lb10 strains had diminished adhesion to intestinal piglet mucus when galactose and mannose was added at 2 mg/mL ( $P = 0.028 \text{ and } 0.026$ , respectively). However, the Lb6 strain showed diminished adhesion only with pig gastric mucus at 2 mg/mL ( $P = 0.013$ ; Figure 3). Asialofetuin, ovalbumin, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-lactose, and sucrose did not inhibit



**Figure 2.** (a) Sodium dodecyl sulfate-PAGE nonreducing gel of piglet mucins stained with Coomassie blue. (b) Western blot analysis showing bacterial interaction with proteins from piglet mucins. (c) Sodium dodecyl sulfate-PAGE nonreducing gel of finishing pig mucins stained with Coomassie blue. (d) Western blot analysis showing bacterial interaction with proteins from finishing pig mucins. In total, 50 µg of mucins per lane were loaded in the gels and transferred to a nitrocellulose membrane. An asterisk (\*) indicates a molecular weight marker. D = duodenal mucins; J = jejunal mucins; I = ileal mucins. The *Lactobacillus* strains have large differences in adhesion patterns. The Lb6 strain adhered to proteins with molecular weights above 50 kDa in piglet mucins, and the Lb9 strain adhered to smaller proteins. Additionally, the Lb9 strain had greater interactions with mucins from young pigs than from older animals. The Lb10 strain showed an adhesion pattern identical to that of the Lb9 strain (data not shown).



**Figure 3.** Inhibition of adhesion of *Lactobacillus salivarius* strains (Lb6, Lb9, Lb10) to 5 µg/mL of intestinal piglet mucins by glycoprotein, neoglycoproteins, or carbohydrates by dot blot assays. A = positive control (intestinal mucin of piglets; 5 µg/mL), B = Galβ(1–4)GlcNAc-BSA (1.25 µg/mL; Calbiochem, San Diego, CA), C = Galα(1–3)Gal-BSA (1.25 µg/mL; Calbiochem), D = pig gastric mucin (2 mg/mL), E = galactose (2 mg/mL), and F = mannose (2 mg/mL). Values with different letters (a, b) differ ( $P < 0.05$ ).



**Figure 4.** Dot blot assays of enzymatic and chemical treatments of *Lactobacillus salivarius* strains (Lb6, Lb9, Lb10) to diminish their adhesion to 5 µg/mL of intestinal piglet mucins. Values with different letters (a, b) differ ( $P < 0.05$ ).

adhesion for any of the strains tested at any concentration tested (data not shown).

Results are variable in receptors identified for bacterial adhesins of *Lactobacillus* strains. Gao and Meng (2004) reported that galactose, arabinose, mannose, and other carbohydrates were able to inhibit hemagglutination reactions with *Lactobacillus* spp. and concluded that simple sugars, not glycoproteins, are the specific receptors for this strain. In other instances, some glycoproteins were observed to inhibit adhesion of *L. reuteri* 1063 to mucus material, whereas monosaccharides did not (Roos and Jonsson, 2002). Fontaine et al. (1994) mentioned that the interaction between probiotic bacteria and intestinal mucus is highly dependent on peptide structures rather than oligosaccharide moieties. It is possible that adhesins are variable for each bacterial strain; thus, receptors may also be variable. We found 2 *L. salivarius* strains that agreed with the results of Gao and Meng (2004) and another that confirmed the results of Roos and Jonsson (2002).

To investigate bacterial components involved in the adherence of strains to intestinal mucins, bacteria were exposed to chemical and enzymatic treatments before adhesion. The Lb9 and Lb10 strains were affected only by proteases ( $P = 0.023$ , and  $0.018$ , respectively; Figure 4). The involvement of proteins in adhesion was strongly supported by these results. In the Lb6 strain, the nature of this lectin-like substance was suggested to be glycoprotein(s) because a decrease in adhesion was

observed after periodate and protease treatments ( $P = 0.038$ ). Some studies have revealed the presence of S-layer and lectin-like adhesin protein arrays in *Lactobacillus* strains (Smith et al., 2001; Talay, 2005). These proteins can be potential mediators in the initial steps involved in adhesion. It is possible that exopolysaccharides or teichoic acids provide sites for this or these proteins to bind (Sun et al., 2007).

Tuomola et al. (2000) observed that adherence of *Lactobacillus gasseri* ADH to human intestinal mucus involved only periodate-sensitive factors. The adhesion of the *Lactobacillus acidophilus* BG2FO4 strain involved both protease-sensitive and periodate-sensitive factors, and the *L. acidophilus* NCFM/2 strain used only protease-sensitive factors.

In conclusion, all 3 tested strains adhered to specific targets in the mucosa of the small intestine of piglets, and this adhesion involved lectin-like proteins. In addition, the intestinal receptors for *Lactobacillus* could vary with the age of the animal, with greater occurrence in piglets; however, this seemed to depend on the strain tested.

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