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Inhibitory Effects of Chick Lactobacilli on Enteropathogenic *Salmonella*

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Abstract: The major source of human salmonellosis are farm animals, which may frequently be intestinal carriers of the organism. Lactobacilli isolated from the intestinal tract with efficiency adhesion were selected and studied in assay competitions with different *Salmonella* serotypes. The growth and lectin production of lactobacilli remained unchanged in several mixed and single culture studies. However, in mixed cultures, the inhibition (bacteriostatic) of viable bacteria of salmonella strains was observed. The adhesion ratio showed significant values for *L. animalis*, *L. fermentum*, *S.* Gallinarum and *S.* Pullorum. These results indicate the remarkable importance of a specific host interaction in the colonization process by microorganisms. *L. animalis* was effective in reducing the attachment of *S.* Gallinarum, *S.* Pullorum and *S.* Enteritidis to host-specific epithelial cells, while *L. fermentum* was able to reduce the attachment of *S.* Pullorum and *S.* Gallinarum. Therefore, we suggest that chicken lactobacilli included in this work may be considered potential probiotic microorganisms, and used in the preparation of a probiotic food.

Key words: Probiotic, chicken, salmonellosis

INTRODUCTION

Avian salmonellosis is an inclusive term designating a large group of acute or chronic diseases of fowl caused by any one or more members of the bacterial genus *Salmonella*, which is a member of the larger family Enterobacteriaceae. Domestic poultry may constitute the largest single reservoir of *Salmonella* organisms existing in nature. Avian salmonellosis is a problem of economic concern to all phases of the poultry industry from production to marketing[1] .

Farm animals are the major source of human salmonellosis, which may frequently be intestinal carriers of the organism. Many investigations have shown that, in particular, pigs and poultry could carry *Salmonella* in the intestinal tract in high percentages and in high numbers without any symptoms of disease[2,3]. During slaughtering, when ruptures in the gut wall can easily occur, the spread of intestinal contents causes fecal contamination of the meat.

It is theoretically possible to raise and fatten animals that are free from *Salmonella*. In fact, this means that the animals must be kept under special pathogen-free conditions, such as: 1) the establishment of *Salmonella*-free breeding stocks; 2) the adoption of an all-in all-out system; 3) the reinforcement to biosecurity, particularly with respect to birds, rodents, insects and dust; 4) the wearing of special clothes and footwear; 5) the production of *Salmonella*-free feed; 6) the establishment of a safe water supply; 7) the use of *Salmonella*-free litter; and 8) the prevention of the spread of slurry or manure in the close environment of the premises[4].

Another approach to reduce *Salmonella* in chickens is the application of intestinal flora from 1-day-old chicks to adult birds, in the hope that the rapid adhesion will reduce the colonization opportunities for *Salmonella*. The process is known as competitive exclusion (CE)[5]. Several authors have shown the protect effect of oral administration of intestinal native microorganisms of salmonella-free adult chickens to chicks against *Salmonella* infection[5-8].

The colonization of the different intestinal tract compartments by specific commensal bacteria serves as a first defense barrier against invading pathogenic microorganisms or toxic substances. Due to the intensive management methods of today, farm animals are very susceptible to enteric bacterial imbalance, leading to inefficient digestion, adsorption of nutrients, retarded

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growth and pathogenic colonization[9]. To improve feed efficiency and growth rates, the some years ago animal foods have been supplemented with antibiotics. However, the potential for development of antibiotic-resistant strains of bacteria which could lead to public health problems has increased the pressure to eliminate or reduce the use of antibiotics in feeds. For these reasons there is wide interest in replacing feed antibiotics with more natural feed additives-*probiotic feed*, live indigenous microorganisms or nonantibiotic substances, which decrease the number of intestinal infections and/or increase production and/or improve food hygiene by contributing to a better gastrointestinal environment[9].

In a previous paper, we showed that *Lactobacillus animalis* CRL1014, *L. fermentum* CRL1015 and *L. fermentum* CRL1016 were isolated from chickens' intestinal tracts based and selected on their adhesive capacity and presence of lectin-like proteins in their cellular surface[10]. The objectives of this work were to study *in vitro* interactions between these probiotic avian microorganisms and pathogen salmonellae in mixed cultures and intestinal cells. Factors to be analyzed included microorganism growth and production of lectin-like structures.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Lactobacillus animalis CRL1014, *L. fermentum* CRL1015 and *L. fermentum* CRL1016 were isolated from faeces of chickens in the Technological Echophysiology Laboratory of CERELA (Tucumán, Argentina), and chosen for their adhesive properties. *Salmonella* Gallinarum and *S.* Pullorum were provided by the Enterobacteria Service and the Service of Special Bacteriology of the Instituto Nacional de Enfermedades Infecciosas Dr. Carlos Malbrán, Buenos Aires, Argentina; *S.* Typhimurium and *S.* Enteritidis by Instituto de Microbiología Dr. Luis Verna, Universidad Nacional de Tucumán, Argentina; and *Saccharomyces cerevisiae* by Planta Piloto de Procesamiento Industriales Microbiológicos (PROIMI), Tucumán, Argentina.

All strains were kept at -20°C in LAPTg broth with 30% glycerol. Lactobacilli were activated and grown in LAPTg medium[11] and Brain Heart Infusion (BHI, Merck).

Associated cultures assays: Different mixed cultures (three lactobacilli with each pathogen) were studied. One hundred ml of LAPTg broth were inoculated with 1 x 10^7 CFU ml^{-1} of individual strains of lactobacilli and 1 x 10^6 CFU ml^{-1} of pathogenic *Salmonella*, and incubated for 48 h at 37°C. Total counts were determined on LAPTg agar. Lactobacilli counts were carried out on Man, Rogosa, and Sharpe (MRS)[12] agar (Merck) and *Salmonella* was counted on MacConkey agar (Merck). All plates were incubated at 37°C for 48 h. After 48 h of incubation, samples were collected to determine of lectin production.

Production of lectin-like substances by lactobacilli in mixed and single cultures: Production of lectin-like substances by lactobacilli at 48 h of incubation in mixed and single cultures was determined with agglutination inhibition assay, described by Eshdat[13].

The agglutination was performed on a microscopic slide by mixing 10 µl of the microbial suspension with 5 µl of phosphate-buffered saline (PBS pH 7.4) and 10 µl of 0.2 mM of specific carbohydrate solutions (glucose for *L. animalis*, *N*-Ac. glucosamine for *L. fermentum* subsp. *cellobiosus*, fucose for *L. fermentum*)[10]. These mixes were incubated at room temperature in 10 µl of a suspension of glutaraldehyde-treated *Saccharomyces cerevisiae* (10^8 cells ml^{-1} PBS) for 1 min.

The yeast cells were prepared by preincubation in PBS with glutaraldehyde (1 mg ml^{-1}) for 1 h at 25°C, washed twice with PBS, incubated for 30 min at 25°C with 10 mg ml^{-1} glycine and washed twice with PBS. The treated yeast cells were stored at 4°C as a suspension in PBS (1 x 10^5 cells ml^{-1}) containing 0.02 % sodium azide.

Animals: Chickens were maintained in our laboratory with free access to feed and water. The animals were deprived of food 16 h before each assay.

Intestinal epithelial cell isolation: Intestinal epithelial cell isolation was by the described by Uni[14]. The intestinal fragments were flushed twice with phosphate-buffered saline (PBS) pH 7.4 with 4x antibiotic (Gentamycin and Amphotericin B), and agitated incubation with PBS 1x antibiotic containing 15% fetal calf serum (FCS). The segment was tied at two ends, and filled to distension with PBS containing 0.25 mM dithiothreitol (DTT) and 1.5 mM EDTA from a syringe. After 30 min of incubation at 37°C, epithelial cells were collected by gently scraping the intestinal wall, and transferred with sterile forceps to a sterile tube containing PBS with 10% FCS and 15% heparin (16 IU ml^{-1}). The cells obtained were collected by pelleting at 100 g for 5 min, washed twice by resuspension and recentrifugation in fresh PBS, and plated out onto gelatin coated plastic 96-well plates. Standard plating media consisted of high glucose DMEM (SIGMA), 1% HEPES, and 15% FCS. Cells were maintained at 37°C and 7.5% CO_2.

***In vitro* adhesion and inhibition adhesion assays:** Lactobacilli and salmonella were cultured in LAPTg broth,

Table 1: Lectin-like production in pure and quadruple cultures

	Inhibitory of yeast agglutination			
	Mannose	Glucose	*N*-Acetyl glucosamine	Fucose
Simple cultures				
a) *L. animalis* CRL1014	+	+	-	-
b) *L. fermentum* CRL1015	+	-	-	+
c) *L. fermentum* CRL1016	+	-	+	-
a + b + c with:				
S. Pullorum	+	+	+	+
S. Gallinarum	+	+	+	+
S. Typhimurium	+	+	+	+
S. Enteritidis	+	+	+	+

(+): positive inhibition; (-): negative inhibition

Table 2: Percentage of adhesion to intestinal epithelial cells

L. animalis CRL1014	62.5±15.3*
L. fermentum CRL1015	47.3±19.4
L. fermentum CRL1016	33.0±9.2
S. Gallinarum	59.4±8.3
S. Pullorum	53.2±12.5
S. Enteritidis	25.4±5.2
S. Typhimurium	12.3±5.8

*(%)
Results are mean ± SD for three replicates

Table 3: Inhibition adhesion of *Salmonella* serotypes to intestinal epithelial cells

	S.Gallinarum	S.Pullorum	S.Enteritidis	S.Typhimurium
L. animalis	65.0±4.5*	46.2±4.7	29.5±5.8	12.3±4.1
L. fermentum	32.3±2.8	41.2±3.2	11.2±2.7	5.4±1.8
L. fermentum subsp. cellobiosus	15.2±3.4	18.3±3.9	9.8±3.6	19.4±3.1

*(%)
Results are mean ± SD for three replicates

Table 4: The mean porcentage of adhesion of *Salmonella* to intestinal epithelial cells before addition

	S. Gallinarum	S. Pullorum	S. Enteritidis	S. Typhimurium
Control	61.6±10.5*	51.8±8.5	28.2±1.9	15.7±5.3
Mannose	27.3±4.6	22.3±2.8	16.2±5.2	8.0±2.4

*(%) Results are mean ± SD for three replicates

to which 1% [methyl, 1,2-^{3}H] thymidine was added (specific activity 117 Ci mmol^{-1}). Cells were collected after 2 h growth at 42°C and washed twice prior to being resuspended in DMEM, 1% HEPES, and 15% FCS.

To study the effect of adhered lactobacilli isolated from chicken on the adhesion of *Salmonella*, 200 µL of lactobacillus suspension were firstly incubated for 1 h at 42°C in polystyrene tissue culture wells with intestinal cells isolated, then the wells were washed twice to remove unbound bacteria. Washed wells were challenged with different labeled enteropathogens for 1 h at 42°C. The wells were washed twice with HEPES-Hanks. Adhesion of salmonella and lactobacilli labeled were used as positive control. Adhering bacteria were released by adding 5 mL of 5% SDS to each well and incubating the plates overnight al 37°C to lyse bacteria. The lysed cells from each well were mixed with 2 ml of scintillation liquid. The adhesion ratio (%) was calculated by comparing the radioactivity of the original bacterial suspension to the final signal from the lysed cells. All assays were performed in triplicate.

Statistical analysis: All experiments were performed in triplicate. Significant differences were tested using Tukey´s test (Minitab Student R12)[15].

RESULTS AND DISCUSSION

In this work, we study the *in vitro* interactions between lactobacilli with adhesive capacity isolated from adult chick intestine and enteropathogens belonging to *Salmonella* genera. Lactobacilli isolated from faeces adult chickens were selected for their efficiency adhesion to the host-specific epithelial cells mediated by the presence of a lectin-like superficial structure[10].

Several mixed cultures between potentially probiotic lactobacilli and *Salmonella* were performed in order to determine production of adhesive structures for each lactic acid bacteria assayed and study growth parameters. The growth and the population of viable cells of lactobacilli remained unchanged in different mixed and single (control) culture studies (Fig. 1 and 2) ($p>0.05$). However, in the mixed cultures, the inhibition of viable bacteria of some *Salmonella* strains was observed; and, in all mixed cultures, the antimicrobial effect was bacteriostatic (Fig. 1). This inhibition could be due to lactic acid production, competition of nutrients and/or synthesis of bacteriocin by lactobacilli. Recently, we have presented evidence that *Lactobacillus animalis* isolated from chicks produce bacteriocin with anti-salmonella activity[16].

In mixed cultures, using agglutination inhibition assays with specific sugars we determined that lectin production by studied lactobacilli was not affected after 48 h of incubation by the presence of enteropathogenic bacteria (Table 1).

Interactions between lactobacilli or pathogens of *Salmonella* genus and intestinal epithelial cells were studied in microplates coated with intestinal cells. The adhesion ratio (%) calculated with radioactivity methods showed significant values ($p<0.05$) for *L. animalis* (68.5 ± 15.3%) and *L. fermentum* (47.3 ± 19.4%) (Table 2). These results confirm the data obtained in a previous work, where *L. animalis* CRL 1014 showed higher adhesion ability to tissue fragments of crop, large and small intestines with prevalence of small intestine than *L. fermentum* and *L. fermentum* subsp. *cellobiosus*[17]; *S.* Gallinarum and *S.* Pullorum showed higher adhesion than *S. enteritidis* and *S.* Tiphymurium (Table 2). These results indicate the remarkable importance of a host-specific

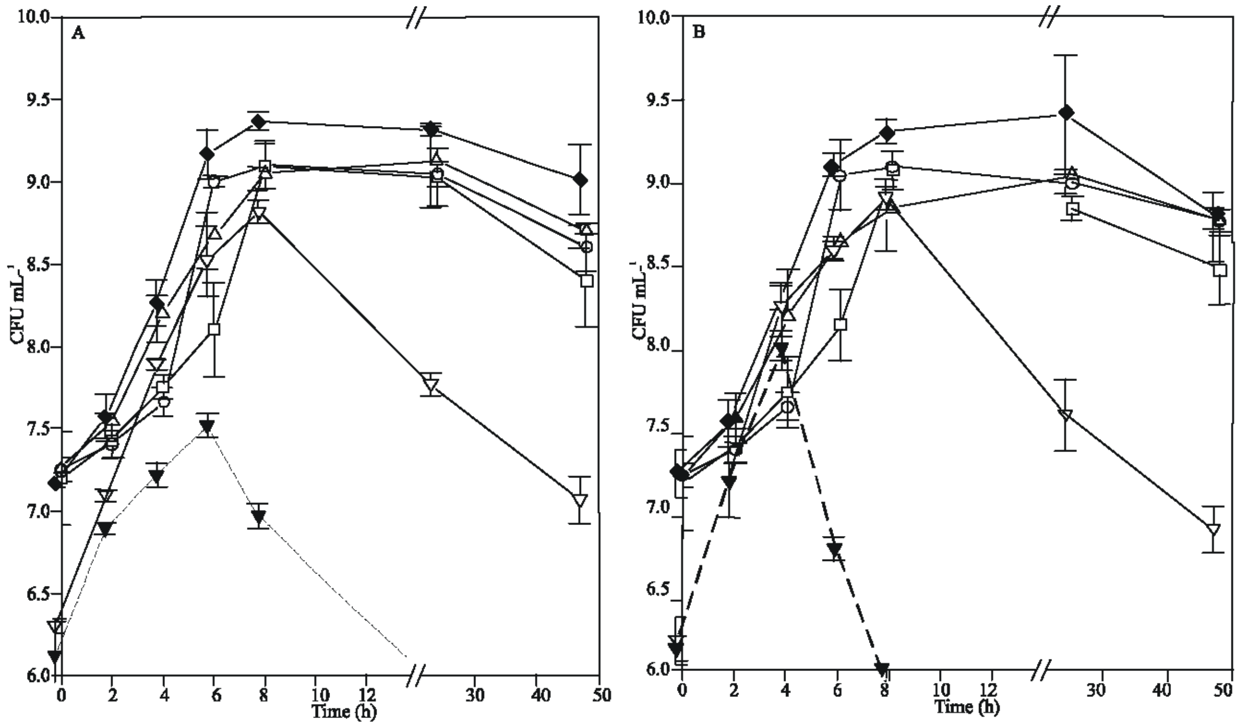

Fig. 1: Associated culture between chicken lactobacilli and A) *Salmonella* Pullorum, B) *S.* Gallinarum. Pure cultures, (£) *Lactobacillus fermentum* CRL1016, (™) *L. fermentum* CRL1015, (r) *L. animalis* CRL1014, (s) *Salmonella*. Mixed culture: (¿) total lactobacilli, (q) *Salmonella*

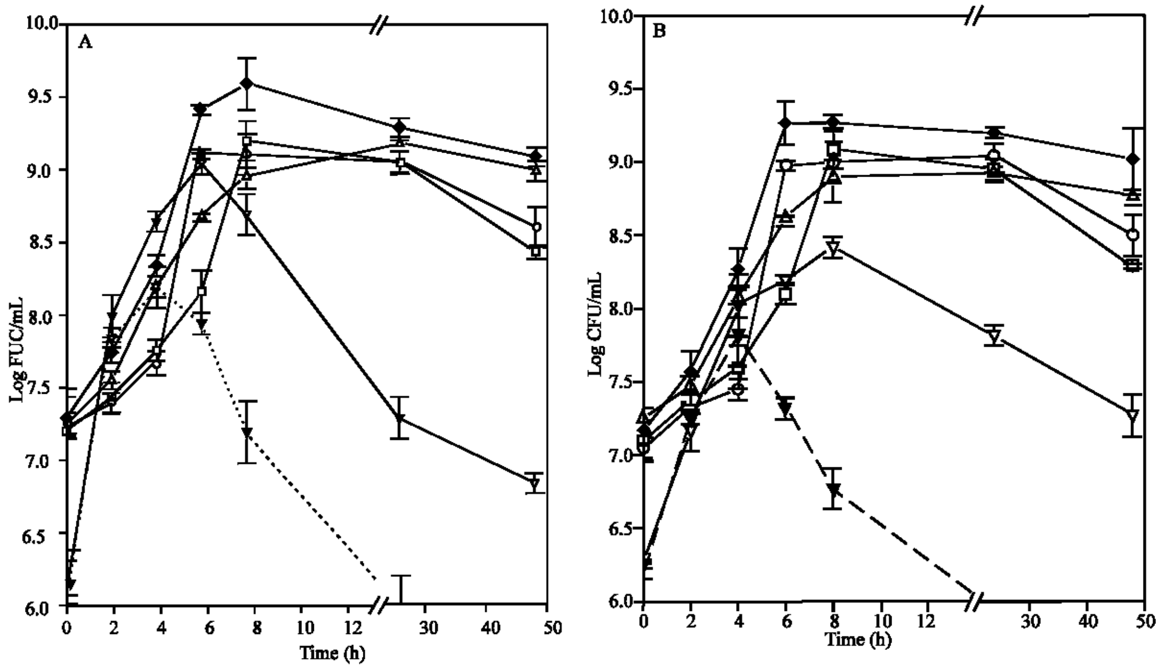

Fig. 2: Associated culture between chicken lactobacilli and A) *Salmonella* Thyphimurium, B) *S.* Enteritidis. Pure cultures, (£) *Lactobacillus fermentum* CRL1016, (™) *L. fermentum* CRL1015, (r) *L. animalis* CRL1014, (s) *Salmonella*. Mixed culture: (¿) total lactobacilli, (q) *Salmonella*

(lactobacilli and salmonella avian strains) interaction in the colonization process of microorganisms.

Adhesion of the LAB probiotics to the epithelial cells may further contribute to competitive exclusion for example, LAB that grow relatively slowly but attach to the intestinal wall can colonize the luminal contents or, if LAB occupy the adhesion receptors on the surface, the harmful bacteria relying on them will be eliminated from the intestinal tract[9].

L. animalis CRL1014 was effective in reducing by 65.0, 46.2 and 29.5% the attachment of *S.* Gallinarum, *S.* Pullorum and *S.* Enteritidis to host specific epithelial cells respectively, while *L. fermentum* CRL1015 was able to reduce by 41.2% the attachment of *S.* Pullorum and 32% for *S.* Gallinarum (Table 3). In previous work, we determined that *L. animalis* CRL1014 and *L. fermentum* CRL1015 were able to adhere to epithelial cells (crop, small and large intestines) with predominance to small intestine, and this adhesion was inhibited in both strains with 0.2M mannose[10]. In order to determine competitive exclusion mechanism by these lactobacilli, we studied the presence of lectin-like substances in external layers of the pathogens assayed. The addition of mannose produced a decrease ($p<0.05$) in the number of *Salmonella* Gallinarum, *S.* Pullorum, *S.* Enteritidis and *S.* Typhimurium attached to intestinal epithelial cells (Table 4). These results are consistent with those obtained by[17]. Therefore, potentially probiotic lactobacilli isolated and selected for their adhesion properties could inhibit *Salmonella* colonization of chicken intestinal epithelium by competitive antagonism.

CONCLUSION

We suggest that isolated chick lactobacilli (*Lactobacillus animalis* CRL1014, *L. fermentum* CRL1015 and *L. fermentum* CRL1016) may be considered potential avian probiotic microorganisms, and used in the preparation of probiotic foods. *In vivo* studies on the interaction between potentially probiotic lactobacilli and pathogens of *Salmonella* genus are in progress in our laboratory.

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