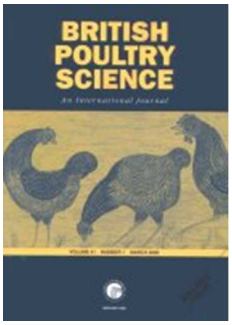

Lactic acid bacteria isolated from poultry protect intestinal epithelial cells of chicks from *in vitro* wheat germ agglutinin-induced cytotoxicity

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Lactic acid bacteria isolated from poultry protect intestinal epithelial cells of chicks from *in vitro* wheat germ agglutinin-induced cytotoxicity

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Running title: Wheat lectin toxicity protection

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Abstract. 1. Whole grain or milled wheat is often used instead of corn in poultry feeding, due to its high content of starch and proteins. Poultry fed with wheat-based diets regularly ingest wheat germ agglutinin (WGA).

2. WGA showed toxic effect *in vitro* on intestinal epithelial cells (IEC) obtained from fourteen-day-old broilers. Cytotoxicity was dependent on time and lectin concentration; lethal dose (LD_{50}) was 8.36 µg/mL for IEC exposed for 2 h to WGA.

3. Complementary sugars to WGA were detected on the surface of one *Enterococcus* and nine *Lactobacillus* strains isolated from poultry. These strains were evaluated as a lectin removal tool intended to cytotoxicity prevention.

4. The incubation of the lactic acid bacteria with WGA before the IEC-lectin interaction caused a remarkable reduction in the percentage of cell death. The protection was attributed to the amount of lectin bound to the bacterial surfaces and was strain-dependent. *L. salivarius* LET 201 and *L. reuteri* LET 210 were significantly more efficient than the other lactic acid bacteria assayed.

5. These results provide bases for the development of probiotic supplements or cell-wall preparations of selected lactic acid bacteria intended to avoid harmful effects of a natural constituent of the grain in wheat-based diets.

INTRODUCTION

Wheat is commonly used as a source of metabolizable energy for poultry (Adebiyi and Olukosi, 2015), mainly in western Canada and parts of Europe but worldwide when corn is scarce. Although the nutrients content of wheat allow the partial or total replacement of corn in the poultry diet, some components of this grain like non-starch polysaccharides have anti-nutritional effects that may be prevented by addition of hydrolytic enzymes to the diet, like xylanase (Kiarie *et al.*, 2014) and *β*-glucanase (Rodríguez *et al.*, 2012). Other component of interest is the WGA lectin (wheat germ agglutinin), whose effects in poultry production have not been still studied.

Plant lectins are proteins or glycoproteins of non immune origin possessing at least one non catalytic domain, which binds reversibly to specific mono- or oligosaccharide through hydrogen bonds and van der Waals interactions (Lis and Sharon, 1998, Peumans and Van Damme, 1995). WGA is a rather small and heat-resistant protein with four binding sites specific for N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid residues (Rudiger and Gabius, 2001, Van Damme *et al.*, 1998). These complementary sugars are ubiquitously expressed by animal cells as they constitute key molecular components of the membrane glycoconjugates. WGA binds to glycosaminoglycans, glycolipids and also some glycoproteins via sialic acid residues (Peters et al., 1979). WGA is relatively stable at low pH and resistant to proteolysis (Van Damme, Peumans, Pusztai and Bardocz, 1998) and thus it can overcome the conditions of gastrointestinal tract and reach active the intestine (Jones *et al.*, 2012). WGA has been proved toxic for Molt4, K562 and PBMC human leukemic cell lines (Ohba *et al.*, 2003), and for Caco2 cells (Dalla Pellegrina *et al.*, 2005), among others. The binding of WGA to enterocytes from the villi of proximal jejune-ileum of chicks 2, 15 and 30 days post hatching (Pohlmeyer *et al.*, 2005), to enterocytes from the ileum of

chicks 2 days post hatching (Zhou *et al.*, 1995), and to enterocytes from the villi of caecal tonsils of adult chickens (Kitagawa *et al.*, 2000) has been previously reported. The interaction lectin-epithelium may alter the activity of digestive enzymes located on the epithelial cells (Rueda *et al.*, 2007), and the integrity and permeability of the intestinal epithelium allowing small molecules to cross the epithelial barrier contributing to the toxicity of WGA to gastrointestinal cells (Dalla Pellegrina, Rizzi, Mosconi, Zoccatelli, Peruffo and Chignola, 2005). In poultry, this may depress the growth of broilers especially during the first days of life.

Poultry industry has explored the benefit of including probiotic cultures in broilers feed as alternative to antibiotic treatments for growth promotion. Probiotics may contribute to xenobiotics metabolism or to avoid deleterious effects of toxic compounds included in the diet by binding them on the bacterial envelopes that express appropriated determinants (Carasi *et al.*, 2012, Turbic *et al.*, 2002, Zárate and Perez Chaia, 2012). The binding of WGA to carbohydrates expressed on the bacterial surface and its removal by the normal transit of the intestinal content may contribute to a healthier broiler feed.

In the present investigation we explore the toxicity of WGA on intestinal epithelial cells of broilers and the ability of lactic acid bacteria isolated from poultry intestine to remove WGA and protect cells from the harmful effects of this lectin.

MATERIALS AND METHODS

Microorganisms and culture conditions

Thirteen strains of lactobacilli and one enterococcus isolated from poultry intestine were used in the study. Suitable properties for probiotic strains selection were previously

determined, such as the growth at the corporal temperature of poultry, tolerance to pH, bile salts and digestive enzymes, adhesion to small bowel mucosa, antibiotics sensitivity and absence of virulence factors (Babot *et al.*, 2014). The strains were grouped as non-adherent (*Lactobacillus salivarius* LET 201, *L. vaginalis* LET 202, *L. vaginalis* LET 203, *L. reuteri* LET 208, *L. reuteri* LET 209, *L. reuteri* LET 212 and *L. reuteri* LET 2013) and adherent (*L. reuteri* LET 204, *L. reuteri* LET 205, *L. reuteri* LET 206, *L. reuteri* LET 207, *L. reuteri* LET 210, *L. reuteri* LET 211 and *Enterococcus faecium* LET 301) bacteria.

All strains were stored at -70 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract and 15% glycerol. MRS broth (de Man *et al.*, 1960) and LAPTg broth (Raibaud *et al.*, 1973) were used to activate cultures of lactobacilli and enterococci, respectively. Prior to use, cultures were incubated at 41.5 ± 0.5 °C for 12 h.

Intestinal epithelial cells extraction

For extraction of intestinal epithelial cells (IEC), fourteen-day-old broiler chicks were slaughtered by cervical dislocation. The birds were immediately eviscerated for collection of ileum which was in turn rinsed repeatedly with ice cold PBS pH 7.40 to eliminate the digested content. The tissue was cut lengthwise and washed once again with cold PBS. Epithelial cells were scrapped from the surface using a sterile microscope slide and collected in ice cold PBS pH 7.40 supplemented with 1% Fetal Bovine Serum (PBS/FBS). The cells were washed twice with PBS/FBS, incubated with 0.25% Trypsin-EDTA (Gibco, Grand Island, USA) at 37 °C for 5 min before cold PBS/FBS was added to stop the enzyme activity. The cells were immediately collected (800 × g, 5 min, 4 °C) and washed once with PBS/FBS prior to adjust their

concentration to 1 × 10^6 cells/mL in RPMI 1640 medium supplemented with 1% FBS (RPMI/FBS). Cells were counted using a Neubauer chamber in a conventional light microscope at 40 × magnification (Zeiss–Axiolab; Cool Zeiss, Jena, Germany).

The animal handling protocols of this investigation were adjusted to the *Ethical Framework of Reference for Biomedical Research in Laboratory Animals, from Farm and Obtained from Nature*, contained in the Resolution N°. 1047/05, Annex II, of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) - Argentina. Experimental procedures for IEC extraction were approved by The Committee of Ethics for Animal Studies of CERELA (CCT Tucumán - CONICET).

WGA cytotoxicity

For cytotoxicity assessment, equal volumes of suspensions of IEC and WGA in RPMI/FBS were mixed and incubated at 41.5 ± 0.5 °C. Final concentrations in the mixtures were 5 × 10^5 cells/mL and 0, 12.5, 25, 50, 100, 150 or 200 µg/mL of WGA. After 1 and 2 h of incubation, 100 µL of cells suspensions were washed with RPMI/FBS and suspended in the same volume of medium. A freshly prepared mixture of Fluorescein diacetate (FDA; Sigma-Aldrich, Argentina) and Propidium iodide (PI) was added to obtain final concentrations of 7.5 µg/mL of FDA and 2.5 µg/mL of PI (Zárate and Perez Chaia, 2009). The samples were incubated on ice in the darkness for 10 min and washed again. Cells with green cytoplasm and normal nuclei (viable) and cells with bright red nuclei (necrotic cells) were counted on ten microscopic fields by fluorescence microscopy with the appropriated filters. The mean value of dead cells on each of two equally prepared suspensions was reported as percentage of the total cells. Three independent assays were performed with the same procedure and the mean value of dead cells (%) vs. WGA concentration (C in µg/mL) was represented with data of 1

and 2 hours of incubation. The maximum death ($\%_{max}$) and the WGA concentration responsible for half of this value (LD_{50}) were determined for each condition using the mathematical expression of a model adapted for toxic chemicals in experimental bioassays (Sánchez-Bayo and Goka, 2007). The mathematical expression to represent the toxicity model was:

$$Death\ (\%) = \frac{Maximun\ death\ (\%_{max}) \times C}{LD_{50} + C}$$

Carbohydrates of the bacterial surfaces

Surface carbohydrates of the strains were assessed using FITC-labeled lectins (Sigma-Aldrich, Buenos-Aires, Argentina) with different carbohydrates specificity (Table). Active cultures of the fourteen strains were adjusted to Absorbance (A_{600nm}) of 0.7, washed three times with a lectin buffer (60.57 g/L Tris, 87 g/L NaCl, 1.11 g/L $CaCl_2$, pH 7.60) described by Leathem and Brooks (1997), suspended in equal volume of buffer containing 20 µg/mL of one of the five FITC-labeled lectin and incubated 1 h at 25 °C (Wanchoo *et al.*, 2009). The same procedure was carried out for each lectin and strain. Cells suspensions were centrifuged (10000 × g, 10 min, 4 °C) at the end of incubation. Harvested cells were washed 4 times, suspended in equal volume of lectin buffer and observed on a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with the appropriated filter at 100 × magnification. The mean number of fluorescent cells was assessed by counting them in 10 microscopic fields and the number of fluorescent bacteria/mL was determined as in Lorenzo-Pisarello *et al.* (2010). The fluorescence intensity of each cells suspension was also measured with a fluorospectrophotometer (Cary Eclipse, Varian Inc., Walnut Creek,

California, USA) and reported as arbitrary units (AU) / bacteria. Each trial was reproduced three times with new active cultures of each strain.

Table somewhere near here

Exopolysaccharide production

The production of exopolysaccharides by the strains was assessed according to Mozzi *et al.* (2001). Briefly, 2.5 µl of India ink were spotted onto microscopic slides and 10 µl of active cultures ($O.D._{560}$ 0.70) were added, the mixtures were covered with coverslips and the excess of liquid was eliminated by gently pressing on them with absorbent paper. Finally, the samples were observed in a light microscope (Zeiss–Axiolab; Carl Zeiss, Jena, Germany). Bright areas surrounding bacteria indicated the production of exopolysaccharide.

Protection of epithelial cells by bacteria

Suspensions of 1×10^8 bacteria/mL of adherent and non-adherent strains with ability to bind WGA were incubated with 50 µg/mL of the lectin in RPMI/FBS for 1 h at 41.5 ± 0.5 °C. Mixtures were centrifuged (10000 × g, 10 min, 4 °C) and the supernatants stored at 4 °C for further use. Freshly prepared IEC were centrifuged, suspended to reach 5×10^5 cells/ mL in the stored supernatants or in RPMI/FBS containing 50 µg/mL of WGA, and incubated during 2 h at 41.5 ± 0.5 °C under 5% CO_2 atmosphere (Nuaire Co., MN, USA). IEC suspended in RPMI/FBS without lectin incubated as described were used as control of spontaneous death. Finally, cells were stained with 7.5 µg/mL of FDA and 2.5 µg/mL of PI for 10 min on ice in a dark room and viable and necrotic cells were counted and reported as already described.

In other trials, suspensions of both adherent bacteria (1×10^8 bacteria/mL) and IEC (5×10^5 cells/mL) were prepared and incubated 30 min at 41.5 ± 0.5 °C under 5%

CO_2 atmosphere to allow adhesion. Then, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS containing 50 µg/mL of WGA. IEC suspended in RPMI/FBS with 50 µg/mL of WGA were used as control of WGA toxicity; IEC suspended in RPMI/FBS were used as control of spontaneous death; IEC incubated with 1 × 10^8 bacteria/mL and then suspended in RPMI/FBS without lectin were used as control of damage produced by the bacterial adhesion. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO_2 atmosphere, cells were stained with FDA/PI and viable and necrotic IEC were counted and reported as already described.

Effect of WGA on the bacterial adhesion to IEC

Intestinal epithelial cells were obtained as above described and incubated with bacterial suspensions of 1 × 10^8 CFU/mL for 1 h at 41.5 ± 0.5 °C under 5% CO_2 atmosphere. After incubation, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS containing 50 µg/mL of WGA. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO_2 atmosphere, cells adhesion to IEC was examined by counting adhered bacteria in 30 IEC, using phase-contrast microscopy. Results were expressed as the percentage of IEC with adhered bacteria (adhesion percentage).

Statistical analysis

Three independent assays were performed for each *in vitro* experiment and the mean values ± SD were obtained for each sample. Significant differences were determined by Tukey´s test after analysis of variance (ANOVA) with OriginPro 8 SR0 v8.0724

(OriginLab Corporation, Northampton, MA, USA). A value of $P < 0.5$ was considered statistically significant.

RESULTS

WGA cytotoxicity

As it is shown in Figure 1, WGA exhibited toxic effect on IEC obtained from chicken intestine. Cells incubated with the lectin at the body temperature of the animals induced cells death in different extent depending on the incubation time and lectin concentration. Increases in the WGA concentration from 0 to 200 µg/mL induced a progressive and negative effect on viability in 1 h incubations. In contrast, the loss of viability was notable with minor variations of the lectin concentration from 0 to 25 µg/mL when the incubation was extended to 2 h. The plot of cytotoxicity, percentage of dead cells vs lectin concentration, showed hyperbolic design with tendency to a maximum effect at high lectin concentrations. Double-reciprocal plots were used to determine the maximum percentages of dead cells ($\%_{max}$) and the lectin concentration that induce the death of half of the cells in the suspension (lethal dose 50, LD_{50}) at each incubation time (Purich and Allison, 2000). Results for LD_{50} were 56.7 and 8.4 µg/mL for cells incubated during 1 and 2 hours, respectively. Maximum death percentages produced by the lectin were 90.6 % and 90.2 %, respectively.

Figure 1 somewhere near here

Characterization of surface carbohydrates and WGA removal

The characterization of surface carbohydrates of the strains was carried out through the use of FITC-labeled lectins. The binding of lectins to the cells surface was confirmed by fluorescence microscopy and the relative abundance of lectin-linked carbohydrates was

registered by fluorospectrophotometry as arbitrary units of fluorescence (AU). Figure 2A represents the AU obtained when *Arachis hypogaea* agglutinin (PNA), *Dolichos biflorus* agglutinin (DBA) or *Ulex europaeus* agglutinin (UEA-I) were incubated with the studied strains; Figure 2B shows the results of incubation with *Phaseolus vulgaris* agglutinin (PHA-P) or WGA. The only strain tested of *L. salivarius* ligated significant amount of PNA and WGA; one of the strains of *L. vaginalis* ligated PNA and UEA-I while *E. faecium* only captured WGA. Nine strains of *L. reuteri* removed WGA, three of them captured also PNA and two strains removed PHA-P. The lectins DBA and UEA-I linked to only one of the strains of *L. reuteri* each. The fluorescence intensity measured evidenced that WGA was captured by lactobacilli and *E. faecium* LET 301 in significantly higher amount than the other lectins tested.

Figure 2 somewhere near here

Exopolysaccharide production

Only *L. reuteri* LET 206, *L. reuteri* LET 209 and *E. faecium* LET 301 out of 14 strains produced exopolysaccharide, as evidenced by the observation of bright areas surrounding bacteria due to colloidal carbon exclusion by exopolysaccharide (data not shown).

Protection of cells by adherent and non-adherent bacteria

Lectin removal by lactic acid bacteria was evaluated as a tool to protect epithelial cells facing cytotoxic lectins. Taking into account that the ability to remove WGA was observed in adherent and non-adherent strains, the protective effect of bacteria on WGA cytotoxicity was evaluated following different protocols depending on the strains studied. Non-adherent bacteria were studied simulating the conditions of free bacteria in the intestinal lumen (Fig. 3). Dead cells due to the toxic effect of WGA were

determined and represented as percentages of total cells in the suspensions. These values were compared with the percentage obtained in samples of IEC incubated with supernatants of lectin-bacteria interaction for each strain under study. As expected, cell death was significantly reduced when IEC were exposed to supernatants of the lectin-bacteria interactions. A remarkable protective effect was observed for *L. salivarius* LET 201, which reduced cell death in almost 54 % while the lowest effect was that of *L. reuteri* LET 209 with a death reduction of almost 10 %. An unexpected result was observed for *L. reuteri* LET 208, which reduced cells death in almost 42 %, as the previously assessed amount of lectin removed by this strain was lower than that for *L. reuteri* LET 209 (Fig. 2). The effective amount of lectin in supernatants of WGA-bacteria interactions and in the WGA solutions used in each assay were determined from the percentages of cellular death measured in the cytotoxicity assays using the $\%_{max}$ and LD_{50} values previously obtained (subsection 3.1). The lectin removed by each strain was assessed as the difference between these values. The suspension of 1×10^8 cells of *L. salivarius* LET 201 removed 46.9 ± 0.4 µg of WGA, while *L. reuteri* LET 209 removed 26.0 ± 0.6 µg of WGA in the conditions used in the trial. Figure 3 somewhere near here

The effect of lectin capture by adherent bacteria was first assessed in a protocol that simulates the conditions of free bacteria in the intestinal lumen (Fig. 3). *L. reuteri* LET 210 exerted the highest protection lowering cell death approximately 45 %. The less efficient strain was *L. reuteri* LET 204 which reduced in almost 22 % the percentage of dead cells. The lectin removal assessed by the percentage of dead cells was 45.6 ± 0.3 µg of lectin for *L. reuteri* LET 210 and 37.2 ± 0.5 µg of lectin for the strain LET 204.

The same strains were assayed for the ability to protect the epithelial cells of lectin toxicity during the bacteria-IEC interaction. As observed in Figure 4, strain *L.*

reuteri LET 210 exerted scarce protection of the cells when it was in intimate relation to them; cell death was reduced in approximately 5 % and represented the removal of 15.2 ± 2.5 µg of lectin. In the contrary, *L. reuteri* LET 204 reduced in almost 17 % the cells death by removal of 33.2 ± 0.3 µg of lectin. Moderate protection was achieved with *L. reuteri* LET 211, *L. reuteri* LET 207 and *E. faecium* LET 301. *L. reuteri* LET 206 was the only strain that increased slightly the cell death when IEC with adhered bacteria were exposed to WGA. The damage was also observed during the bacteria-IEC interaction without exposure to WGA (data not shown). Figure 4 somewhere near here

As shown in Figures 3 and 4, comparisons of the protective effect of the strains were performed for each protocol used. The statistical analysis of the reduction of cell death obtained by each strain in the protocol that simulates free bacteria in the intestinal lumen, demonstrated that the highest protection was exerted by *L. salivarius* LET 201, a non-adherent strain, followed by *L. reuteri* LET 210, an adherent one (Fig. 3). The statistical analysis of results of the protection achieved by the strains adhered to IEC indicated that *L. reuteri* LET 204 was the most efficient one.

Effect of WGA on the bacterial adhesion to IEC

The incubation with WGA induced the detachment of bacteria adhered to IEC, as it is shown in Figure 5. The phenomenon was not attributed to effects of the incubation time but to competition of WGA for binding sites of bacteria on IEC as *L. reuteri* LET 205, an adherent bacterium without ability to bind WGA, was not detached.

Figure 5 somewhere near here

DISCUSSION

The binding of WGA to enterocytes of adult chickens and enterocytes from the villi of jejuni and ileum of young chicks was previously reported (Pohlmeyer, Jorns,

Schumacher, Van Damme, Peumans, Pfuller and Neumann, 2005, Zhou, Deng and Ding, 1995). However, the effects of WGA on cell viability have not been previously analyzed in poultry.

In this study, WGA showed cytotoxicity towards IEC of fourteen-day-old chicks, which was dependent on the time of exposition and concentration used. The percentage of cell death caused by the incubation of WGA with IEC was used to establish the values of LD_{50} for 1 and 2 h of exposure (56.7 and 8.4 µg/mL, respectively). These were comparable to the reported on other cells types. Indeed, the LD_{50} for WGA on the human leukemic cell lines Molt4, K562 and PBMC was 5.0 µg/mL for the first and higher than 72.0 for the last two, after 24 h of incubation (Ohba, Bakalova and Muraki, 2003).

The binding of lectins by Gram (+) bacteria was previously demonstrated. Zárate and Perez Chaia (2009) reported the capture of *Canavalia ensiformis* agglutinin (Con A), *Arachis hypogaea* agglutinin (PNA) and *Artocarpus intergrifolia* lectin (AIL) by several strains of *Propionibacterium* and *Bifidobacterium longum*. Moreover, Babot et al. (2014) demonstrated the binding of Con A by lactic acid bacteria isolated from chicken intestinal content. In the present study, the capture of several lectins by enterococcus and lactobacilli strains of poultry origin was shown. The affinity of lectins for carbohydrates expressed on the surface of lactobacilli and the *Enterococcus* strain evidenced the expression of diverse molecules according to the bacterial species studied, with some differences depending on strains. The results for *L. salivarius* LET 201 suggested the presence of GlcNAc-β-1,4-GlcNAc, NeuNAc and α-L-Fucose as surface carbohydrates. Only one strain of *L. vaginalis* expressed Gal-β-1,3-GalNac and minor amount of α-L-Fucose. With exception of the strains LET 205 and LET 213, the other strains of *L. reuteri* showed significant fluorescence when WGA was used,

indicating the presence of GlcNAc-β-1,4-GlcNAc and/or NeuNAc as surface carbohydrates. Minor amount of Gal-β-1,3-GalNac was also detected in the strains LET 209, LET 211 and LET 213. Among the five lectins assayed, only WGA interacted with *E. faecium* LET 301, evidencing the existence of GlcNAc-β-1,4-GlcNAc and/or NeuNAc on its surface.

The production of EPS, free or capsular, as well as its quantity and composition is strain-dependent, as reported by Raftis *et al.* (2011), who studied this capability on 33 strains of *L. salivarius* from different origin. Nevertheless, there was not production of EPS by *L. salivarius* LET 201. Several authors reported the production of glucose or fructose homopolysaccharides by strains of *L. reuteri*, *L. pontis*, *L. panis*, *L. acidophilus* and *L. frumenti* (Tieking *et al.*, 2003a, Tieking *et al.*, 2003b, Wang *et al.*, 2010). Despite the binding of Con A to *L. reuteri* LET 205 revealed the expression of glucose or mannose residues on the bacterial surface (Babot et al., 2014), no EPS production was observed for this strain in the present study. Only two of the studied strains, *L. reuteri* LET 206 and *L. reuteri* LET 209, showed the properties of EPS production and WGA binding. However, they captured lower amount of WGA than other non-EPS-producing strains of the same species. Therefore, the production of EPS was not a relevant property for WGA binding by the strains studied.

On the other hand, the capture of different amount of lectins by strains of a same species would indicate a distinctive expression of carbohydrates on their surface. This agrees with the results reported by Baintner *et al.* (1993), who studied the binding of 15 lectins by microorganisms isolated from sheep rumen and found lectin capture by most cultures, but differences on the quantity of lectin bound to the surface of strains of the same species. In our study, the strains binding WGA evidenced higher fluorescence intensity than after capture of other lectins. Moreover, all the strains of *L. reuteri* were

able to bind WGA but the amount of lectin attached to each strain was significantly different.

Adherent and non-adherent strains of lactic acid bacteria could avoid the interaction eukaryotic cells-WGA by different mechanisms depending on the location of these bacteria within the intestine. The protection against cytotoxicity was assayed considering that the capture of WGA by non-adherent bacteria could take place in the intestinal lumen, thus reducing the amount of free lectin able to interact with IEC. The protection mediated by adherent bacteria was assayed allowing the adhesion of bacteria to IEC prior to the addition of WGA to interfere in the interaction between the lectin and eukaryotic cells. Results showed that binding of WGA depended on the strain assayed and the conditions used to study this property. Among non-adherent bacteria, *L. salivarius* LET 201 exerted the highest protection. *L. reuteri* LET 208 was more efficient for the binding of lectin in RPMI medium at 41.5 ± 0.5 °C than in lectin buffer at 25 °C when compared with the strain *L. reuteri* LET 209, suggesting that environmental factors could be involved in the interaction of WGA with some strains. On the other hand, the adherent strain *L. reuteri* LET 210 was the less efficient when IEC, bacteria and WGA were incubated together in the same medium, simulating the environment near to the intestinal epithelium. This finding suggested that despite the binding of bacteria to IEC surfaces, in some cases the determinants involved in the interaction IEC-WGA remain exposed in the cellular surface. The failure in blocking the access to these determinants due to the lower size of bacteria related to eukaryotic cells or the binding of bacteria to IEC by surface molecules other than WGA receptors could be the reasons of these results. Conversely, many adherent strains succeeded in the protection of IEC against WGA cytotoxicity through the protocol that simulates de capture and removal of WGA in the intestinal lumen. Indeed, *L. reuteri* LET 210 was

able to attract higher amount of lectin than others strains of the same species and was more efficient in protecting IEC. The suspension of 1×10^8 cells of LET 210 removed almost all the WGA added when it was incubated with the lectin during 1 h in RPMI medium prior to the cytotoxicity assay, simulating the conditions in the intestinal lumen. Besides, the percentage of enterocytes with at least one bound bacterium decreased significantly after incubation with WGA for almost all of the adherent strains, assayed in the same conditions. These detached bacteria would still be able to bind the lectin in the intestinal lumen and be eliminated along with the WGA adsorbed to their surface during the normal transit of the intestinal content.

CONCLUSIONS

WGA showed cytotoxicity in broiler enterocytes in a range of concentrations that may be found in the poultry digesta. Several lactic acid bacteria isolated from poultry intestine captured the lectin in *in vitro* assays. *L. salivarius* LET 201 and *L. reuteri* LET 210 were able to remove WGA more efficiently and exerted higher protective effect than the other strains assayed. This finding provides bases for the development of probiotic supplements or cell-wall preparations of these selected strains intended to avoid harmful effects of a natural constituent of the grain in wheat-based diets.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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FITC-labeled dietary lectins

Lectin	Source	Specificity
Con A	*Canavalia ensiformis* (jack bean)	α−D-mannose, α−D-glucose
DBA	*Dolichos biflorus* (horse gram)	GalNAc-α-1,3-GalNAc
PHA-P	*Phaseolus vulgaris* (kidney bean)	Gal-β-1,4-GalNAc-β-1,2-Man
PNA	*Arachis hypogaea* (peanut)	Gal-β-1,3-GalNac
UEA-I	*Ulex europaeus* (furze)	α-L-Fucose
WGA	*Wheat germ agglutinin* (wheat)	GlcNAc-β-1,4-GlcNAc, NeuNAc

Figure captions

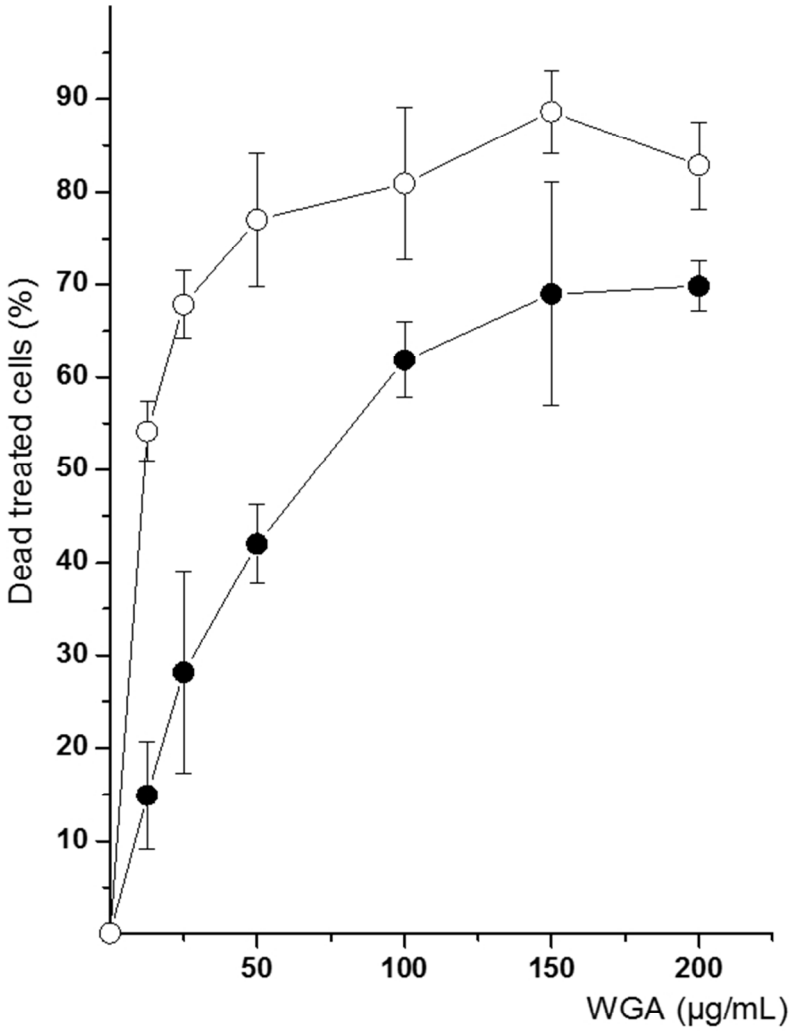
Figure 1. WGA cytotoxicity on IEC of fourteen-day-old broiler chickens after 1 (-●-) and 2 h (-○-) of exposure. Results are expressed as mean values of three independent assays ± standard deviations (SD).

Figure 2. Relative abundance of lectin-linked carbohydrates on the surface of bacteria. (A) Binding of PNA (■), DBA (▩) and UEA-I (▨). (B) Binding of PHA-P (□) and WGA (▨) by the strains *L. salivarius* LET201, *L. vaginalis* LET 202 and 203, *L. reuteri* LET 204, LET 205, LET 206, LET 207, LET 208, LET 209, LET 210, LET 211, LET 212 and LET 213; *E. faecium* LET 301. Results are expressed as mean values of three independent assays ± SD for each strain and lectin used.

Figure 3. Protection of WGA cytotoxicity by removal of lectin by strains of lactobacilli and *Enterococcus*. Percentage of dead cells after IEC incubation with WGA (▩) and supernatant of bacteria-WGA interaction (▩), and the reduction in cell death (□) due to the lectin removal are represented as mean values of three independent assays ± SD. Values of reduction in cell death with no common superscript letter differ significantly at $P < 0.5$.

Figure 4. Protection of WGA cytotoxicity by attachment of lactobacilli and *Enterococcus* to IEC. Percentage of dead cells after IEC incubation with WGA (▩), after incubation of bacteria-bound IEC with WGA (▩), and the reduction in cell death (□) are represented as mean values of three independent assays ± SD. Values of reduction in cell death with no common superscript letter differ significantly at $P < 0.5$.

Figure 5. Influence of WGA on the adhesion of bacteria to IEC. Results are represented as mean values of three independent assays ± SD. An asterisk indicates significant differences ($p < 0.5$) between adhesion (%) before (■) and after (□) incubation with the lectin.



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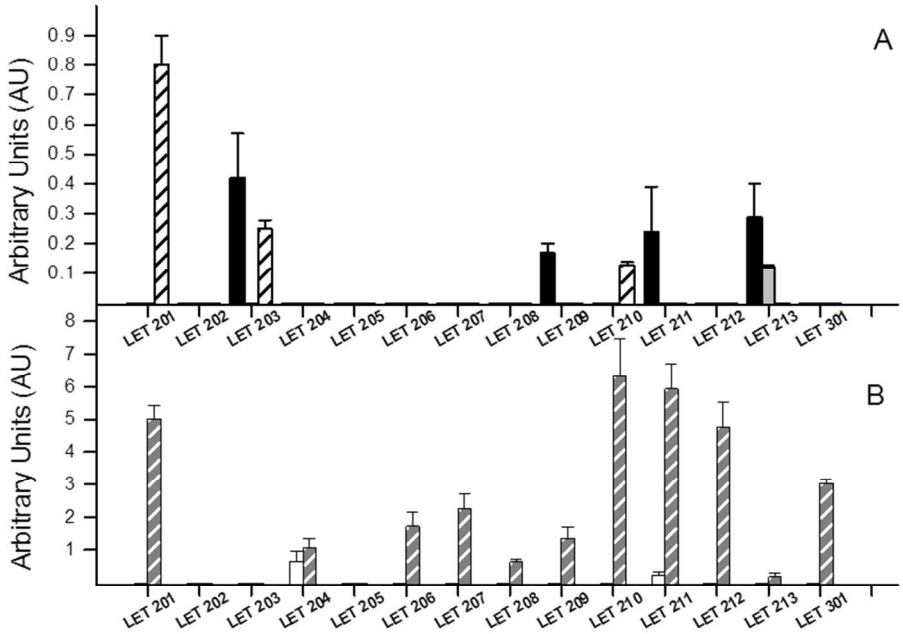

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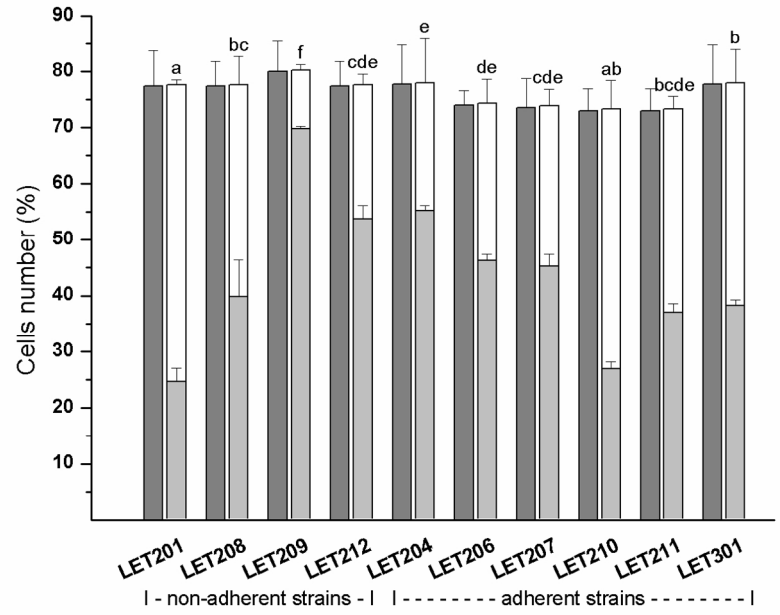

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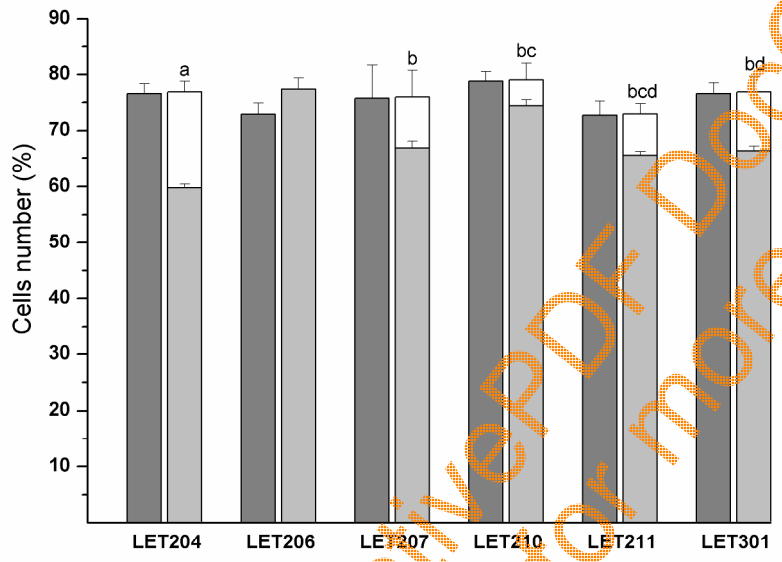

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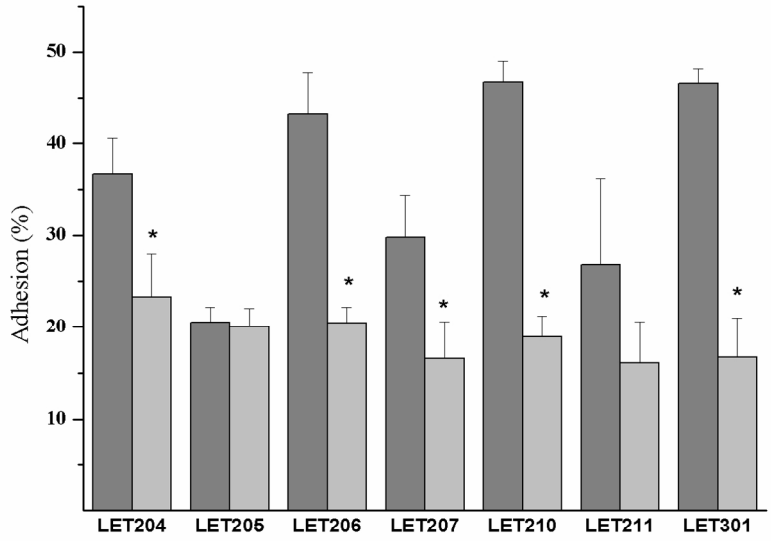

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Lactic acid bacteria isolated from poultry protect intestinal epithelial cells of chicks from *in vitro* wheat germ agglutinin ~~(WGA)~~ induced cytotoxicity

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Running title: Wheat lectin toxicity protection

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Abstract. 1. ~~The wheat as~~ Whole grain or milled ~~or whole grain~~ wheat is often used instead of corn in poultry feeding, due to its high content of starch and proteins. Poultry fed with wheat-based diets ~~ingest~~ regularly ingest wheat germ agglutinin (WGA).

2. ~~Considering previous reports on the toxicity of WGA against several cell line types, its toxicity to~~ WGA showed toxic effect *in vitro* on intestinal epithelial cells (IEC) ~~of poultry was studied in enterocytes~~ obtained from fourteen-day-old broilers. ~~WGA showed cytotoxic effect on IEC that~~Cytotoxicity was dependent on time and lectin concentration; lethal dose (LD_{50}) was 8.36 µg/mL for IEC exposed ~~during~~for 2 h to WGA.

~~3. A screening of lactic acid bacteria that express the complementary sugars to WGA on their cell surfaces was performed and strains were evaluated as a tool to remove the lectin avoiding the interaction with IEC.~~3. Complementary sugars to WGA were detected ~~in~~ on the surface of one *Enterococcus* and nine *Lactobacillus* strains ~~of different species and one *Enterococcus* strain, all of them~~ isolated from poultry~~, with and without the ability to adhere to tissues~~. These strains were evaluated as a lectin removal tool intended to cytotoxicity prevention.

4. ~~The~~ The incubation of the lactic acid bacteria with WGA before the IEC-lectin interaction caused a remarkable reduction in the percentage of cell death ~~caused by WGA was reduced by incubation of these strains with the lectin prior the IEC-WGA interaction or after bacteria-IEC adhesion. The extension of the protective effect against the damage caused by WGA depended on the strain studied and~~. The protection was attributed to the amount of lectin bound to the bacterial ~~surfaces~~surfaces and was strain-dependent. *L. salivarius* LET 201 and *L. reuteri* LET 210 were significantly more efficient than the other lactic acid bacteria assayed.

5. These results provide bases for the development of probiotic supplements or cell-wall preparations of selected lactic acid bacteria intended to avoid harmful effects of a natural constituent of the grain in wheat-based diets.

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INTRODUCTION

Wheat is commonly used as a source of metabolizable energy for poultry (Adebiyi and Olukosi, 2015), mainly in western Canada and parts of Europe but worldwide when corn is scarce. Although the nutrients content of wheat allow the partial or total replacement of corn in the poultry diet, some components of this grain like non-starch polysaccharides have anti-nutritional effects that may be prevented by addition of hydrolytic enzymes to the diet, like xylanase (Kiarie *et al.*, 2014) and *β*-glucanase (Rodríguez *et al.*, 2012). Other component of interest is the WGA lectin (wheat germ agglutinin), whose effects in poultry production have not been still studied.

Plant lectins are proteins or glycoproteins of non immune origin possessing at least one non catalytic domain, which binds reversibly to specific mono- or oligosaccharide through hydrogen bonds and van der Waals interactions (Lis and Sharon, 1998, Peumans and Van Damme, 1995). WGA is a rather small and heat-resistant protein with four binding sites specific for N-acetyl-D-glucosamine and N-

acetyl-D-neuraminic acid residues (Rudiger and Gabius, 2001, Van Damme *et al.*, 1998). These complementary sugars are ubiquitously expressed by animal cells as they constitute key molecular components of the membrane glycoconjugates. WGA binds to glycosaminoglycans, glycolipids and also some glycoproteins via sialic acid residues (Peters et al., 1979). WGA is relatively stable at low pH and resistant to proteolysis (Van Damme, Peumans, Pusztai and Bardocz, 1998) and thus it can overcome the conditions of gastrointestinal tract and reach active the intestine (Jones *et al.*, 2012). WGA has been proved toxic for Molt4, K562 and PBMC human leukemic cell lines (Ohba *et al.*, 2003), and for Caco2 cells (Dalla Pellegrina *et al.*, 2005), among others. The binding of WGA to enterocytes from the villi of proximal jejune-ileum of chicks 2, 15 and 30 days post hatching (Pohlmeyer *et al.*, 2005), to enterocytes from the ileum of chicks 2 days post hatching (Zhou *et al.*, 1995), and to enterocytes from the villi of caecal tonsils of adult chickens (Kitagawa *et al.*, 2000) has been previously reported. The interaction lectin-epithelium may alter the activity of digestive enzymes located on the epithelial cells (Rueda *et al.*, 2007), and the integrity and permeability of the intestinal epithelium allowing small molecules to cross the epithelial barrier contributing to the toxicity of WGA to gastrointestinal cells (Dalla Pellegrina, Rizzi, Mosconi, Zoccatelli, Peruffo and Chignola, 2005). In poultry, this may depress the growth of broilers especially during the first days of life.

Poultry industry has explored the benefit of including probiotic cultures in broilers feed as alternative to antibiotic treatments for growth promotion. Probiotics may contribute to xenobiotics metabolism or to avoid deleterious effects of toxic compounds included in the diet by binding them on the bacterial envelopes that express appropriated determinants (Carasi *et al.*, 2012, Turbic *et al.*, 2002, Zárate and Perez Chaia, 2012). The binding of WGA to carbohydrates expressed on the bacterial surface

and its removal by the normal transit of the intestinal content may contribute to a healthier broiler feed.

In the present investigation we explore the toxicity of WGA on intestinal epithelial cells of broilers and the ability of lactic acid bacteria isolated from poultry intestine to remove WGA and protect cells from the harmful effects of this lectin.

MATERIALS AND METHODS

Microorganisms and culture conditions

Thirteen strains of lactobacilli and one enterococcus isolated from poultry intestine were used in the study. Suitable properties for probiotic strains selection were previously determined, such as the growth at the corporal temperature of poultry, tolerance to pH, bile salts and digestive enzymes, adhesion to small bowel mucosa, antibiotics sensitivity and absence of virulence factors (Babot *et al.*, 2014). The strains were grouped as non-adherent (*Lactobacillus salivarius* LET 201, *L. vaginalis* LET 202, *L. vaginalis* LET 203, *L. reuteri* LET 208, *L. reuteri* LET 209, *L. reuteri* LET 212 and *L. reuteri* LET 2013) and adherent (*L. reuteri* LET 204, *L. reuteri* LET 205, *L. reuteri* LET 206, *L. reuteri* LET 207, *L. reuteri* LET 210, *L. reuteri* LET 211 and *Enterococcus faecium* LET 301) bacteria.

All strains were stored at -70 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract and 15% glycerol. MRS broth (de Man *et al.*, 1960) and LAPTg broth (Raibaud *et al.*, 1973) were used to activate cultures of lactobacilli and enterococci, respectively. Prior to use, cultures were incubated at 41.5 ± 0.5 °C for 12 h.

Intestinal epithelial cells extraction

For extraction of intestinal epithelial cells (IEC), fourteen-day-old broiler chicks were slaughtered by cervical dislocation. The birds were immediately eviscerated for collection of ileum which was in turn rinsed repeatedly with ice cold PBS pH 7.40 to eliminate the digested content. The tissue ~~were~~was cut lengthwise and washed once again with cold PBS. Epithelial cells were scrapped from the surface using a sterile microscope slide and collected in ice cold PBS pH 7.40 supplemented with 1% Fetal Bovine Serum (PBS/FBS). The cells were washed twice with PBS/FBS, incubated with 0.25% Trypsin-EDTA (Gibco, Grand Island, USA) at 37 °C for 5 min before cold PBS/FBS was added to stop the enzyme activity. The cells were immediately collected (800 × g, 5 min, 4 °C) and washed once with PBS/FBS prior to adjust their concentration to 1×10^6 cells/mL in RPMI 1640 medium supplemented with 1% FBS (RPMI/FBS). Cells were counted using a Neubauer chamber in a conventional light microscope at 40 × magnification (Zeiss–Axiolab; Cool Zeiss, Jena, Germany).

The animal handling protocols of this investigation were adjusted to the *Ethical Framework of Reference for Biomedical Research in Laboratory Animals, from Farm and Obtained from Nature*, contained in the Resolution N°. 1047/05, Annex II, of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) - Argentina. Experimental procedures for IEC extraction were approved by The Committee of Ethics for Animal Studies of CERELA (CCT Tucumán - CONICET).

WGA cytotoxicity

For cytotoxicity assessment, equal volumes of ~~suspension~~suspensions of IEC and WGA in RPMI/FBS were mixed and incubated at 41.5 ± 0.5 °C. Final concentrations in the mixtures were 5×10^5 cells/mL and 0~~–~~, 12.5, 25, 50, 100, 150 or 200 µg/mL of WGA.

After 1 and 2 h of incubation, 100 µL of cells suspensions were washed with RPMI/FBS and suspended in the same volume of medium. A freshly prepared mixture of Fluorescein diacetate (FDA; Sigma-Aldrich, Argentina) and Propidium iodide (PI) was added to obtain final concentrations of 7.5 µg/mL of FDA and 2.5 µg/mL of PI (Zárate and Perez Chaia, 2009). The samples were incubated on ice in the darkness for 10 min and washed again. Cells with green cytoplasm and normal nuclei (viable) and cells with bright red nuclei (necrotic cells) were counted on ten microscopic fields by fluorescence microscopy with the appropriated filters ~~and the percentage~~. The mean value of dead cells on each of two equally prepared suspensions was reported ~~. Dead~~ as percentage of the total cells. Three independent assays were performed with the same procedure and the mean value of dead cells (%) vs. WGA concentration (C in µg/mL) was represented with data of 1 and 2 hours of incubation ~~; the maximun~~. The maximum death ($\%_{max}$) and the WGA concentration responsible for half of this value (LD_{50}) were determined for each condition using the mathematical expression of a model adapted for toxic chemicals in experimental bioassays (Sánchez-Bayo and Goka, 2007). The mathematical expression to represent the toxicity model was:

$$Death\ (\%) = \frac{Maximun\ death\ (\%_{max}) \times C}{LD_{50} + C}$$

Carbohydrates of the bacterial surfaces

Surface carbohydrates of the strains were assessed using FITC-labeled lectins (Sigma-Aldrich, Buenos-Aires, Argentina) with different carbohydrates specificity (Table). Active cultures of the fourteen strains were adjusted to Absorbance (A_{600nm}) of 0.7, washed three times with a lectin buffer (60.57 g/L Tris, 87 g/L NaCl, 1.11 g/L $CaCl_2$, pH 7.60) described by Leathem and Brooks (1997), suspended in equal volume of

buffer containing 20 µg/mL of one of the five FITC-labeled lectin and incubated 1 h at 25 °C (Wanchoo *et al.*, 2009). The same procedure was carried out for each lectin and strain. Cells suspensions were centrifuged (10000 × g, 10 min, 4 °C) at the end of incubation. Harvested cells were washed 4 times, suspended in equal volume of lectin buffer and observed on a conventional fluorescence microscope (Carl Zeiss Axio Scope A1, Gottingen, Germany) fitted with the appropriated filter at 100 × magnification. The mean number of fluorescent cells was assessed by counting them in 10 microscopic fields and the number of fluorescent bacteria/mL was determined as ~~(Lorenzo-Pisarello *et al.*, 2010).~~in Lorenzo-Pisarello *et al.* (2010). The fluorescence intensity of each cells suspension was also measured with a fluorospectrophotometer (Cary Eclipse, Varian Inc., Walnut Creek, California, USA) and reported as arbitrary units (AU) / bacteria. Each trial was reproduced three times with new active cultures of each strain.

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Exopolysaccharide production

The production of exopolysaccharides by the strains was assessed according to Mozzi *et al.* (2001). Briefly, 2.5 µl of India ink were spotted onto microscopic slides and 10 µl of active cultures ($O.D._{560}$ 0.70) were added, the mixtures were covered with coverslips and the excess of liquid was eliminated by gently pressing on them with absorbent paper. Finally, the samples were observed in a light microscope (Zeiss–Axiolab; ~~Cool~~Carl Zeiss, Jena, Germany). Bright areas surrounding bacteria indicated the production of exopolysaccharide.

Protection of epithelial cells by bacteria

Suspensions of 1×10^8 bacteria/mL of adherent and non-adherent strains with ability to bind WGA were incubated with 50 µg/mL of the lectin in RPMI/FBS for 1 h at 41.5 ±

0.5 °C. Mixtures were centrifuged (10000 × g, 10 min, 4 °C) and the supernatants stored at 4 °C for further use. Freshly prepared IEC were centrifuged, suspended to reach 5 × 10^5 cells/ mL in the stored supernatants or in RPMI/FBS containing 50 µg/mL of WGA, and incubated during 2 h at 41.5 ± 0.5 °C under 5% CO_2 atmosphere~~.~~ (Nuaire Co., MN, USA). IEC suspended in RPMI/FBS without lectin incubated as described were used as control of spontaneous death. Finally, cells were stained with 7.5 µg/mL of FDA and 2.5 µg/mL of PI for 10 min on ice in a dark room and viable and necrotic cells were counted and reported as already described.

In other trials, suspensions of both adherent bacteria (1 × 10^8 bacteria/mL) and IEC (5 × 10^5 cells/mL) were prepared and incubated 30 min at 41.5 ± 0.5 °C under 5% CO_2 atmosphere to allow adhesion. Then, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS containing 50 µg/mL of WGA. IEC suspended in RPMI/FBS with 50 µg/mL of WGA were used as control of WGA toxicity; IEC suspended in RPMI/FBS were used as control of spontaneous death; IEC incubated with 1 × 10^8 bacteria/mL and then suspended in RPMI/FBS without lectin were used as control of damage produced by the bacterial adhesion. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO_2 atmosphere, cells were stained with FDA/PI and viable and necrotic IEC were counted and reported as already described.

Effect of WGA on the bacterial adhesion to IEC

Intestinal epithelial cells were obtained as above described and incubated with bacterial suspensions of 1 × 10^8 CFU/mL for 1 h at 41.5 ± 0.5 °C ~~in a mixture of~~under 5~~-~~% CO_2 ~~and 95 % O_2-~~atmosphere. After incubation, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS

containing 50 µg/mL of WGA. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO_2 atmosphere, cells adhesion to IEC was examined by counting adhered bacteria in 30 IEC, using phase-contrast microscopy. Results were expressed as the percentage of IEC with adhered bacteria (adhesion percentage).

Statistical analysis

Three independent assays were performed for each *in vitro* experiment and the mean values ± SD were obtained for each sample. Significant differences were determined by Tukey´s test after analysis of variance (ANOVA) with ~~Minitab Statistic Program, release 12 for Windows.~~OriginPro 8 SR0 v8.0724 (OriginLab Corporation, Northampton, MA, USA). A value of $P < 0.~~05~~5$ was considered statistically significant.

RESULTS

WGA cytotoxicity

As it is shown in Figure 1, WGA exhibited toxic effect on IEC obtained from chicken intestine. Cells incubated with the lectin at the body temperature of the animals induced cells death in different extent depending on the incubation time and lectin concentration. Increases in the WGA concentration from 0 to 200 µg/mL induced a progressive and negative effect on viability in 1 h incubations. In contrast, the loss of viability was notable with minor variations of the lectin concentration from 0 to 25 µg/mL when the incubation was extended to 2 h. The plot of cytotoxicity, percentage of dead cells vs lectin concentration, showed hyperbolic design with tendency to a maximum effect at high lectin concentrations. Double-reciprocal plots were used to determine the maximum percentages of dead cells ($\%_{max}$) and the lectin ~~concentrations~~concentration

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~~Figure 1 somewhere near here~~

that induce the death of half of the cells in the suspension (lethal dose 50, LD_{50}) at each incubation time (Purich and Allison, 2000). Results for LD_{50} were 56.7 and 8.4 µg/mL for cells incubated during 1 and 2 hours, respectively. Maximum death percentages produced by the lectin were 90.6 % and 90.2 %, respectively.

Characterization of surface carbohydrates and WGA removal

The characterization of surface carbohydrates of the strains was carried out through the use of FITC-labeled lectins. The binding of lectins to the cells surface was confirmed by fluorescence microscopy and the relative abundance of lectin-linked carbohydrates was registered by fluorospectrophotometry as arbitrary units of fluorescence (AU). Figure 2A represents the AU obtained when *Arachis hypogaea* agglutinin (PNA), *Dolichos biflorus* agglutinin (DBA) or *Ulex europaeus* agglutinin (UEA-I) were incubated with the studied strains; Figure 2B shows the results of incubation with *Phaseolus vulgaris* agglutinin (PHA-P) or WGA. The only strain tested of *L. salivarius* ligated significant amount of PNA and WGA; one of the strains of *L. vaginalis* ligated PNA and UEA-I while *E. faecium* only captured WGA. Nine strains of *L. reuteri* removed WGA, three of them captured also PNA and two strains removed PHA-P. The lectins DBA and UEA-I linked to only one of the strains of *L. reuteri* each. The fluorescence intensity measured evidenced that WGA was captured by lactobacilli and *E. faecium* LET 301 in significantly higher amount than the other lectins tested.

Figure 2 somewhere near here

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Exopolysaccharide production

Only *L. reuteri* LET 206, *L. reuteri* LET 209 and *E. faecium* LET 301 out of 14 strains produced exopolysaccharide, as evidenced by the observation of bright areas

surrounding bacteria due to colloidal carbon exclusion by exopolysaccharide (data not shown).

Protection of cells by adherent and non-adherent bacteria

Lectin removal by lactic acid bacteria was evaluated as a tool to protect epithelial cells facing cytotoxic lectins. Taking into account that the ability to remove WGA was observed in adherent and non-adherent strains, the protective effect of bacteria on WGA cytotoxicity was evaluated following different protocols depending on the strains studied. Non-adherent bacteria were studied simulating the conditions of free bacteria in the intestinal lumen (Fig. 3). Dead cells due to the toxic effect of WGA were determined and represented as percentages of total cells in the suspensions. These values were compared with the percentage obtained in samples of IEC incubated with supernatants of lectin-bacteria interaction for each strain under study. As expected, cell death was significantly reduced when IEC were exposed to supernatants of the lectin-bacteria interactions. A remarkable protective effect was observed for *L. salivarius* LET 201, which reduced cell death in almost 54 % while the lowest effect was that of *L. reuteri* LET 209 with a death reduction of almost 10 %. An unexpected result was observed for *L. reuteri* LET 208, which reduced cells death in almost 42 %, as the previously assessed amount of lectin removed by this strain was lower than ~~in~~that for *L. reuteri* LET 209 (Fig. 2). The effective ~~amounts~~amount of lectin in supernatants of WGA-bacteria interactions and in the WGA solutions used in each assay were determined from the percentages of cellular death measured in the cytotoxicity assays using the $\%_{max}$ and LD_{50} values previously obtained (subsection 3.1). The lectin removed by each strain was assessed as the difference between these values. –The suspension of 1×10^8 cells of *L. salivarius* LET 201 removed 46.9 ± 0.4 µg of WGA,

while *L. reuteri* LET 209 removed 26.0 ± 0.6 µg of WGA in the conditions used in the trial. ~~where near here~~

The effect of ~~binding the~~ lectin capture by adherent bacteria was first assessed in a protocol that simulates the conditions of free bacteria in the intestinal lumen (Fig. ~~4A~~3). *L. reuteri* LET 210 exerted the highest protection lowering ~~45.5 % of the~~ cell ~~dead~~ death approximately 45 %. The less efficient strain was *L. reuteri* LET 204 which reduced in almost 22 ~~1~~ % the percentage of dead cells. The lectin removal assessed by the percentage of ~~death~~dead cells was 45.6 ± 0.3 µg of lectin for *L. reuteri* LET 210 and 37.2 ± 0.5 µg of lectin for the strain LET 204. ~~Figure 4a somewhere near here~~

The same strains were assayed for the ability to protect the epithelial cells of lectin toxicity during the bacteria-IEC interaction. As observed in Figure ~~4B~~4, strain *L. reuteri* LET 210 exerted scarce protection of the cells when it was in intimate relation to them; cell death was reduced in approximately 5 % and represented the removal of 15.2 ± 2.5 µg of lectin. In the contrary, *L. reuteri* LET 204 reduced in almost 17 % the cells death by removal of 33.2 ± 0.3 µg of lectin. Moderate protection was achieved with *L. reuteri* LET 211, *L. reuteri* LET 207 and *E. faecium* LET 301. *L. reuteri* LET 206 was the only strain that increased slightly the cell death when IEC with adhered bacteria were exposed to WGA. The damage was also observed during the bacteria-IEC interaction without exposure to WGA (data not shown ~~Figure 4b somewhere near here~~

Figure 4 somewhere near here

As shown in Figures 3 and 4, comparisons of the protective effect of the strains were performed for each protocol used. The statistical analysis of the reduction of cell death obtained by each strain in the protocol that simulates free bacteria in the intestinal lumen, demonstrated that the highest protection was exerted by *L. salivarius* LET 201, a non-adherent strain, followed by *L. reuteri* LET 210, an adherent one (Fig. 3). The

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statistical analysis of results of the protection achieved by the strains adhered to IEC indicated that *L. reuteri* LET 204 was the most efficient one.

Effect of WGA on the bacterial adhesion to IEC

The incubation with WGA induced the detachment of bacteria adhered to IEC, as it is shown in Figure 5. The phenomenon was not attributed to effects of the incubation time but to competition of WGA for binding sites of bacteria on IEC as *L. reuteri* LET 205, an adherent bacterium without ability to bind WGA, was not detached.

~~**Figure 5 somewhere near here**~~

DISCUSSION

~~Almost 500 lectin producing plant species have been described and the tridimensional structure of almost 200 of these lectins is currently known (Sharon and Lis, 2004). They bind to different complementary sugars expressed on the surface of animal and insects cells and exert effects that seem to be dependent on the cellular type and the animal species.~~

~~Plant lectins are associated to practical applications such as detection of carbohydrates on cells surface, mapping neuronal pathways, glycoproteins biosynthesis, among others (Singh and Sarathi, 2012), but they are also related to toxicity and human diseases. The toxicity of a few lectins has been studied (Wu and Sun, 2012), and most of these studies have been done using tumoral cell lines. Several researches have shown that these proteins may inhibit tumor growth, especially by causing cytotoxicity and apoptosis and thus could be considered as potential therapeutic agents (Mody et al., 1995), although their contribution in this field is still under study.~~

~~Lectins from vegetal origin naturally present in human foodstuffs are resistant to the gastrointestinal tract enzymes. After arriving to the gut they bind to the mucosa~~

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~~depending on their specificity to carbohydrates expressed on the cell surface and may cause damage to the enterocytes membrane. This could be one of the factors responsible for Crohn´s disease and Irritable Bowel Syndrome (IBS) among other human diseases in individuals with a particular sensitivity (Singh and Sarathi, 2012).~~ The binding of WGA to enterocytes of adult chickens and enterocytes from the villi of ~~jejune~~jejuni and ileum of young chicks was previously reported (Pohlmeyer, Jorns, Schumacher, Van Damme, Peumans, Pfuller and Neumann, 2005, Zhou, Deng and Ding, 1995). However, the effects of WGA on ~~the~~ cell viability have not been previously analyzed in poultry.

In this study, WGA showed cytotoxicity towards IEC of fourteen-day-old chicks ~~two week old~~, which was dependent on the time of exposition and concentration used. The percentage of cell death caused by the incubation of WGA with IEC ~~allowed~~was used to establish the ~~assessment~~values of ~~the~~ LD_{50} for 1 and 2 h of exposure (56.7 and 8.4 µg/mL, respectively). These ~~values~~ were comparable to the ~~effect of WGA~~reported on other cells types. Indeed, the LD_{50} for WGA on the human leukemic cell lines Molt4, K562 and PBMC was 5.0 µg/mL for the first and higher than 72.0 for the last two, after 24 h of incubation (Ohba, Bakalova and Muraki, 2003).

~~Lactic acid bacteria are Gram (+) microorganisms, thus they show a peptidoglycan rich cell wall which accounts for almost 40% of its weight (Shockman and Barrett, 1983). This structure contains one or more accessory polymers, such as teichoic and teichuronic acids, and other neutral or acidic polysaccharides (Munson and Glaser, 1981, Rogers *et al.*, 1980, Ward, 1981). Repetitive units of N-acetyl-muramic acid and N acetyl glucosamine joint through β 1,4 glycosidic links are the main constituent of this polymer (Rogers, Perkins and Ward, 1980, Schleifer and Kandler, 1972, Tipper and Wright, 1979). The production of extracellular polymers seems to be widely distributed among bacteria. Among them are capsular polysaccharides, which~~

~~form a cohesive layer covalently bound to the cellular surface, and exopolysaccharides (EPS), which are either liberated to the extracellular medium or remain softly joint to the cellular surface (Madigan *et al.*, 1997). The binding of lectins by Gram (+) strains was previously demonstrated. Zárate and Perez Chaia (2009) reported the capture of *Canavalia ensiformis* agglutinin (Con A), PNA~~The binding of lectins by Gram (+) bacteria was previously demonstrated. Zárate and Perez Chaia (2009) reported the capture of *Canavalia ensiformis* agglutinin (Con A), *Arachis hypogaea* agglutinin (PNA) and *Artocarpus intergrifolia* lectin (AIL) by several strains of *Propionibacterium* and *Bifidobacterium longum*. Moreover, Babot et al. (2014) demonstrated the binding of Con A by lactic acid bacteria ~~obtained~~isolated from chicken intestinal content. ~~*L .reuteri* LET 205 and LET 210 and *E. faecium* LET 301 captured the lectin in different extent suggesting that α−D-mannose and/or α−D-glucose, complementary sugars of Con A, are differently expressed on the cells surface. In the same way, other sugars exposed on the surface of the studied strains, either as capsular polysaccharides, glycoproteins or glycolipids bound to their cell wall could be complementary to lectins from different vegetal origin.~~

In the present study, the ~~binding~~capture of several lectins by ~~lactic acid bacteria~~enterococcus and lactobacilli strains of poultry origin was shown. The ~~lectins~~ affinity of lectins for ~~surface~~ carbohydrates expressed on the surface of lactobacilli and the *Enterococcus* strain evidenced the expression of diverse molecules according to the bacterial species studied, with some differences depending on strains. The results for *L. salivarius* LET 201 suggested the presence of GlcNAc-β-1,4-GlcNAc, NeuNAc and α-L-Fucose as surface carbohydrates. Only one strain of *L. vaginalis* expressed Gal-β-1,3-GalNac and minor amount of α-L-Fucose. With exception of the ~~strain~~strains LET 205 and LET 213, the other strains of *L. reuteri* showed significant fluorescence when WGA

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was used, indicating the presence of GlcNAc-β-1,4-GlcNAc and/or NeuNAc as surface carbohydrates. Minor amount of Gal-β-1,3-GalNac was also detected in the strains LET 209, LET 211 and LET 213. Among the five lectins assayed, only WGA interacted with *E. faecium* LET 301, evidencing the existence of GlcNAc-β-1,4-GlcNAc and/or NeuNAc on its surface.

The production of EPS, free or capsular, as well as its quantity and composition is strain-dependent, as reported by Raftis *et al.* (2011), who studied this capability on 33 strains of *L. salivarius* from different origin. Nevertheless, there was not production of EPS by *L. salivarius* LET 201. Several authors reported the production of glucose or fructose homopolysaccharides by strains of *L. reuteri*, *L. pontis*, *L. panis*, *L. acidophilus* and *L. frumenti* (Tieking *et al.*, 2003a, Tieking *et al.*, 2003b, Wang *et al.*, 2010). Despite the binding of Con A to *L. reuteri* LET 205 revealed the expression of glucose or mannose residues on the bacterial surface (Babot et al., 2014), no EPS production was observed for this strain in the present study. Only two of the studied strains, *L. reuteri* LET 206 and *L. reuteri* LET 209, showed the properties of EPS production and WGA binding. However, they captured lower amount of WGA than other non-EPS-producing strains of the same species ~~that not produce EPS.~~. Therefore, ~~for the strains that bound WGA in our study,~~ the production of EPS was not a relevant property ~~towards this end~~for WGA binding by the strains studied.

On the other hand, the capture of different ~~amounts~~amount of lectins by strains of a same species would indicate a distinctive expression of carbohydrates on their surface. This agrees with the results ~~previously informed by Baintner *et al.* (1993),~~reported by Baintner *et al.* (1993), who studied the binding of 15 lectins by microorganisms isolated from sheep rumen and found lectin capture by most cultures, but differences on the quantity of lectin bound to the surface of strains of the same

species. In our ~~investigations~~study, the strains binding WGA evidenced higher fluorescence intensity than ~~interacting with~~ after capture of other lectins. Moreover, all the strains of *L. reuteri* ~~species~~ were able to ~~capture~~bind WGA but the amount of lectin attached to each strain was significantly different.

Adherent and non-adherent strains of lactic acid bacteria could avoid the interaction eukaryotic cells-WGA by different mechanisms depending on the location of these bacteria within the intestine. The protection against cytotoxicity was assayed considering that the ~~lectin~~ capture of WGA by non-adherent bacteria could take place in the intestinal lumen, thus reducing the amount of free ~~WGA~~lectin able to interact with IEC. The protection mediated by adherent bacteria~~,~~ was assayed allowing the adhesion of bacteria to IEC prior to the addition of WGA to interfere in the interaction between the lectin and eukaryotic cells. Results showed that binding of WGA depended on the strain assayed and the conditions used to study this property. ~~–~~Among non-adherent bacteria, *L. salivarius* LET 201 exerted the highest protection. *L. reuteri* LET 208~~,~~ was more efficient for the binding of lectin in RPMI medium at 41.5 ± 0.5 °C than in lectin buffer at 25 °C when compared with the strain *L. reuteri* LET 209, suggesting that environmental factors could be involved in the interaction of WGA with some strains. On the other hand, the adherent strain *L. reuteri* LET 210 was the less efficient when IEC, bacteria and WGA were incubated together in the same medium, simulating the environment near to the intestinal epithelium. This finding suggested that despite the binding of bacteria to IEC surfaces, in some cases the determinants involved in the interaction IEC-WGA remain exposed in the cellular surface. The failure in blocking the access to these determinants due to the lower size of bacteria related to eukaryotic cells or the binding of bacteria to IEC by surface molecules other than WGA receptors could be the reasons of these results. Conversely, many adherent strains succeeded in

the protection of IEC against WGA cytotoxicity through the protocol that simulates de capture and removal of WGA in the intestinal lumen. Indeed, *L. reuteri* LET 210 was able to attract higher amount of lectin than others strains of the same species and was more efficient in protecting IEC. The suspension of 1×10^8 cells of LET 210 removed almost all the WGA added when it was incubated with the lectin during 1 h in RPMI medium prior to the cytotoxicity assay, simulating the conditions in the intestinal lumen. Besides, the percentage of enterocytes with at least one bound bacterium decreased significantly after incubation with WGA for almost all of the adherent strains, assayed in the same conditions. These detached bacteria would still be able to bind the lectin in the intestinal lumen and be eliminated along with the WGA adsorbed to their surface during the normal transit of the intestinal content.

~~CONCLUSLIONS~~

CONCLUSIONS

WGA showed cytotoxicity in broiler enterocytes in a range of concentrations that may be found in the poultry digesta. ~~The stability of lectins during food processing conditions and in the intestinal environment, have encouraged investigating on biological methods intended to prevent damages to the intestinal mucosa. The use of~~ Several lactic acid bacteria isolated from poultry ~~origin to avoid the epithelium~~ intestine captured the lectin in *in vitro* assays. *L. salivarius* LET 201 and *L. reuteri* LET 210 were able to remove WGA ~~interaction, as~~more efficiently and exerted higher protective effect than the other strains assayed. This finding provides bases for the development of probiotic supplements or cell-wall preparations~~, is a safe and effective proposal to this end. Taking into account that wheat is an alternative energy source for poultry feed due to its content~~ of these selected strains intended to avoid harmful effects of ~~starch and~~

proteins, a natural constituent of the removal of antinutritional factors as wheat germ agglutinin is of high interest for improving nutrition and health of animals fed with grain in wheat-based diets. This may be relevant especially in countries with high availability of wheat and limited in corn.

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DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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FITC-labeled dietary lectins

Lectin	Source	Specificity
Con A	*Canavalia ensiformis* (jack bean)	α−D-mannose, α−D-glucose
DBA	*Dolichos biflorus* (horse gram)	GalNAc-α-1,3-GalNAc
PHA-P	*Phaseolus vulgaris* (kidney bean)	Gal-β-1,4-GalNAc-β-1,2-Man
PNA	*Arachis hypogaea* (peanut)	Gal-β-1,3-GalNac
UEA-I	*Ulex europaeus* (furze)	α-L-Fucose
WGA	*Wheat germ agglutinin* (wheat)	GlcNAc-β-1,4-GlcNAc, NeuNAc

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Figure captions

Figure 1. WGA cytotoxicity on IEC of fourteen-day-old broiler chickens after 1 (-●-) and 2 h (-○-) of exposure. Results are expressed as mean values of three independent assays ± standard deviations (SD).

Figure 2. Relative abundance of lectin-linked carbohydrates on the surface of bacteria. (A) Binding of PNA (■), DBA (□) and UEA-I (◪). (B) Binding of PHA-P (□) and WGA (◪) by the strains *L. salivarius* LET201, *L. vaginalis* LET 202 and 203, *L. reuteri* LET 204, LET 205, LET 206, LET 207, LET 208, LET 209, LET 210, LET 211, LET 212 and LET 213; *E. faecium* LET 301. Results are expressed as mean values ~~±~~ ~~SD~~of three independent assays ± SD for each strain and lectin used.

Figure 3. Protection of WGA cytotoxicity by ~~non-adherent~~ removal of lectin by strains~~.~~ of lactobacilli and *Enterococcus*. Percentage of dead cells after IEC incubation with WGA ( ) and ~~after their incubation with~~ supernatant of bacteria-WGA interaction (~~)~~), and the reduction in cell death ( ) due to the lectin removal are represented as mean values of three independent assays ± SD. ~~Significant differences between results for each strain and its control are indicated with an asterisk (p <~~Values of reduction in cell death with no common superscript letter differ significantly at P < 0.~~05)~~.5.

Figure 4. Protection of WGA cytotoxicity by ~~adherent strains. (A) Percentage of dead cells after~~attachment of lactobacilli and *Enterococcus* to IEC ~~incubation with WGA ( ) and after their incubation with supernatant of bacteria-WGA interaction ( ). (B).~~ Percentage of dead cells after IEC incubation with WGA (~~) and~~), after incubation of bacteria-bound IEC with WGA (~~). Results~~), and the reduction in cell death ( ) are

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represented as mean values of three independent assays ± SD. ~~Significant differences between results for each strain and its control are indicated (p ≤~~Values of reduction in cell death with no common superscript letter differ significantly at P < 0.~~05).~~5.

Figure 5. Influence of WGA on the adhesion of bacteria to IEC. Results are represented as mean values of three independent assays ± SD. An asterisk indicates significant differences (p ~~=~~≤ 0.~~05~~5) between adhesion (%) before ( ) and after (-) incubation with the lectin.

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