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Research Paper

Identification and adhesion profile of *Lactobacillus spp.* strains isolated from poultry

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Abstract

In the aviculture industry, the use of *Lactobacillus* spp. as a probiotic has been shown to be frequent and satisfactory, both in improving bird production indexes and in protecting intestine against colonization by pathogenic bacteria. Adhesion is an important characteristic in selecting *Lactobacillus* probiotic strains since it impedes its immediate elimination to enable its beneficial action in the host. This study aimed to isolate, identify and characterize the *in vitro* and *in vivo* adhesion of *Lactobacillus* strains isolated from birds. The *Lactobacillus* spp. was identified by PCR and sequencing and the strains and its adhesion evaluated *in vitro* via BMM cell matrix and *in vivo* by inoculation in one-day-old birds. Duodenum, jejunum, ileum and cecum were collected one, four, 12 and 24 h after inoculation. The findings demonstrate greater adhesion of strains in the cecum and an important correlation between *in vitro* and *in vivo* results. It was concluded that BMM utilization represents an important technique for triage of *Lactobacillus* for subsequent *in vivo* evaluation, which was shown to be efficient in identifying bacterial adhesion to the enteric tract.

Key words: *Lactobacillus*, identification, intestinal adhesion.

Introduction

Probiotics are defined as microorganisms that, when administered in suitable quantities, confer health benefits to the host (FAO/WHO, 2002). In the aviculture industry, supplementation with probiotics, especially *Lactobacillus*, has been shown efficient in augmenting weight gain, improving the alimentary conversion rate, and diminishing mortality in birds of production (Huang *et al.*, 2004). Due to the growing interest in the utilization of these microorganisms as probiotics, their correct identification becomes necessary (Moreira *et al.*, 2005).

Given that many *Lactobacillus* species have similar nutritional and growth requirements, it is often difficult to apply classical microbiology methods to identify them with precision. Several research studies have focused on the application of molecular biology techniques to achieve rapid detection and differentiation of this specie. The use of primers and probes that rDNA sequences coding for the 16S and 23S rRNA has been validated as a means of identification. (Dubernet *et al.*, 2002).

The adhesion of *Lactobacillus* to the epithelium was defined as a characteristic of interest for selection of probiotic strains, since it represents the first step in the formation of a barrier to prevent colonization by undesirable microorganisms due to competition for nutrients and adherence sites, in addition to preventing its immediate elimination by intestinal peristalsis (Collado *et al.*, 2005); but despite this, information on its adhesion to the intestinal epithelium of poultry remains scarce (Morelli, 2002).

The use of *in vitro* models, including epithelial cells, mucosal components and extracellular matrix components such as laminin, fibronectin, collagen and proteoglycans, found in the commercial product Basement Membrane Matrix - BMM (BD - Becton Dickinson, USA), could be very useful, once it demonstrate the adhesion capacity of *Lactobacillus*, both individually and collectively (Horie *et al.*, 2002; Vellez *et al.*, 2007).

Although none of the previously cited methods *in vitro* reflect the complex interactions that occur in the mucosa of the gastrointestinal tract *in vivo*, they represent a

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rapid method for characterizing and selecting strains of *Lactobacillus*, and according to Muñoz-Provencio *et al.* (2009), in most cases there is a high correlation between results found *in vitro* and those obtained from *in vivo* tests.

The staining of *Lactobacillus* by the fluorescence technique is a highly accurate and reproducible technique for tracking and quantifying bacterial cells, especially via *in vivo* models (Bianchi *et al.*, 2004), and one option is to utilize carboxyfluorescein succinimidyl amino ester (CFDA SE), which is colourless and nonfluorescent until its acetate group is cleaved by intracellularesterase to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester (Bouzaine *et al.*, 2005) that allows easily distinguish form from the native bacterial population of the intestine, without having their adhesion, survival capacity or membrane properties altered by the staining agent (Fuller *et al.*, 2000).

The present work aimed to identify, by polymerase chain reaction (PCR), strains of *Lactobacillus spp.* isolated from chicken, to evaluate their adhesion capacity *in vitro* and *in vivo* in one-day-old birds and posterior sequencing.

Material and Methods

Isolation of *Lactobacillus* spp.strains

The isolation and genus identification of *Lactobacillus* strains were done as described before by Barros *et al.* (2009) using crop and cecum of thirty Cobb breeders, aged 65 weeks collected aseptically.

Confirmation of *Lactobacillus* spp. identification by Polymerase Chain Reaction (PCR) technique

The bacterial strains that presented characteristics compatible with the *Lactobacillus* genus were submitted to confirmation by means of PCR. The following strains were utilized as positive reaction controls: *Lactobacillus fermentum* CCT0559-ATCC9338, *L. reuteri* CCT3433 - ATCC23272, *L. acidophilus* CCT3258-ATCC4356, *L. casei ssp. casei* CCT 1465- ATCC 393, *L. delbrueckii ssp. lactis* CCT7520-ATCC7830 and *L. helveticus* - CCT 3747 - ATCC 15009.

For this purpose, DNA was extracted from the strains utilizing the kit QIAamp DNA blood Mini Kit (Qiagen, USA) according to the manufacturer's instructions. The following primers: *Forw* R16-1 (5'-CTT GTA CAC ACC GCC CGT CA- 3') and *Rev* LbLMA1- (5'-CTC AAA ACT AAA CAA AGT TTC -3'), the reaction and cycle employed were according to previously described by Dubernet *et al.* (2002) in order to amplify a product of approximately 250 pb.

In vitro adhesion in cell matrix type BMM

Each *Lactobacillus* strain was submitted to an adhesion test, as described by Bouzaine *et al.* (2005) utilizing the cell matrix type Basement Membrane Matrix (BMM).

To prepare the slides with BMM matrix, the same was diluted (1:20) in PBS; and 35 uL was deposited on Falcon® (BD) slide at 4 °C. The slides were maintained in repose at 37 °C for two h and then incubated with phosphate buffer solution (PBS) with the addition of 2% bovine serum albumin (BSA) for one hour at room temperature.

The preparation of the *Lactobacillus* strains consisted of incubating the bacterial cells in MRS broth for 24 h at 37 °C, centrifuging (13,000 x *g* for 3 min) and washing twice with one mL of PBS. Subsequently, 200 µL of this solution at the concentration of 10^7 UFC mL^{-1} was deposited on slides previously prepared with BMM and incubated for two h at room temperature. After incubation, the slides were washed three times with PBS, submitted to Gram staining and read in an optical microscope.

Three repetitions were performed for each strain tested, with eight fields being counted in each one in a rectangular area of 1.7 x 1.0 cm. The area for counting in each microscopic field was 3.8 x 10^{-2} mm^2, utilizing a 100x objective. The counts were performed by double-blind study. A strain of *Escherichia coli* isolated from chicken and a *Bacillus subtilis* 168 were utilized as positive and negative control, respectively.

In vivo adhesion of *Lactobacillus spp.* in intestinal epithelium of birds

Culturing and bacterial staining with carboxyfluorescein succinimidyl amino ester (CFDA SE).

Based on the *in vitro* adhesion test, the five *Lactobacillus spp.* strains that had obtained the best adhesion results in BMM were selected for the *in vivo* test.

The strains of *Lactobacillus spp.* and *Bacillus subtilis* were cultured in MRS broth and Luria broth (LB), respectively, until they reached a stationary phase (16 h). Subsequently, they were centrifuged at 9300 x *g* for five minutes, washed twice in PBS and resuspended in PBS (pH 7.5) to obtain a concentration of 10^{10} cfu mL^{-1}. The strains were stained with Vybrant CFDA SE Tracer (Invitrogen) according to the manufacturer's recommendations using 50 µmol L^{-1} of CFDA SE solution at 37 °C for 30 min. The stained cells were then centrifuged, washed twice in PBS and re-suspended to its initial volume in the proper culture broth (MRS or LB) and incubated again at 37 °C for 30 min. The stained cultures were protected from light and maintained at 4 °C until inoculation in birds.

Experimental design

168 one-day-old Ross lineage breeders were utilized, divided into seven groups composed of 24 animals each. In each group the following different bacterial strains stained with CFDA SE were inoculated: negative control group (*Bacillus subtillis*), positive control group (*L. reuteri* CCT3433 - ATCC23272) and five other groups (strains 311, 262, 258, 231 and 206).

Prior to the *in vivo* assay a pilot test was performed with a group in which the birds did not receive any type of stained microorganism, to certify that only the inoculated strains would be detected by fluorescence.

The inoculum was administered orally, with the aid of a gavage needle, directly into the crop. Alimentation was suspended 12 h before this procedure. The collection moments were at one, four, 12 and 24 h after treatment.

Preparation of intestine fragments from birds

At each of the four moments after bacterial inoculation, six birds per group were humanely euthanized by cervical dislocation, and cranial one-centimeter portions of duodenum, jejunum, ileum and cecum were collected. All visible residues were carefully removed to avoid damaging the intestinal mucosa. The collected fragments were immediately frozen via submersion in liquid nitrogen and stored at -80 °C until the histological preparation. The fragments were collected with the aid of a micro-cryostat and prepared on slides containing five histological cuts (8 to 10 μm each). The slides were protected from light and stored at -20 °C until analysis.

To quantify the attached *Lactobacillus spp.* and *Bacillus subtilis*, two random microscopic fields were observed in each cut, totaling ten counting fields per slide/fragment. The counting area in each microscopic field was 3.8×10^{-2} mm^2. The reading was performed in a fluorescence microscope and the results were expressed as the median number of stained bacteria per histological cut.

Sequencing

The PCR products of the five best adhesive strains at *in vitro* test were cloned at pGem vector (Promega), accordingly to the manufacture's recommendation, utilizing vector' primers *Fw*-M13 5'-CACGACGTTGTAAAACGAC-3' e *rev*-M13 - 5'-GGATAACAATTTCACACAGG-3'. The sequences of the 16S/23S ribosomal RNA intergenic spacer region of phylogenetically related species: *L. acidophilus* (U32971), *L. amylovorus* (AF182732), *L. casei subsp. casei* (Z75478), *L. crispatus* (AF074857), *L. delbrueckii subsp. bulgaricus* (Z75475), *L. fermentum* (AF080099), *L. gasseri* (AF074859), *L. hamsteri* (AF113601), *L. helveticus* (Z75482), *L. jensenii* (AB035486), *L. johnsonii* (AF074860), *L. plantarum* (U97139), *L. reuteri* (AF080100), *L. rhamnosus* (AF121201), *L. sakei* (U97137), *L. salivarius* (AF113600), *L. vaginalis* (AF182731) were retrieved from GenBank (www.ncbi.nlm.nih.gov) and used to perform multiple alignments with the our strains sequences using Clustal W (Tamura *et al.*, 2011).

Statistical analysis

The results obtained in vitro were transformed to $\log_{10}$ form; the mean and standard deviation and subsequently the confidence interval were calculated to determine the upper and lower limits, which were compared with the mean of the positive control. A 5% significance level was adopted (Sampaio, 2002).

For the *in vivo* counts we used a non-parametric analysis of variance (Dunn test), utilizing the median values of bacterial counts, with a two-factorial scheme (Zar, 2009). A 5% significance level was adopted in all tests and the datas were submitted to the software SIGMASAT (Surhone *et al.*, 2010).

Results

Identification of *Lactobacillus spp.*

Of the strains analyzed, 123 were compatible with the genus *Lactobacillus* in the aforementioned tests and were then submitted to PCR. Of this total, only 73 strains were positive (see Figure 1).

In vitro adhesion in cellular matrix type BMM

After identification of the genus *Lactobacillus* by PCR, each of the strains (73) was submitted to *in vitro* adhesion test in BMM. Table 1 displays the counts of the microorganisms adhering to the BMM matrix.

The visualization of the *Escherichia coli* strain utilized as a positive control and the *Bacillus subtilis* 168 strain employed as a negative control is represented in Figure 2, A and B.

In the present study, it was possible to determine that the strains of *Lactobacillus spp.* isolated from the bird intestines are capable of adhering to BMM (Figure 3 A and B). We can observe greater adhesion capacity in strains 206, 231, 258, 262 and 311 with mean counts of bacteria adhering in BMM closer to the positive control (Table 1). In this manner, these five strains were selected for the *in vivo* adhesion test.

Adhesion *in vivo* of *Lactobacillus spp.* strains

The median counts of adherent cells in the *in vivo* test of *Bacillus subtillis* (negative control), *Lactobacillus reuteri* (positive control) and of the test of strains 206, 231, 258, 262 and 311 are shown in Table 2.

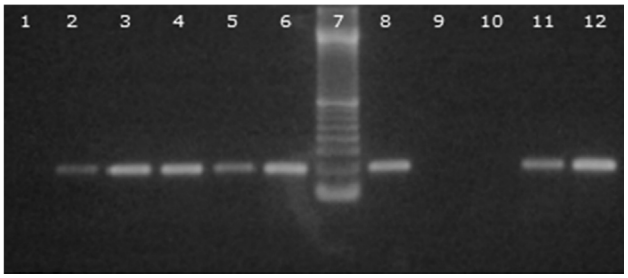

Figure 1 - Agarose gel 1.5%. 1-Negative control; 2- *Lactobacillus acidophilus* CCT3258; 3-*L. casei* CCT 1465; 4-*L. delbrueckii* CCT 7520; 5-*L. fermentum* CCT 0559; 6-*L. helveticus* CCT 3747; 8-*L. reuteri* CCT3433; 9 and 10-Samples negative for *Lactobacillus* spp.; 11 and 12-Samples positive for *Lactobacillus* spp.; 7-Molecular weight 100 bp.

Table 1 - Result of the *in vitro* adhesion test represented by mean number of bacterial *Lactobacillus* spp cells adhering to BMM matrix.

Identification of strains	Mean	Identification of strains	Mean
E. coli (positive control)	206.12	250	2.62
B. subtilis (negative control)	7.08	251	6.79
203	6.00	252	2.33
204	2.70	253	2.08
206	111.70	254	2.00
208	71.70	255	3.33
210	3.37	256	6.16
211	6.87	257	4.25
212	56.29	258	106.25
214	19.5	259	3.83
215	65.66	260	3.33
216	42.65	261	18.54
217	13.16	262	231.41
218	40.29	263	5.87
219	49.16	264	2.75
220	29.16	265	3.33
221	17.70	266	3.66
222	6.75	267	5.70
224	8.12	268	2.79
226	5.83	271	7.25
227	19.91	273	1.12
230	13.66	305	2.04
231	254.87	306	1.08
234	7.08	307	2.04
237	10.04	308	16.16
238	21.00	309	1.83
239	26.45	310	23.70
240	12.79	311	109.54
241	15.25	312	9.91
242	11.12	318	2.29
243	6.45	319	2.08
244	18.29	320	7.04
245	11.83	321	9.95
246	2.20	322	12.95
247	1.66	323	4.66
248	5.5	324	5.16
249	2.91	325	1.54

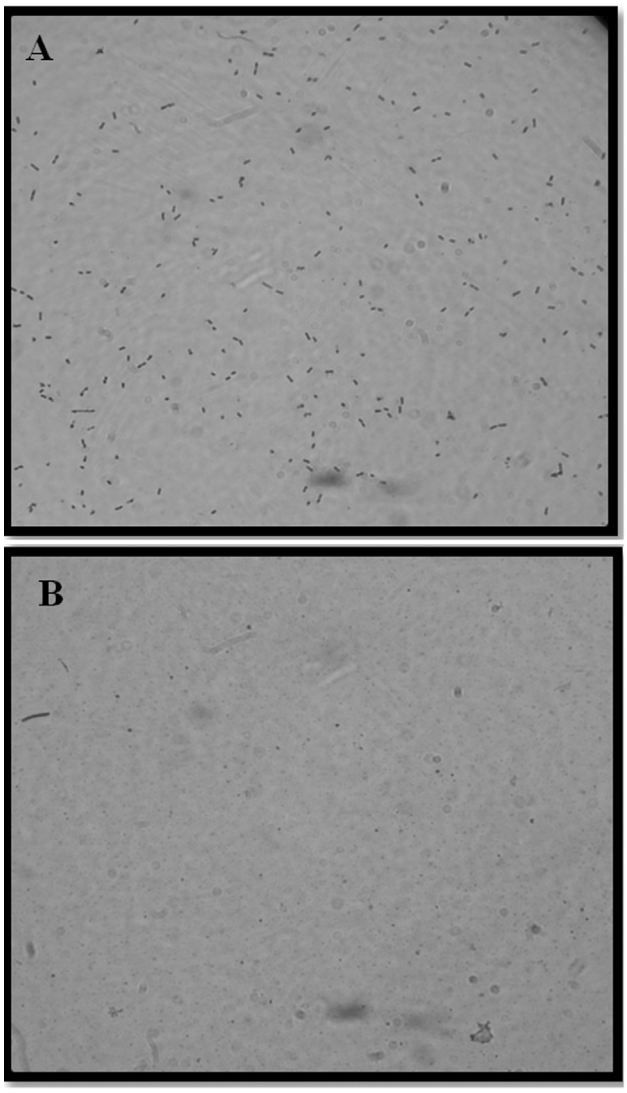

Figure 2 - Optical microscopic image (100x) of *Escherichia coli* (A) and *Bacillus subtillis* (B), stained by Gram, demonstrating high and low adherence of strains in BMM matrix by *in vitro* adhesion tests, respectively.

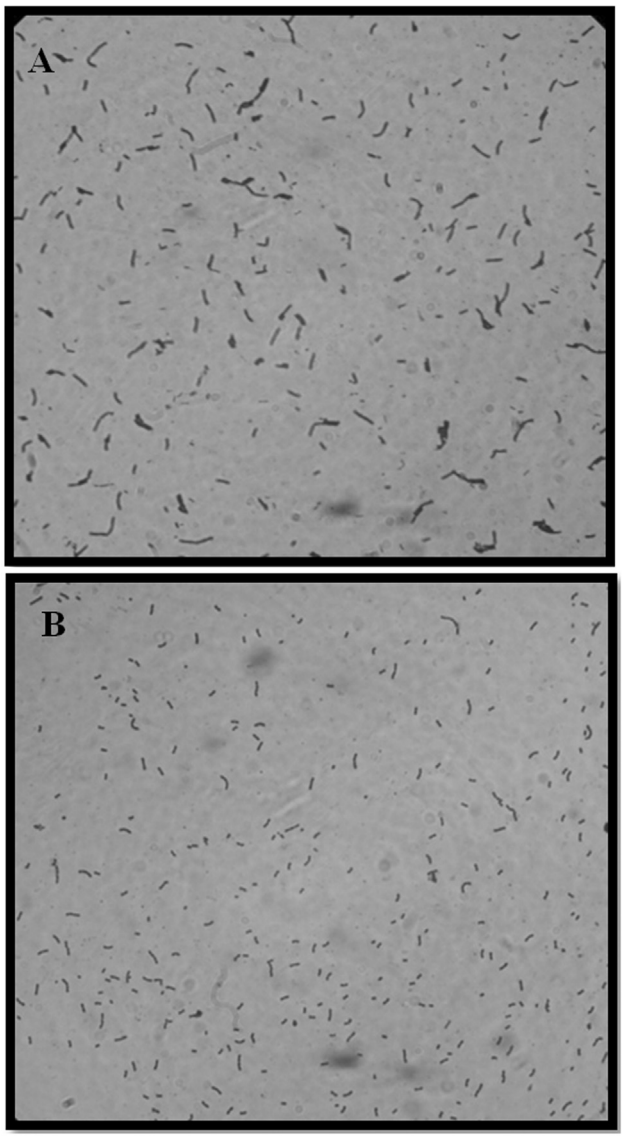

Figure 3 - Optical microscopic image (100x) of Strains 231 (A) and 262 (B), stained by Gram, demonstrating high adherence rate in BMM matrix in *in vitro* adhesion tests.

As expected, in the group in which the birds did not receive stained microorganisms, no type of fluorescence or staining was found. The visualization of the *Lactobacillus spp.* strains stained in different intestinal segments is represented in Figure 4.

The greatest bacterial count was found in strain 262 in the cecum, four hours after inoculation. However, a decrease in the counts was observed at the moments 12 and 24 h after inoculation.

In the four different intestinal segments during the time of the experiment, it was observed that all strains presented a greater quantity of *Lactobacillus* adhering at 1 h and 4 h than at 24 h, but this was not found in the cecum fragments of the treatments with strains 231 and 206.

The positive control presented reduced counts in all segments as time elapsed, demonstrating low adherence to intestinal epithelium. In general, the cecum was the segment that demonstrated the greatest quantity of adhering *Lactobacillus*, with the exception of strain 258. But this strain showed stability in values that did not differ significantly ($p > 0.05$) during the collections at 12 and 24 h (see Table 2), demonstrating a homogenous distribution throughout the intestine, but in a lesser quantity when compared to the other *Lactobacillus* strains.

In all the strains, the bacterial counts observed in the duodenum at 12 and 24 h were similar of the negative control, demonstrating low adherence of the *Lactobacillus* evaluated in this segment.

The results obtained with the sequencing alignment showed a highly conserved region between nucleotides 369-391 corroborating with Dubernet *et al.* (2002). The strains 206, 231, 258, 262, 311 and 316 showed an identity

Table 2 - Median count of bacteria adhering to intestinal epithelium, by *in vivo* assay, considering the strain, time after inoculation and intestinal segment.

Strain	Time (h)	Intestinal Segment			
		Duodenum	Jejunum	Ileum	Cecum
Control -*B. subtillis*	1	12.2 $^{ab\,x}$*	3.8 $^{b\,x}$	49.6 $^{a\,x}$	1.3 $^{b\,x}$
	4	0.7 $^{a\,x}$	0.8 $^{a\,x}$	0.4 $^{a\,x}$	0.8 $^{a\,x}$
	12	0.8 $^{a\,x}$	0.3 $^{a\,x}$	0.0 $^{a\,y}$	0.5 $^{a\,x}$
	24	1.9 $^{a\,x}$	1.0 $^{ab\,x}$	0.7 $^{b\,y}$	0.5 $^{b\,x}$
Control +*L. reuteri*	1	215.5 $^{ab\,x}$	300.7 $^{a\,x}$	301.9 $^{a\,x}$	57.3 $^{b\,y}$
	4	22.9 $^{b\,y}$	3.0 $^{b\,y}$	5.2 $^{b\,y}$	104.0 $^{a\,xy}$
	12	6.4 $^{b\,y}$	4.3 $^{b\,y}$	3.4 $^{b\,y}$	190.4 $^{a\,x}$
	24	3.8 $^{a\,y}$	10.2 $^{a\,y}$	2.8 $^{a\,y}$	9.9 $^{a\,z}$
311	1	146.7 $^{a\,x}$	38.7 $^{ab\,x}$	14.5 $^{bc\,x}$	2.1 $^{c\,x}$
	4	19.4 $^{a\,y}$	7.5 $^{a\,xy}$	3.4 $^{a\,x}$	4.3 $^{a\,x}$
	12	1.3 $^{a\,y}$	1.4 $^{ab\,y}$	1.4 $^{ab\,x}$	10.1 $^{b\,x}$
	24	1.4 $^{ab\,y}$	1.4 $^{ab\,y}$	0.7 $^{b\,x}$	2.6 $^{a\,x}$
262	1	251 $^{a\,x}$	328.4 $^{a\,x}$	192.2 $^{a\,x}$	14.1 $^{b\,z}$
	4	21.3 $^{b\,y}$	105.3 $^{b\,y}$	125.4 $^{ab\,x}$	534.0 $^{a\,x}$
	12	1.4 $^{b\,z}$	1.5 $^{b\,z}$	1.2 $^{b\,y}$	155.5 $^{a\,y}$
	24	0.5 $^{b\,z}$	0.8 $^{b\,z}$	0.6 $^{b\,y}$	59.3 $^{a\,yz}$
258	1	163.0 $^{a\,y}$	102.9 $^{a\,y}$	67.4 $^{a\,x}$	0.7 $^{b\,y}$
	4	63.6 $^{a\,y}$	8.9 $^{a\,x}$	5.8 $^{a\,y}$	55.8 $^{a\,x}$
	12	0.5 $^{a\,x}$	0.7 $^{a\,x}$	0.4 $^{a\,y}$	5.7 $^{a\,y}$
	24	0.9 $^{a\,x}$	0.1 $^{a\,x}$	0.1 $^{a\,y}$	1.7 $^{a\,y}$
231	1	17.2 $^{ab\,x}$	25.5 $^{ab\,x}$	42.7 $^{a\,x}$	0.0 $^{b\,y}$
	4	65.2 $^{ab\,y}$	192.2 $^{a\,y}$	18.0 $^{b\,xy}$	150.8 $^{ab\,x}$
	12	18.4 $^{ab\,x}$	16.6 $^{ab\,x}$	7.7 $^{b\,y}$	53.2 $^{a\,x}$
	24	24.0 $^{ab\,x}$	12.6 $^{ab\,x}$	5.6 $^{b\,y}$	71.4 $^{a\,x}$
206	1	155.3 $^{a\,y}$	368.7 $^{a\,z}$	172.6 $^{a\,x}$	0.2 $^{b\,z}$
	4	85.5 $^{a\,y}$	46.8 $^{a\,xy}$	33.8 $^{a\,y}$	125.8 $^{a\,xy}$
	12	22.9 $^{b\,x}$	67.0 $^{ab\,y}$	34.2 $^{b\,y}$	252.9 $^{a\,x}$
	24	18.8 $^{a\,x}$	9.1 $^{ab\,x}$	1.4 $^{b\,z}$	33.9 $^{a\,y}$

* ^{a,b,c} Two medians followed by at least one different superscript, in the same row, do differ significantly ($p \geq 0.05$), comparing the same strain and same time in different intestinal segments (Line).

^{x,y,z} Two medians followed by at least one different superscript, in the same columm, do differ significantly ($p \geq 0.05$), comparing different times in the same strain and same intestinal segment (Column).

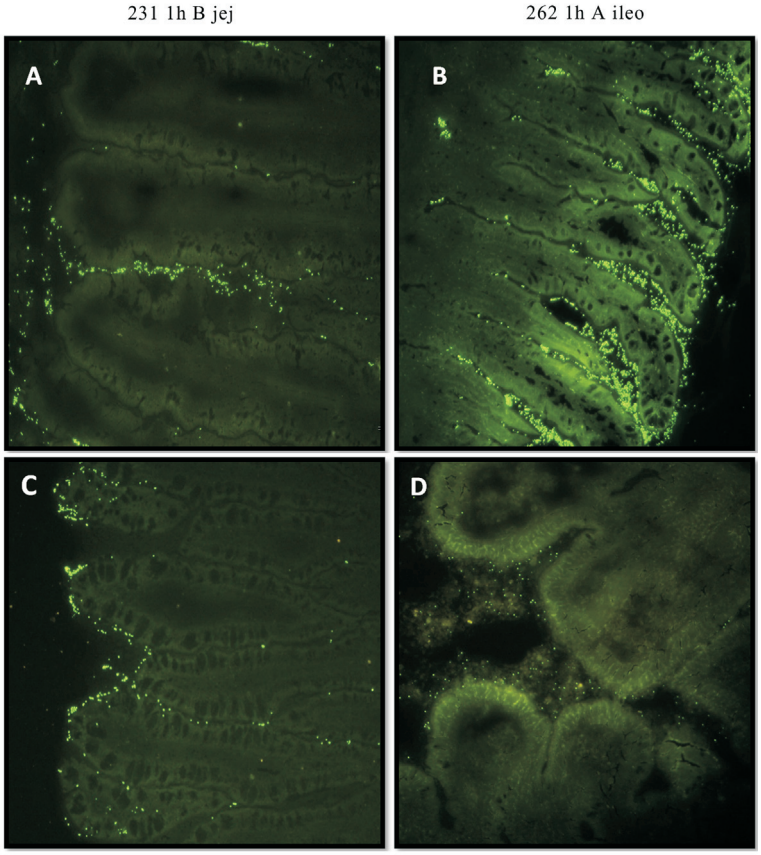

Figure 4 - cFDA-staining *Lactobacillus* adhered to intestinal epithelium observed by fluorescent microscope (magnification 40). A- duodenum one hour after strain 258 inoculation; B - jejunum one hour after strain 231 inoculation; C - ileum four hours after positive control *Lactobacillus reuteri* inoculation D- cecum four hours after strain 206 inoculation.

of 100% with up to eight sequences of L. reuteri (CP002844.1; AP007281.1; CP000705.1; EU547293.1, EU547292.1, EU547290.1, EF412989.1; EF412988.1), allowing us to conclude that all strains analyzed belong to this specie.

Discussion

The difference found between the PCR and biochemical analyses corroborates De Martinis (2002), who considers the utilization of a fermentation pattern of carbohydrates unsatisfactory as a single criterion for identifying lactic acid bacteria, since frequent variations occur in the fermentations, enabling subjectivity in their interpretations.

Adhesion to intestinal mucosa can confer an important competitive advantage for bacterial maintenance in the gastrointestinal tract and it is generally accepted that adhesive properties contribute to the efficacy of probiotic strains (Servin and Coconnier, 2003).

Bouzaine *et al.* in 2005, utilizing the same methodology employed in the present study for the evaluation of *in vitro* adhesion, reported respective mean counts for the strains *Lactobacillus rhamnosus TB1* and *Lactobacillus reuteri* LRT1 greater than 2000 and 500 cels per field, while those of the strains *Lactobacillus johnsonii* LJT2 and *Lactobacillus salivarius* LST1 were less than 250 cells per field. But Horie *et al.* in 2002 evaluated the adhesion capacity in BMM of *Lactobacillus crispatus* JCM 5810 and found an average of 70 bacterial cells per microscopic field.

The counts reported in previous studies are higher than most of the values observed in our study (except for the strains selected for the *in vivo* adhesion test), which may be explained by the fact that the physical-chemical nature of the external membrane of the cell wall, the conformation of macromolecules of the bacterial surface and the susceptibility to external factors (time, medium and general culture conditions) determine the propensity of *Lactobacillus* to adhere to a surface, factors that vary even within the same specie (Schar-Zammaretti and Ubbinik, 2003).

The CFDA SE cell tracer, utilized in the *in vivo* assay, is a compound capable of staining cells without compromising their viability or altering their adhesive characteristics, although during bacterial growth, when the bacterial concentration is expected to rise, the number of cells stained can decrease due to dilution of intracellular CFDA SE after cellular division (Fuller *et al.*, 2000). Thus, to avoid discrepant results between the quantities of adhering bacteria *vs.* the number effectively stained, the present

study opted to evaluate adhesion for a maximum period of 24 h after bacterial inoculation in the birds.

Lee *et al.* (2004) in an investigational study of the growth and colonization of *Lactobacillus casei shirota* stained by CFDA SE in rat intestines; determined that the *Lactobacillus* strain utilized presented in the first collection, at 24 h, greater adhesion to the jejunum, in contrast to the data found in the present study, in which the strains, except for 258, presented greater adhesion to ceca at 24 h after inoculation of birds.

According to Bouzaine *et al.* (2005) *Lactobacillus rhamnosus TB1* presented a low bacterial count in intestinal epithelium of birds, despite having demonstrated greater affinity for adhesion in the rectum, ileum and jejunum. The cecum was the segment that presented the least adhesion by *Lactobacillus rhamnosus TB1*, in agreement with the results of Edelman *et al.* (2002), who also observed low adhesion of different Lactobacillus species in the cecal epithelium of birds.

In our present work it was possible to identify a tendency among the strains evaluated to colonize the ceca, with less capacity to establish themselves in the duodenum or jejunum, especially in the 24 h period after inoculation, corroborating the results of Fuller and Turvey (1971) who reported a massive bacterial colonization of the ceca.

We verified that a single strain (258) presented homogeneity in intestinal colonization but also the lowest counts in the four intestinal segments evaluated at 24 h after inoculation. In contrast, strain 231 presented the highest adhesion values at 24 h, but showed variations in its adhesive capacity in the different intestinal segments, at the moments analyzed.

These data confirm the strain-dependent nature of the intestinal adhesion capacity, since they may have differences in the structural conformation and composition of the bacterial cell wall, with these characteristics varying within the same genus and even within the same species of *Lactobacillus* (Servin and Coconnier, 2003; Deepika and Charalampopoulos, 2010).

Strain 231 also presented better adherence in the *in vitro* test utilizing BMM, and satisfactory results in the *in vivo* adhesion tests, significantly superior to the other ones and especially to the positive control (Table 2), demonstrating the relevance and correspondence of the *in vitro* with the *in vivo* results and suggesting that the *in vitro* technique employed may be utilized for the selection of possible probiotic strains.

Early studies on chicken microbiota found that *Lactobacillus salivarius*, *L. reuteri*, and *L. acidophilus* inhabited the crop and the chicken digestive tract. Our results show that the five strains sequenced are compatible with *Lactobacillus reuteri* specie, and agrees with the fact that this is the most abundant *Lactobacillus* species in the chicken gastrointestinal tract (Abbas Hilmi *et al.*, 2007).

From the results obtained in the present study it can be concluded that the PCR technique is an important tool for identifying *Lactobacillus* spp., and can be utilized to validate the result of biochemical identification tests.

It may also be concluded that the *in vitro* adhesion process of *Lactobacillus*, utilizing the Basement Membrane Matrix (BMM) permits the selection of probiotic strains with adhesion capacity; also, the staining of *Lactobacillus* by CFDA SE is shown to be suitable for the visualization and counting of this bacterium adhering to the intestinal epithelium of birds, with both techniques being efficient for evaluating the adherence capacity of probiotic strains.

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