

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

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Manuscripts

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6 2 **Lactic acid bacteria isolated from poultry protect intestinal epithelial cells of**  
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8 **chicks from *in vitro* wheat germ agglutinin-induced cytotoxicity**  
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37 16 **Running title: Wheat lectin toxicity protection**  
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4 24 **Abstract.** 1. Whole grain or milled wheat is often used instead of corn in poultry  
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6 25 feeding, due to its high content of starch and proteins. Poultry fed with wheat-based  
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8 26 diets regularly ingest wheat germ agglutinin (WGA).  
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10 27 2. WGA showed toxic effect *in vitro* on intestinal epithelial cells (IEC) obtained from  
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12 28 fourteen-day-old broilers. Cytotoxicity was dependent on time and lectin concentration;  
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14 29 lethal dose (LD<sub>50</sub>) was 8.36 µg/mL for IEC exposed for 2 h to WGA.  
15  
16 30 3. Complementary sugars to WGA were detected on the surface of one *Enterococcus*  
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18 31 and nine *Lactobacillus* strains isolated from poultry. These strains were evaluated as a  
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20 32 lectin removal tool intended to cytotoxicity prevention.  
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22 33 4. The incubation of the lactic acid bacteria with WGA before the IEC-lectin interaction  
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24 34 caused a remarkable reduction in the percentage of cell death. The protection was  
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26 35 attributed to the amount of lectin bound to the bacterial surfaces and was strain-  
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28 36 dependent. *L. salivarius* LET 201 and *L. reuteri* LET 210 were significantly more  
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30 37 efficient than the other lactic acid bacteria assayed.  
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32 38 5. These results provide bases for the development of probiotic supplements or cell-wall  
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34 39 preparations of selected lactic acid bacteria intended to avoid harmful effects of a  
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36 40 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  $\beta$ -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 jejunum-ileum of chicks 2, 15 and 30 days post hatching (Pohlmeyer *et al.*, 2005), to enterocytes from the ileum of

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4 74 chicks 2 days post hatching (Zhou *et al.*, 1995), and to enterocytes from the villi of  
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6 75 caecal tonsils of adult chickens (Kitagawa *et al.*, 2000) has been previously reported.  
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8 76 The interaction lectin-epithelium may alter the activity of digestive enzymes located on  
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10 77 the epithelial cells (Rueda *et al.*, 2007), and the integrity and permeability of the  
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12 78 intestinal epithelium allowing small molecules to cross the epithelial barrier  
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14 79 contributing to the toxicity of WGA to gastrointestinal cells (Dalla Pellegrina, Rizzi,  
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16 80 Mosconi, Zoccatelli, Peruffo and Chignola, 2005). In poultry, this may depress the  
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18 81 growth of broilers especially during the first days of life.  
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22 82 Poultry industry has explored the benefit of including probiotic cultures in  
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24 83 broilers feed as alternative to antibiotic treatments for growth promotion. Probiotics  
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26 84 may contribute to xenobiotics metabolism or to avoid deleterious effects of toxic  
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28 85 compounds included in the diet by binding them on the bacterial envelopes that express  
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30 86 appropriated determinants (Carasi *et al.*, 2012, Turbic *et al.*, 2002, Zárate and Perez  
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32 87 Chaia, 2012). The binding of WGA to carbohydrates expressed on the bacterial surface  
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34 88 and its removal by the normal transit of the intestinal content may contribute to a  
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36 89 healthier broiler feed.  
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40 90 In the present investigation we explore the toxicity of WGA on intestinal  
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42 91 epithelial cells of broilers and the ability of lactic acid bacteria isolated from poultry  
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44 92 intestine to remove WGA and protect cells from the harmful effects of this lectin.  
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## 48 94 MATERIALS AND METHODS

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### 50 96 **Microorganisms and culture conditions**

51 97 Thirteen strains of lactobacilli and one enterococcus isolated from poultry intestine were  
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53 98 used in the study. Suitable properties for probiotic strains selection were previously  
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4 99 determined, such as the growth at the corporal temperature of poultry, tolerance to pH,  
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6 100 bile salts and digestive enzymes, adhesion to small bowel mucosa, antibiotics sensitivity  
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8 101 and absence of virulence factors (Babot *et al.*, 2014). The strains were grouped as non-  
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10 102 adherent (*Lactobacillus salivarius* LET 201, *L. vaginalis* LET 202, *L. vaginalis* LET  
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12 103 203, *L. reuteri* LET 208, *L. reuteri* LET 209, *L. reuteri* LET 212 and *L. reuteri* LET  
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14 104 2013) and adherent (*L. reuteri* LET 204, *L. reuteri* LET 205, *L. reuteri* LET 206, *L.*  
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16 105 *reuteri* LET 207, *L. reuteri* LET 210, *L. reuteri* LET 211 and *Enterococcus faecium*  
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18 106 LET 301) bacteria.

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21 107 All strains were stored at -70 °C in 10% (w/v) reconstituted non-fat milk (NFM)  
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23 108 supplemented with 0.5% yeast extract and 15% glycerol. MRS broth (de Man *et al.*,  
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25 109 1960) and LAPTg broth (Raibaud *et al.*, 1973) were used to activate cultures of  
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27 110 lactobacilli and enterococci, respectively. Prior to use, cultures were incubated at 41.5 ±  
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29 111 0.5 °C for 12 h.  
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### 34 113 **Intestinal epithelial cells extraction**

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36 114 For extraction of intestinal epithelial cells (IEC), fourteen-day-old broiler chicks were  
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38 115 slaughtered by cervical dislocation. The birds were immediately eviscerated for  
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40 116 collection of ileum which was in turn rinsed repeatedly with ice cold PBS pH 7.40 to  
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42 117 eliminate the digested content. The tissue was cut lengthwise and washed once again  
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44 118 with cold PBS. Epithelial cells were scrapped from the surface using a sterile  
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46 119 microscope slide and collected in ice cold PBS pH 7.40 supplemented with 1% Fetal  
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48 120 Bovine Serum (PBS/FBS). The cells were washed twice with PBS/FBS, incubated with  
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50 121 0.25% Trypsin-EDTA (Gibco, Grand Island, USA) at 37 °C for 5 min before cold  
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52 122 PBS/FBS was added to stop the enzyme activity. The cells were immediately collected  
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54 123 (800 × g, 5 min, 4 °C) and washed once with PBS/FBS prior to adjust their  
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4 124 concentration to  $1 \times 10^6$  cells/mL in RPMI 1640 medium supplemented with 1% FBS  
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6 125 (RPMI/FBS). Cells were counted using a Neubauer chamber in a conventional light  
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8 126 microscope at  $40 \times$  magnification (Zeiss–Axiolab; Cool Zeiss, Jena, Germany).  
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11 127 The animal handling protocols of this investigation were adjusted to the *Ethical*  
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13 128 *Framework of Reference for Biomedical Research in Laboratory Animals, from Farm*  
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15 129 *and Obtained from Nature*, contained in the Resolution N°. 1047/05, Annex II, of  
16  
17 130 CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) - Argentina.  
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19 131 Experimental procedures for IEC extraction were approved by The Committee of Ethics  
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21 132 for Animal Studies of CERELA (CCT Tucumán - CONICET).  
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#### 24 133 25 26 134 **WGA cytotoxicity**

27  
28 135 For cytotoxicity assessment, equal volumes of suspensions of IEC and WGA in  
29  
30 136 RPMI/FBS were mixed and incubated at  $41.5 \pm 0.5$  °C. Final concentrations in the  
31  
32 137 mixtures were  $5 \times 10^5$  cells/mL and 0, 12.5, 25, 50, 100, 150 or 200 µg/mL of WGA.  
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34 138 After 1 and 2 h of incubation, 100 µL of cells suspensions were washed with  
35  
36 139 RPMI/FBS and suspended in the same volume of medium. A freshly prepared mixture  
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38 140 of Fluorescein diacetate (FDA; Sigma-Aldrich, Argentina) and Propidium iodide (PI)  
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40 141 was added to obtain final concentrations of 7.5 µg/mL of FDA and 2.5 µg/mL of PI  
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42 142 (Zárate and Perez Chaia, 2009). The samples were incubated on ice in the darkness for  
43  
44 143 10 min and washed again. Cells with green cytoplasm and normal nuclei (viable) and  
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46 144 cells with bright red nuclei (necrotic cells) were counted on ten microscopic fields by  
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48 145 fluorescence microscopy with the appropriated filters. The mean value of dead cells on  
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50 146 each of two equally prepared suspensions was reported as percentage of the total cells.  
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52 147 Three independent assays were performed with the same procedure and the mean value  
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54 148 of dead cells (%) vs. WGA concentration (C in µg/mL) was represented with data of 1  
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4 149 and 2 hours of incubation. The maximum death (%<sub>max</sub>) and the WGA concentration  
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6 150 responsible for half of this value (LD<sub>50</sub>) were determined for each condition using the  
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8 151 mathematical expression of a model adapted for toxic chemicals in experimental  
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10 152 bioassays (Sánchez-Bayo and Goka, 2007). The mathematical expression to represent  
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12 153 the toxicity model was:

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

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### 20 21 156 **Carbohydrates of the bacterial surfaces**

22  
23 157 Surface carbohydrates of the strains were assessed using FITC-labeled lectins (Sigma-  
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25 158 Aldrich, Buenos-Aires, Argentina) with different carbohydrates specificity (Table).  
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27 159 Active cultures of the fourteen strains were adjusted to Absorbance (A<sub>600nm</sub>) of 0.7,  
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29 160 washed three times with a lectin buffer (60.57 g/L Tris, 87 g/L NaCl, 1.11 g/L CaCl<sub>2</sub>,  
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31 161 pH 7.60) described by Leathem and Brooks (1997), suspended in equal volume of  
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33 162 buffer containing 20 µg/mL of one of the five FITC-labeled lectin and incubated 1 h at  
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35 163 25 °C (Wanchoo *et al.*, 2009). The same procedure was carried out for each lectin and  
36  
37 164 strain. Cells suspensions were centrifuged (10000 × g, 10 min, 4 °C) at the end of  
38  
39 165 incubation. Harvested cells were washed 4 times, suspended in equal volume of lectin  
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41 166 buffer and observed on a conventional fluorescence microscope (Carl Zeiss Axio Scope  
42  
43 167 A1, Gottingen, Germany) fitted with the appropriated filter at 100 × magnification. The  
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45 168 mean number of fluorescent cells was assessed by counting them in 10 microscopic  
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47 169 fields and the number of fluorescent bacteria/mL was determined as in Lorenzo-  
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49 170 Pisarello *et al.* (2010). The fluorescence intensity of each cells suspension was also  
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51 171 measured with a fluorospectrophotometer (Cary Eclipse, Varian Inc., Walnut Creek,  
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4 172 California, USA) and reported as arbitrary units (AU) / bacteria. Each trial was  
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6 173 reproduced three times with new active cultures of each strain.  
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Table somewhere near here
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11 175 **Exopolysaccharide production**

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13 176 The production of exopolysaccharides by the strains was assessed according to Mozzi *et*  
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15 177 *al.* (2001). Briefly, 2.5 µl of India ink were spotted onto microscopic slides and 10 µl of  
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17 178 active cultures (O.D.<sub>560</sub> 0.70) were added, the mixtures were covered with coverslips  
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19 179 and the excess of liquid was eliminated by gently pressing on them with absorbent  
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21 180 paper. Finally, the samples were observed in a light microscope (Zeiss–Axiolab; Carl  
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23 181 Zeiss, Jena, Germany). Bright areas surrounding bacteria indicated the production of  
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25 182 exopolysaccharide.  
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31 184 **Protection of epithelial cells by bacteria**

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33 185 Suspensions of  $1 \times 10^8$  bacteria/mL of adherent and non-adherent strains with ability to  
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35 186 bind WGA were incubated with 50 µg/mL of the lectin in RPMI/FBS for 1 h at  $41.5 \pm$   
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37 187  $0.5$  °C. Mixtures were centrifuged ( $10000 \times g$ , 10 min, 4 °C) and the supernatants stored  
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39 188 at 4 °C for further use. Freshly prepared IEC were centrifuged, suspended to reach  $5 \times$   
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41 189  $10^5$  cells/mL in the stored supernatants or in RPMI/FBS containing 50 µg/mL of WGA,  
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43 190 and incubated during 2 h at  $41.5 \pm 0.5$  °C under 5% CO<sub>2</sub> atmosphere (Nuair Co., MN,  
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45 191 USA). IEC suspended in RPMI/FBS without lectin incubated as described were used as  
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47 192 control of spontaneous death. Finally, cells were stained with 7.5 µg/mL of FDA and  
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49 193 2.5 µg/mL of PI for 10 min on ice in a dark room and viable and necrotic cells were  
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51 194 counted and reported as already described.  
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55 195 In other trials, suspensions of both adherent bacteria ( $1 \times 10^8$  bacteria/mL) and  
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57 196 IEC ( $5 \times 10^5$  cells/mL) were prepared and incubated 30 min at  $41.5 \pm 0.5$  °C under 5%

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4 197 CO<sub>2</sub> atmosphere to allow adhesion. Then, the mixtures were centrifuged (120 × g, 5  
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6 198 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS  
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8 199 containing 50 µg/mL of WGA. IEC suspended in RPMI/FBS with 50 µg/mL of WGA  
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10 200 were used as control of WGA toxicity; IEC suspended in RPMI/FBS were used as  
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12 201 control of spontaneous death; IEC incubated with 1 × 10<sup>8</sup> bacteria/mL and then  
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14 202 suspended in RPMI/FBS without lectin were used as control of damage produced by the  
15  
16 203 bacterial adhesion. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO<sub>2</sub> atmosphere,  
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18 204 cells were stained with FDA/PI and viable and necrotic IEC were counted and reported  
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20 205 as already described.  
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#### 26 207 **Effect of WGA on the bacterial adhesion to IEC**

28 208 Intestinal epithelial cells were obtained as above described and incubated with bacterial  
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30 209 suspensions of 1 × 10<sup>8</sup> CFU/mL for 1 h at 41.5 ± 0.5 °C under 5% CO<sub>2</sub> atmosphere.  
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32 210 After incubation, the mixtures were centrifuged (120 × g, 5 min, 4 °C), washed twice  
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34 211 with RPMI and suspended in the initial volume of RPMI/FBS containing 50 µg/mL of  
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36 212 WGA. After 2 h of incubation at 41.5 ± 0.5 °C under 5% CO<sub>2</sub> atmosphere, cells  
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38 213 adhesion to IEC was examined by counting adhered bacteria in 30 IEC, using phase-  
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40 214 contrast microscopy. Results were expressed as the percentage of IEC with adhered  
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42 215 bacteria (adhesion percentage).  
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#### 48 217 **Statistical analysis**

50 218 Three independent assays were performed for each *in vitro* experiment and the mean  
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52 219 values ± SD were obtained for each sample. Significant differences were determined by  
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54 220 Tukey's test after analysis of variance (ANOVA) with OriginPro 8 SR0 v8.0724  
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4 221 (OriginLab Corporation, Northampton, MA, USA). A value of  $P < 0.5$  was considered  
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6 222 statistically significant.  
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## 10 224 RESULTS

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### 13 226 **WGA cytotoxicity**

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15 227 As it is shown in Figure 1, WGA exhibited toxic effect on IEC obtained from chicken  
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17 228 intestine. Cells incubated with the lectin at the body temperature of the animals induced  
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19 229 cells death in different extent depending on the incubation time and lectin concentration.  
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21 230 Increases in the WGA concentration from 0 to 200  $\mu\text{g}/\text{mL}$  induced a progressive and  
22  
23 231 negative effect on viability in 1 h incubations. In contrast, the loss of viability was  
24  
25 232 notable with minor variations of the lectin concentration from 0 to 25  $\mu\text{g}/\text{mL}$  when the  
26  
27 233 incubation was extended to 2 h. The plot of cytotoxicity, percentage of dead cells vs  
28  
29 234 lectin concentration, showed hyperbolic design with tendency to a maximum effect at  
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31 235 high lectin concentrations. Double-reciprocal plots were used to determine the  
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33 236 maximum percentages of dead cells ( $\%_{\text{max}}$ ) and the lectin concentration that induce the  
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35 237 death of half of the cells in the suspension (lethal dose 50,  $\text{LD}_{50}$ ) at each incubation time  
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37 238 (Purich and Allison, 2000). Results for  $\text{LD}_{50}$  were 56.7 and 8.4  $\mu\text{g}/\text{mL}$  for cells  
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39 239 incubated during 1 and 2 hours, respectively. Maximum death percentages produced by  
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41 240 the lectin were 90.6 % and 90.2 %, respectively.

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Figure 1 somewhere near here

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### 242 **Characterization of surface carbohydrates and WGA removal**

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

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4 246 registered by fluorospectrophotometry as arbitrary units of fluorescence (AU). Figure  
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6 247 2A represents the AU obtained when *Arachis hypogaea* agglutinin (PNA), *Dolichos*  
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8 248 *biflorus* agglutinin (DBA) or *Ulex europaeus* agglutinin (UEA-I) were incubated with  
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10 249 the studied strains; Figure 2B shows the results of incubation with *Phaseolus vulgaris*  
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12 250 agglutinin (PHA-P) or WGA. The only strain tested of *L. salivarius* ligated significant  
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14 251 amount of PNA and WGA; one of the strains of *L. vaginalis* ligated PNA and UEA-I  
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16 252 while *E. faecium* only captured WGA. Nine strains of *L. reuteri* removed WGA, three  
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18 253 of them captured also PNA and two strains removed PHA-P. The lectins DBA and  
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20 254 UEA-I linked to only one of the strains of *L. reuteri* each. The fluorescence intensity  
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22 255 measured evidenced that WGA was captured by lactobacilli and *E. faecium* LET 301 in  
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24 256 significantly higher amount than the other lectins tested.  
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Figure 2 somewhere near here

### 258 Exopolysaccharide production

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

### 264 Protection of cells by adherent and non-adherent bacteria

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

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4 271 determined and represented as percentages of total cells in the suspensions. These  
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6 272 values were compared with the percentage obtained in samples of IEC incubated with  
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8 273 supernatants of lectin-bacteria interaction for each strain under study. As expected, cell  
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10 274 death was significantly reduced when IEC were exposed to supernatants of the lectin-  
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12 275 bacteria interactions. A remarkable protective effect was observed for *L. salivarius* LET  
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14 276 201, which reduced cell death in almost 54 % while the lowest effect was that of *L.*  
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16 277 *reuteri* LET 209 with a death reduction of almost 10 %. An unexpected result was  
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18 278 observed for *L. reuteri* LET 208, which reduced cells death in almost 42 %, as the  
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20 279 previously assessed amount of lectin removed by this strain was lower than that for *L.*  
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22 280 *reuteri* LET 209 (Fig. 2). The effective amount of lectin in supernatants of WGA-  
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24 281 bacteria interactions and in the WGA solutions used in each assay were determined  
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26 282 from the percentages of cellular death measured in the cytotoxicity assays using the  
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28 283 %<sub>max</sub> and LD<sub>50</sub> values previously obtained (subsection 3.1). The lectin removed by each  
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30 284 strain was assessed as the difference between these values. The suspension of  $1 \times 10^8$   
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32 285 cells of *L. salivarius* LET 201 removed  $46.9 \pm 0.4$  µg of WGA, while *L. reuteri* LET  
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34 286 209 removed  $26.0 \pm 0.6$  µg of WGA in the conditions used in the trial. Figure 3 somewhere near here

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39 287 The effect of lectin capture by adherent bacteria was first assessed in a protocol  
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41 288 that simulates the conditions of free bacteria in the intestinal lumen (Fig. 3). *L. reuteri*  
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43 289 LET 210 exerted the highest protection lowering cell death approximately 45 %. The  
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45 290 less efficient strain was *L. reuteri* LET 204 which reduced in almost 22 % the  
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47 291 percentage of dead cells. The lectin removal assessed by the percentage of dead cells  
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49 292 was  $45.6 \pm 0.3$  µg of lectin for *L. reuteri* LET 210 and  $37.2 \pm 0.5$  µg of lectin for the  
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51 293 strain LET 204.

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55 294 The same strains were assayed for the ability to protect the epithelial cells of  
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57 295 lectin toxicity during the bacteria-IEC interaction. As observed in Figure 4, strain *L.*

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4 296 *reuteri* LET 210 exerted scarce protection of the cells when it was in intimate relation to  
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6 297 them; cell death was reduced in approximately 5 % and represented the removal of 15.2  
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8 298  $\pm 2.5$   $\mu\text{g}$  of lectin. In the contrary, *L. reuteri* LET 204 reduced in almost 17 % the cells  
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10 299 death by removal of  $33.2 \pm 0.3$   $\mu\text{g}$  of lectin. Moderate protection was achieved with *L.*  
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12 300 *reuteri* LET 211, *L. reuteri* LET 207 and *E. faecium* LET 301. *L. reuteri* LET 206 was  
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14 301 the only strain that increased slightly the cell death when IEC with adhered bacteria  
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16 302 were exposed to WGA. The damage was also observed during the bacteria-IEC  
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18 303 interaction without exposure to WGA (data not shown). **Figure 4 somewhere near here**

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22 304 As shown in Figures 3 and 4, comparisons of the protective effect of the strains  
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24 305 were performed for each protocol used. The statistical analysis of the reduction of cell  
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26 306 death obtained by each strain in the protocol that simulates free bacteria in the intestinal  
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28 307 lumen, demonstrated that the highest protection was exerted by *L. salivarius* LET 201, a  
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30 308 non-adherent strain, followed by *L. reuteri* LET 210, an adherent one (Fig. 3). The  
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32 309 statistical analysis of results of the protection achieved by the strains adhered to IEC  
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34 310 indicated that *L. reuteri* LET 204 was the most efficient one.  
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### 312 **Effect of WGA on the bacterial adhesion to IEC**

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

**Figure 5 somewhere near here**

### 318 DISCUSSION

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

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4 321 Schumacher, Van Damme, Peumans, Pfuller and Neumann, 2005, Zhou, Deng and  
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6 322 Ding, 1995). However, the effects of WGA on cell viability have not been previously  
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8 323 analyzed in poultry.  
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10 324 In this study, WGA showed cytotoxicity towards IEC of fourteen-day-old  
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12 325 chicks, which was dependent on the time of exposition and concentration used. The  
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14 326 percentage of cell death caused by the incubation of WGA with IEC was used to  
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16 327 establish the values of LD<sub>50</sub> for 1 and 2 h of exposure (56.7 and 8.4 µg/mL,  
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18 328 respectively). These were comparable to the reported on other cells types. Indeed, the  
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20 329 LD<sub>50</sub> for WGA on the human leukemic cell lines Molt4, K562 and PBMC was 5.0  
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22 330 µg/mL for the first and higher than 72.0 for the last two, after 24 h of incubation (Ohba,  
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24 331 Bakalova and Muraki, 2003).  
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28 332 The binding of lectins by Gram (+) bacteria was previously demonstrated. Zárte  
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30 333 and Perez Chaia (2009) reported the capture of *Canavalia ensiformis* agglutinin (Con  
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32 334 A), *Arachis hypogaea* agglutinin (PNA) and *Artocarpus intergrifolia* lectin (AIL) by  
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34 335 several strains of *Propionibacterium* and *Bifidobacterium longum*. Moreover, Babot et  
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36 336 al. (2014) demonstrated the binding of Con A by lactic acid bacteria isolated from  
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38 337 chicken intestinal content. In the present study, the capture of several lectins by  
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40 338 enterococcus and lactobacilli strains of poultry origin was shown. The affinity of lectins  
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42 339 for carbohydrates expressed on the surface of lactobacilli and the *Enterococcus* strain  
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44 340 evidenced the expression of diverse molecules according to the bacterial species  
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46 341 studied, with some differences depending on strains. The results for *L. salivarius* LET  
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48 342 201 suggested the presence of GlcNAc-β-1,4-GlcNAc, NeuNAc and α-L-Fucose as  
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50 343 surface carbohydrates. Only one strain of *L. vaginalis* expressed Gal-β-1,3-GalNAc and  
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52 344 minor amount of α-L-Fucose. With exception of the strains LET 205 and LET 213, the  
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54 345 other strains of *L. reuteri* showed significant fluorescence when WGA was used,  
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4 346 indicating the presence of GlcNAc- $\beta$ -1,4-GlcNAc and/or NeuNAc as surface  
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6 347 carbohydrates. Minor amount of Gal- $\beta$ -1,3-GalNac was also detected in the strains LET  
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8 348 209, LET 211 and LET 213. Among the five lectins assayed, only WGA interacted with  
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10 349 *E. faecium* LET 301, evidencing the existence of GlcNAc- $\beta$ -1,4-GlcNAc and/or  
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12 350 NeuNAc on its surface.

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15 351 The production of EPS, free or capsular, as well as its quantity and composition  
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17 352 is strain-dependent, as reported by Raftis *et al.* (2011), who studied this capability on 33  
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19 353 strains of *L. salivarius* from different origin. Nevertheless, there was not production of  
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21 354 EPS by *L. salivarius* LET 201. Several authors reported the production of glucose or  
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23 355 fructose homopolysaccharides by strains of *L. reuteri*, *L. pontis*, *L. panis*, *L. acidophilus*  
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25 356 and *L. frumenti* (Tieking *et al.*, 2003a, Tieking *et al.*, 2003b, Wang *et al.*, 2010). Despite  
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27 357 the binding of Con A to *L. reuteri* LET 205 revealed the expression of glucose or  
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29 358 mannose residues on the bacterial surface (Babot *et al.*, 2014), no EPS production was  
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31 359 observed for this strain in the present study. Only two of the studied strains, *L. reuteri*  
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33 360 LET 206 and *L. reuteri* LET 209, showed the properties of EPS production and WGA  
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35 361 binding. However, they captured lower amount of WGA than other non-EPS-producing  
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37 362 strains of the same species. Therefore, the production of EPS was not a relevant  
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39 363 property for WGA binding by the strains studied.

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42 364 On the other hand, the capture of different amount of lectins by strains of a same  
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44 365 species would indicate a distinctive expression of carbohydrates on their surface. This  
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46 366 agrees with the results reported by Baintner *et al.* (1993), who studied the binding of 15  
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48 367 lectins by microorganisms isolated from sheep rumen and found lectin capture by most  
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50 368 cultures, but differences on the quantity of lectin bound to the surface of strains of the  
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52 369 same species. In our study, the strains binding WGA evidenced higher fluorescence  
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54 370 intensity than after capture of other lectins. Moreover, all the strains of *L. reuteri* were  
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4 371 able to bind WGA but the amount of lectin attached to each strain was significantly  
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6 372 different.

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8 373 Adherent and non-adherent strains of lactic acid bacteria could avoid the  
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10 374 interaction eukaryotic cells-WGA by different mechanisms depending on the location of  
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12 375 these bacteria within the intestine. The protection against cytotoxicity was assayed  
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14 376 considering that the capture of WGA by non-adherent bacteria could take place in the  
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16 377 intestinal lumen, thus reducing the amount of free lectin able to interact with IEC. The  
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18 378 protection mediated by adherent bacteria was assayed allowing the adhesion of bacteria  
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20 379 to IEC prior to the addition of WGA to interfere in the interaction between the lectin  
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22 380 and eukaryotic cells. Results showed that binding of WGA depended on the strain  
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24 381 assayed and the conditions used to study this property. Among non-adherent bacteria, *L.*  
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26 382 *salivarius* LET 201 exerted the highest protection. *L. reuteri* LET 208 was more  
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28 383 efficient for the binding of lectin in RPMI medium at  $41.5 \pm 0.5$  °C than in lectin buffer  
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30 384 at 25 °C when compared with the strain *L. reuteri* LET 209, suggesting that  
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32 385 environmental factors could be involved in the interaction of WGA with some strains.  
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34 386 On the other hand, the adherent strain *L. reuteri* LET 210 was the less efficient when  
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36 387 IEC, bacteria and WGA were incubated together in the same medium, simulating the  
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38 388 environment near to the intestinal epithelium. This finding suggested that despite the  
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40 389 binding of bacteria to IEC surfaces, in some cases the determinants involved in the  
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42 390 interaction IEC-WGA remain exposed in the cellular surface. The failure in blocking  
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44 391 the access to these determinants due to the lower size of bacteria related to eukaryotic  
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46 392 cells or the binding of bacteria to IEC by surface molecules other than WGA receptors  
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48 393 could be the reasons of these results. Conversely, many adherent strains succeeded in  
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50 394 the protection of IEC against WGA cytotoxicity through the protocol that simulates de  
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52 395 capture and removal of WGA in the intestinal lumen. Indeed, *L. reuteri* LET 210 was  
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4 396 able to attract higher amount of lectin than others strains of the same species and was  
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6 397 more efficient in protecting IEC. The suspension of  $1 \times 10^8$  cells of LET 210 removed  
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8 398 almost all the WGA added when it was incubated with the lectin during 1 h in RPMI  
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10 399 medium prior to the cytotoxicity assay, simulating the conditions in the intestinal  
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12 400 lumen. Besides, the percentage of enterocytes with at least one bound bacterium  
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14 401 decreased significantly after incubation with WGA for almost all of the adherent strains,  
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16 402 assayed in the same conditions. These detached bacteria would still be able to bind the  
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18 403 lectin in the intestinal lumen and be eliminated along with the WGA adsorbed to their  
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20 404 surface during the normal transit of the intestinal content.  
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#### 406 CONCLUSIONS

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28 407 WGA showed cytotoxicity in broiler enterocytes in a range of concentrations that may  
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30 408 be found in the poultry digesta. Several lactic acid bacteria isolated from poultry  
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32 409 intestine captured the lectin in *in vitro* assays. *L. salivarius* LET 201 and *L. reuteri* LET  
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34 410 210 were able to remove WGA more efficiently and exerted higher protective effect  
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36 411 than the other strains assayed. This finding provides bases for the development of  
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38 412 probiotic supplements or cell-wall preparations of these selected strains intended to  
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40 413 avoid harmful effects of a natural constituent of the grain in wheat-based diets.  
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## DISCLOSURE STATEMENT

7  
8 423 No potential conflict of interest was reported by the authors.  
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**FITC-labeled dietary lectins**

Lectin	Source	Specificity
Con A	<i>Canavalia ensiformis</i> (jack bean)	$\alpha$ -D-mannose, $\alpha$ -D-glucose
DBA	<i>Dolichos biflorus</i> (horse gram)	GalNAc- $\alpha$ -1,3-GalNAc
PHA-P	<i>Phaseolus vulgaris</i> (kidney bean)	Gal- $\beta$ -1,4-GalNAc- $\beta$ -1,2-Man
PNA	<i>Arachis hypogaea</i> (peanut)	Gal- $\beta$ -1,3-GalNAc
UEA-I	<i>Ulex europaeus</i> (furze)	$\alpha$ -L-Fucose
WGA	<i>Wheat germ agglutinin</i> (wheat)	GlcNAc- $\beta$ -1,4-GlcNAc, NeuNAc

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528 **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).

531 **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.

537 **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  $P < 0.5$ .

543 **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  $P < 0.5$ .

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4 549 **Figure 5.** Influence of WGA on the adhesion of bacteria to IEC. Results are represented  
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6 550 as mean values of three independent assays  $\pm$  SD. An asterisk indicates significant  
7  
8 551 differences ( $p < 0.5$ ) between adhesion (%) before (■) and after (□) incubation with  
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10 552 the lectin.  
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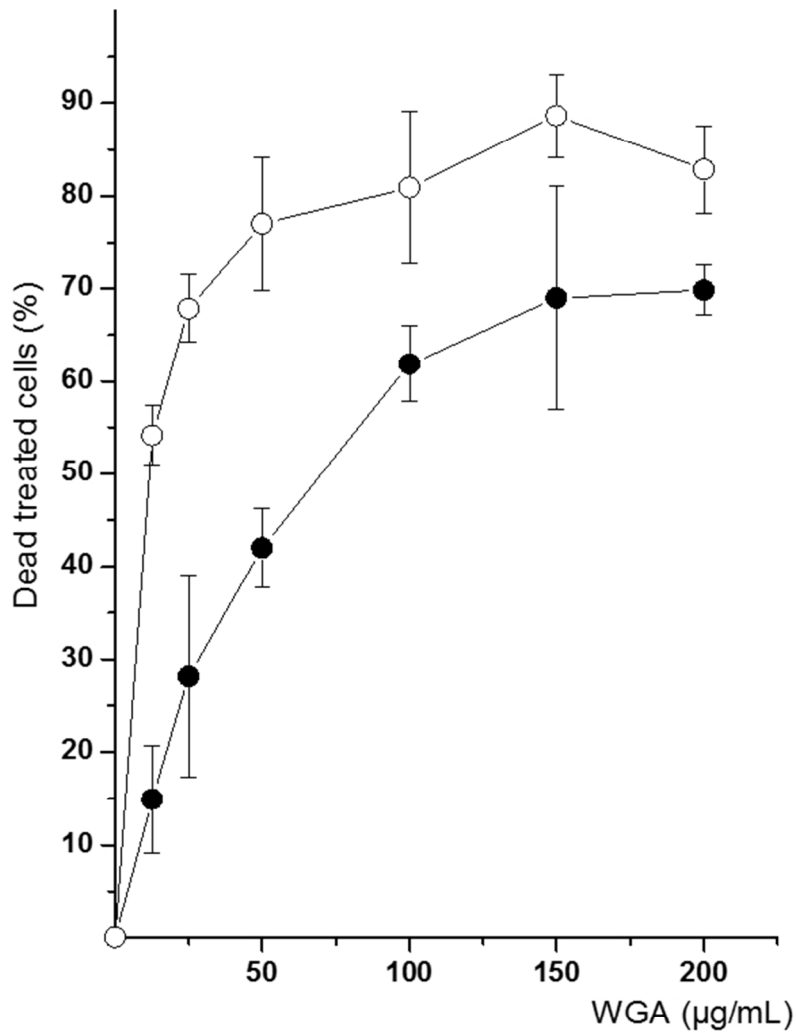


Figure 1. WGA cytotoxicity on IEC of fourteen-day-old broiler chickens after 1 (●) and 2 h (○) of exposure. Results are expressed as mean values of three independent assays  $\pm$  standard deviations (SD).  
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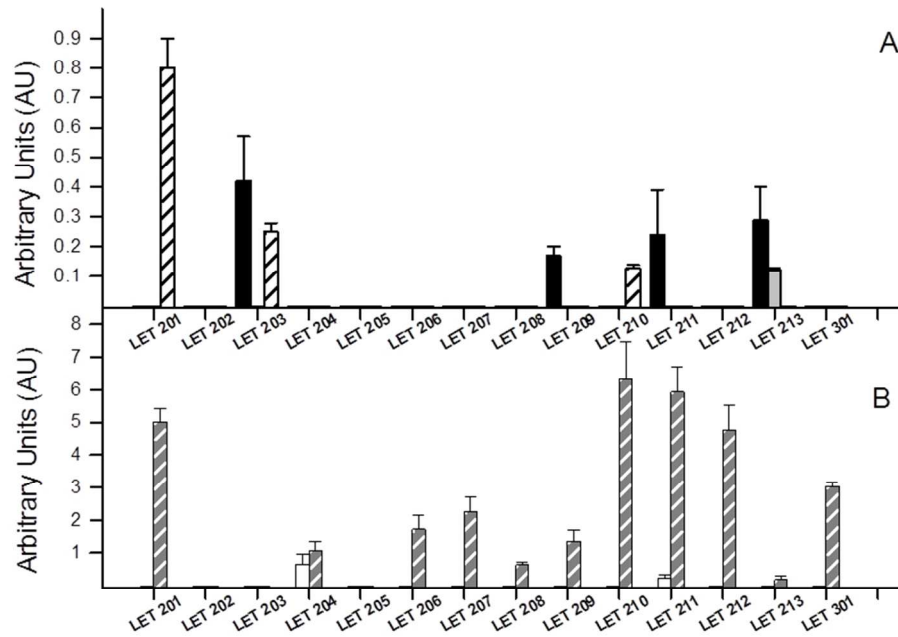


Figure 2. Relative abundance of lectin-linked carbohydrates on the surface of bacteria. (A) Binding of PNA ( ), DBA ( ) and UEA-I ( ). (B) Binding of PHA-P ( ) and WGA ( ) by the strains *L. salivarius* LET201, *L. vaginalis* LET 202 and 203, *L. reuteri* LET 204, LET 205, LET 206, LET 207, LET 208, 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  $\pm$  SD for each strain and lectin used.

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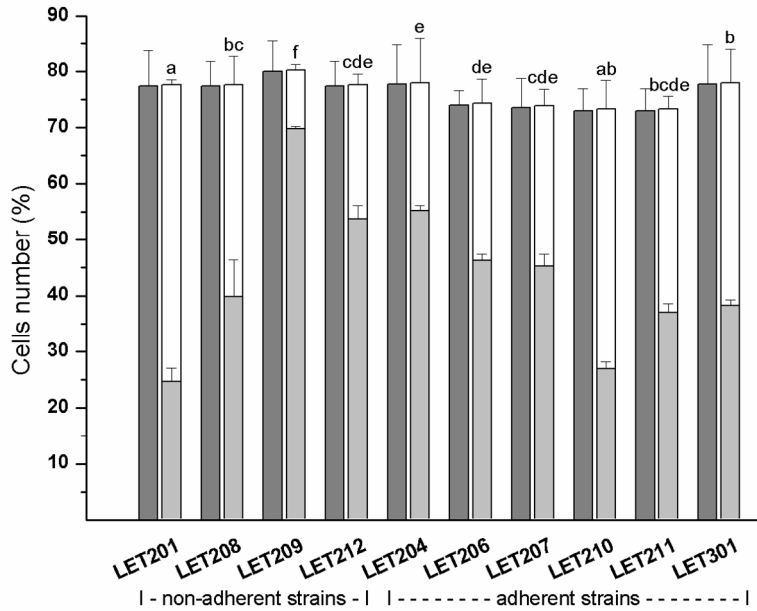


Figure 3. Protection of WGA cytotoxicity by removal of lectin by strains of lactobacilli and *Enterococcus*. Percentage of dead cells after IEC incubation with WGA ( ) and supernatant of bacteria-WGA interaction ( ), and the reduction in cell death ( ) due to the lectin removal are represented as mean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

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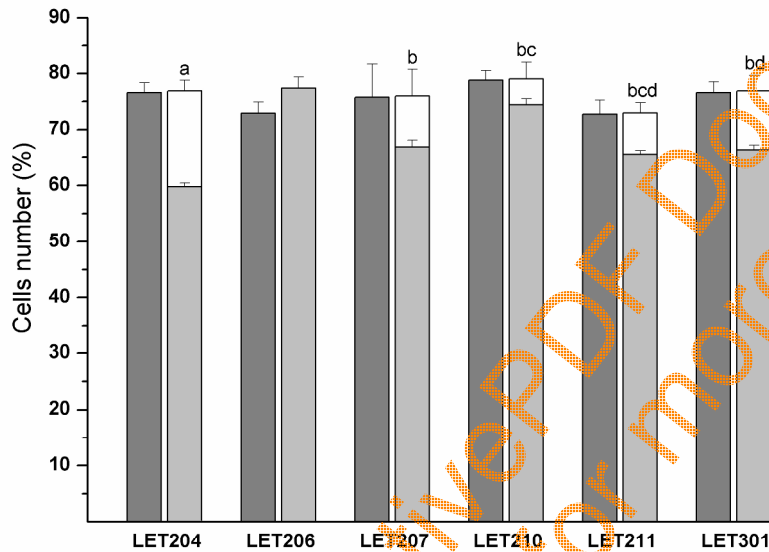


Figure 4. Protection of WGA cytotoxicity by attachment of lactobacilli and *Enterococcus* to IEC. Percentage of dead cells after IEC incubation with WGA ( ), after incubation of bacteria-bound IEC with WGA ( ), and the reduction in cell death ( ) are represented as mean values of three independent assays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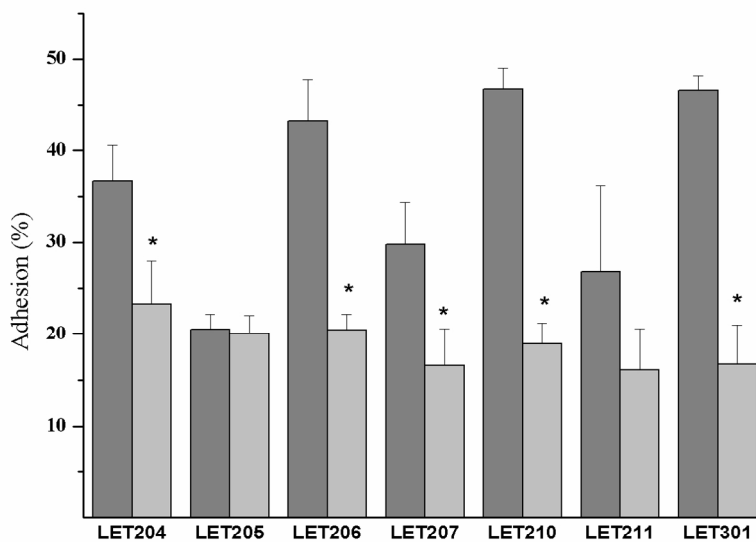


Figure 5. Influence of WGA on the adhesion of bacteria to IEC. Results are represented as mean values of three independent assays  $\pm$  SD. An asterisk indicates significant differences ( $p < 0.5$ ) between adhesion (%) before ( ) and after ( ) incubation with the lectin.  
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3 **Lactic acid bacteria isolated from poultry protect intestinal epithelial cells of**4 **chicks from *in vitro* wheat germ agglutinin (~~WGA~~)-induced cytotoxicity**

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8 J.D. BABOT<sup>1</sup>, E. ARGAÑARAZ-MARTÍNEZ<sup>1,2</sup>, M.J. LORENZO-PISARELLO<sup>2,3</sup>,9 M.C. APELLA<sup>1,2</sup> AND A. PEREZ CHAIA<sup>1,2</sup>

10

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18 **Running title: Wheat lectin toxicity protection**

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21 Correspondence to: Dr. Jaime Daniel Babot, [Dr. Adriana Perez Chaia](mailto:Dr.AdrianaPerezChaia), Centro de

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(International Sort)25 [apchaia@cerela.org.ar](mailto:apchaia@cerela.org.ar)

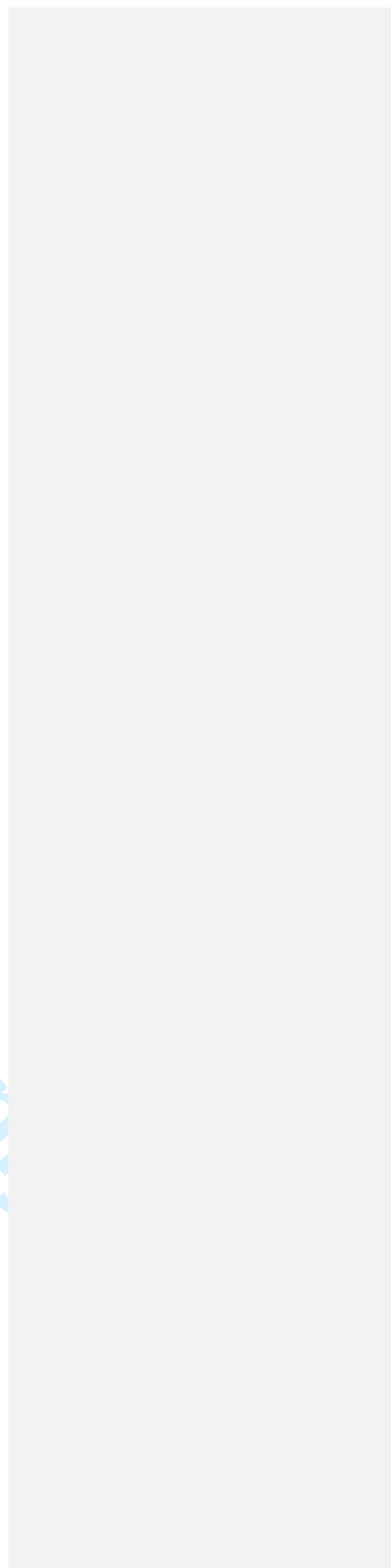


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7 **Abstract.** 1. ~~The wheat as Whole grain or milled or whole grain wheat~~ is often used  
8 instead of corn in poultry feeding, due to its high content of starch and proteins. Poultry  
9 fed with wheat-based diets ~~ingest~~ regularly ingest wheat germ agglutinin (WGA).  
10  
11 2. ~~Considering previous reports on the toxicity of WGA against several cell line types,~~  
12 ~~its toxicity to WGA showed toxic effect in vitro on~~ intestinal epithelial cells (IEC) ~~of~~  
13 ~~poultry was studied in enterocytes~~ obtained from fourteen-day-old broilers. ~~WGA~~  
14 ~~showed cytotoxic effect on IEC that~~ Cytotoxicity was dependent on time and lectin  
15 concentration; lethal dose (LD<sub>50</sub>) was 8.36 µg/mL for IEC exposed ~~duringfor~~ 2 h to  
16 WGA.  
17  
18 3. ~~A screening of lactic acid bacteria that express the complementary sugars to WGA on~~  
19 ~~their cell surfaces was performed and strains were evaluated as a tool to remove the~~  
20 ~~lectin avoiding the interaction with IEC.~~ 3. Complementary sugars to WGA were  
21 detected ~~in~~ on the surface of one *Enterococcus* and nine *Lactobacillus* strains of  
22 ~~different species and one *Enterococcus* strain, all of them~~ isolated from poultry, ~~with~~  
23 ~~and without the ability to adhere to tissues. These strains were evaluated as a lectin~~  
24 ~~removal tool intended to cytotoxicity prevention.~~  
25  
26 4. ~~The~~ The incubation of the lactic acid bacteria with WGA before the IEC-lectin  
27 interaction caused a remarkable reduction in the percentage of cell death ~~caused by~~  
28 ~~WGA was reduced by incubation of these strains with the lectin prior the IEC WGA~~  
29 ~~interaction or after bacteria-IEC adhesion. The extension of the protective effect against~~  
30 ~~the damage caused by WGA depended on the strain studied and. The protection~~ was  
31 attributed to the amount of lectin bound to the bacterial ~~surfaces~~ surfaces and was strain-  
32 dependent. *L. salivarius* LET 201 and *L. reuteri* LET 210 were significantly more  
33 efficient than the other lactic acid bacteria assayed.  
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51 5. These results provide bases for the development of probiotic supplements or cell-wall  
52 preparations of selected lactic acid bacteria intended to avoid harmful effects of a  
53 natural constituent of the grain in wheat-based diets.

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## INTRODUCTION

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

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

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7 76 acetyl-D-neuraminic acid residues (Rudiger and Gabius, 2001, Van Damme *et al.*,  
8  
9 77 1998). These complementary sugars are ubiquitously expressed by animal cells as they  
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11 78 constitute key molecular components of the membrane glycoconjugates. WGA binds to  
12  
13 79 glycosaminoglycans, glycolipids and also some glycoproteins via sialic acid residues  
14  
15 80 (Peters *et al.*, 1979). WGA is relatively stable at low pH and resistant to proteolysis  
16  
17 81 (Van Damme, Peumans, Pusztai and Bardocz, 1998) and thus it can overcome the  
18  
19 82 conditions of gastrointestinal tract and reach active the intestine (Jones *et al.*, 2012).  
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21 83 WGA has been proved toxic for Molt4, K562 and PBMC human leukemic cell lines  
22  
23 84 (Ohba *et al.*, 2003), and for Caco2 cells (Dalla Pellegrina *et al.*, 2005), among others.  
24  
25 85 The binding of WGA to enterocytes from the villi of proximal jejunum-ileum of chicks 2,  
26  
27 86 15 and 30 days post hatching (Pohlmeyer *et al.*, 2005), to enterocytes from the ileum of  
28  
29 87 chicks 2 days post hatching (Zhou *et al.*, 1995), and to enterocytes from the villi of  
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31 88 caecal tonsils of adult chickens (Kitagawa *et al.*, 2000) has been previously reported.  
32  
33 89 The interaction lectin-epithelium may alter the activity of digestive enzymes located on  
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35 90 the epithelial cells (Rueda *et al.*, 2007), and the integrity and permeability of the  
36  
37 91 intestinal epithelium allowing small molecules to cross the epithelial barrier  
38  
39 92 contributing to the toxicity of WGA to gastrointestinal cells (Dalla Pellegrina, Rizzi,  
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41 93 Mosconi, Zoccatelli, Peruffo and Chignola, 2005). In poultry, this may depress the  
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43 94 growth of broilers especially during the first days of life.

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45 95 Poultry industry has explored the benefit of including probiotic cultures in  
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47 96 broilers feed as alternative to antibiotic treatments for growth promotion. Probiotics  
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49 97 may contribute to xenobiotics metabolism or to avoid deleterious effects of toxic  
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51 98 compounds included in the diet by binding them on the bacterial envelopes that express  
52  
53 99 appropriated determinants (Carasi *et al.*, 2012, Turbic *et al.*, 2002, Zárate and Perez  
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55 100 Chaia, 2012). The binding of WGA to carbohydrates expressed on the bacterial surface  
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7 101 and its removal by the normal transit of the intestinal content may contribute to a  
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9 102 healthier broiler feed.

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11 103 In the present investigation we explore the toxicity of WGA on intestinal  
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13 104 epithelial cells of broilers and the ability of lactic acid bacteria isolated from poultry  
14  
15 105 intestine to remove WGA and protect cells from the harmful effects of this lectin.  
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## 18 107 MATERIALS AND METHODS

19 108

### 20 109 **Microorganisms and culture conditions**

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23 110 Thirteen strains of lactobacilli and one enterococcus isolated from poultry intestine were  
24  
25 111 used in the study. Suitable properties for probiotic strains selection were previously  
26  
27 112 determined, such as the growth at the corporal temperature of poultry, tolerance to pH,  
28  
29 113 bile salts and digestive enzymes, adhesion to small bowel mucosa, antibiotics sensitivity  
30  
31 114 and absence of virulence factors (Babot *et al.*, 2014). The strains were grouped as non-  
32  
33 115 adherent (*Lactobacillus salivarius* LET 201, *L. vaginalis* LET 202, *L. vaginalis* LET  
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35 116 203, *L. reuteri* LET 208, *L. reuteri* LET 209, *L. reuteri* LET 212 and *L. reuteri* LET  
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37 117 2013) and adherent (*L. reuteri* LET 204, *L. reuteri* LET 205, *L. reuteri* LET 206, *L.*  
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39 118 *reuteri* LET 207, *L. reuteri* LET 210, *L. reuteri* LET 211 and *Enterococcus faecium*  
40  
41 119 LET 301) bacteria.

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44 120 All strains were stored at -70 °C in 10% (w/v) reconstituted non-fat milk (NFM)  
45  
46 121 supplemented with 0.5% yeast extract and 15% glycerol. MRS broth (de Man *et al.*,  
47  
48 122 1960) and LAPTg broth (Raibaud *et al.*, 1973) were used to activate cultures of  
49  
50 123 lactobacilli and enterococci, respectively. Prior to use, cultures were incubated at 41.5 ±  
51  
52 124 0.5 °C for 12 h.

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54 125

### 126 **Intestinal epithelial cells extraction**

127 For extraction of intestinal epithelial cells (IEC), fourteen-day-old broiler chicks were  
128 slaughtered by cervical dislocation. The birds were immediately eviscerated for  
129 collection of ileum which was in turn rinsed repeatedly with ice cold PBS pH 7.40 to  
130 eliminate the digested content. The tissue ~~were~~was cut lengthwise and washed once  
131 again with cold PBS. Epithelial cells were scrapped from the surface using a sterile  
132 microscope slide and collected in ice cold PBS pH 7.40 supplemented with 1% Fetal  
133 Bovine Serum (PBS/FBS). The cells were washed twice with PBS/FBS, incubated with  
134 0.25% Trypsin-EDTA (Gibco, Grand Island, USA) at 37 °C for 5 min before cold  
135 PBS/FBS was added to stop the enzyme activity. The cells were immediately collected  
136 (800 × g, 5 min, 4 °C) and washed once with PBS/FBS prior to adjust their  
137 concentration to 1 × 10<sup>6</sup> cells/mL in RPMI 1640 medium supplemented with 1% FBS  
138 (RPMI/FBS). Cells were counted using a Neubauer chamber in a conventional light  
139 microscope at 40 × magnification (Zeiss–Axiolab; Cool Zeiss, Jena, Germany).

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

### 147 **WGA cytotoxicity**

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

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151 After 1 and 2 h of incubation, 100  $\mu$ L of cells suspensions were washed with  
152 RPMI/FBS and suspended in the same volume of medium. A freshly prepared mixture  
153 of Fluorescein diacetate (FDA; Sigma-Aldrich, Argentina) and Propidium iodide (PI)  
154 was added to obtain final concentrations of 7.5  $\mu$ g/mL of FDA and 2.5  $\mu$ g/mL of PI  
155 (Zárate and Perez Chaia, 2009). The samples were incubated on ice in the darkness for  
156 10 min and washed again. Cells with green cytoplasm and normal nuclei (viable) and  
157 cells with bright red nuclei (necrotic cells) were counted on ten microscopic fields by  
158 fluorescence microscopy with the appropriated filters ~~and the percentage.~~ The mean  
159 value of dead cells on each of two equally prepared suspensions was reported. ~~Dead as~~  
160 percentage of the total cells. Three independent assays were performed with the same  
161 procedure and the mean value of dead cells (%) vs. WGA concentration (C in  $\mu$ g/mL)  
162 was represented with data of 1 and 2 hours of incubation; ~~the maximum.~~ The maximum  
163 death (%<sub>max</sub>) and the WGA concentration responsible for half of this value (LD<sub>50</sub>) were  
164 determined for each condition using the mathematical expression of a model adapted for  
165 toxic chemicals in experimental bioassays (Sánchez-Bayo and Goka, 2007). The  
166 mathematical expression to represent the toxicity model was:

$$167 \text{ Death (\%)} = \frac{\text{Maximun death (\%}_{\text{max}}) \times C}{LD_{50} + C}$$

168

#### 169 **Carbohydrates of the bacterial surfaces**

170 Surface carbohydrates of the strains were assessed using FITC-labeled lectins (Sigma-  
171 Aldrich, Buenos-Aires, Argentina) with different carbohydrates specificity (Table).  
172 Active cultures of the fourteen strains were adjusted to Absorbance ( $A_{600\text{nm}}$ ) of 0.7,  
173 washed three times with a lectin buffer (60.57 g/L Tris, 87 g/L NaCl, 1.11 g/L CaCl<sub>2</sub>,  
174 pH 7.60) described by Leathem and Brooks (1997), suspended in equal volume of

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7 175 buffer containing 20 µg/mL of one of the five FITC-labeled lectin and incubated 1 h at  
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9 176 25 °C (Wanchoo *et al.*, 2009). The same procedure was carried out for each lectin and  
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11 177 strain. Cells suspensions were centrifuged (10000 × g, 10 min, 4 °C) at the end of  
12  
13 178 incubation. Harvested cells were washed 4 times, suspended in equal volume of lectin  
14  
15 179 buffer and observed on a conventional fluorescence microscope (Carl Zeiss Axio Scope  
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17 180 A1, Gottingen, Germany) fitted with the appropriated filter at 100 × magnification. The  
18  
19 181 mean number of fluorescent cells was assessed by counting them in 10 microscopic  
20  
21 182 fields and the number of fluorescent bacteria/mL was determined as (~~Lorenzo-Pisarello~~  
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23 183 ~~*et al.*, 2010~~); Lorenzo-Pisarello *et al.* (2010). The fluorescence intensity of each cells  
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25 184 suspension was also measured with a fluorospectrophotometer (Cary Eclipse, Varian  
26  
27 185 Inc., Walnut Creek, California, USA) and reported as arbitrary units (AU) / bacteria.

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29 186 Each trial was reproduced three times with new active cultures of each strain.

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31 187 

<u>Table somewhere near here</u>
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### 32 33 188 **Exopolysaccharide production**

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35 189 The production of exopolysaccharides by the strains was assessed according to Mozzi *et*  
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37 190 *al.* (2001). Briefly, 2.5 µl of India ink were spotted onto microscopic slides and 10 µl of  
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39 191 active cultures (O.D.<sub>560</sub> 0.70) were added, the mixtures were covered with coverslips  
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41 192 and the excess of liquid was eliminated by gently pressing on them with absorbent  
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43 193 paper. Finally, the samples were observed in a light microscope (Zeiss–AxioLab;  
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45 194 ~~Carl~~ Zeiss, Jena, Germany). Bright areas surrounding bacteria indicated the  
46  
47 195 production of exopolysaccharide.

### 48 49 50 51 197 **Protection of epithelial cells by bacteria**

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53 198 Suspensions of  $1 \times 10^8$  bacteria/mL of adherent and non-adherent strains with ability to  
54  
55 199 bind WGA were incubated with 50 µg/mL of the lectin in RPMI/FBS for 1 h at  $41.5 \pm$



0.5 °C. Mixtures were centrifuged ( $10000 \times g$ , 10 min, 4 °C) and the supernatants stored at 4 °C for further use. Freshly prepared IEC were centrifuged, suspended to reach  $5 \times 10^5$  cells/mL in the stored supernatants or in RPMI/FBS containing 50 µg/mL of WGA, and incubated during 2 h at  $41.5 \pm 0.5$  °C under 5% CO<sub>2</sub> atmosphere- ([Nuair 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 ( $1 \times 10^8$  bacteria/mL) and IEC ( $5 \times 10^5$  cells/mL) were prepared and incubated 30 min at  $41.5 \pm 0.5$  °C under 5% CO<sub>2</sub> atmosphere to allow adhesion. Then, the mixtures were centrifuged ( $120 \times g$ , 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  $1 \times 10^8$  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  $41.5 \pm 0.5$  °C under 5% CO<sub>2</sub> 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  $1 \times 10^8$  CFU/mL for 1 h at  $41.5 \pm 0.5$  °C ~~in a mixture of~~ under 5-% CO<sub>2</sub> ~~and 95-% O<sub>2</sub>-atmosphere~~. After incubation, the mixtures were centrifuged ( $120 \times g$ , 5 min, 4 °C), washed twice with RPMI and suspended in the initial volume of RPMI/FBS

225 containing 50 µg/mL of WGA. After 2 h of incubation at  $41.5 \pm 0.5$  °C under 5% CO<sub>2</sub>  
226 atmosphere, cells adhesion to IEC was examined by counting adhered bacteria in 30  
227 IEC, using phase-contrast microscopy. Results were expressed as the percentage of IEC  
228 with adhered bacteria (adhesion percentage).

### 230 Statistical analysis

231 Three independent assays were performed for each *in vitro* experiment and the mean  
232 values  $\pm$  SD were obtained for each sample. Significant differences were determined by  
233 Tukey's test after analysis of variance (ANOVA) with ~~Minitab-Statistic-Program;~~  
234 ~~release-12-for-Windows-OriginPro-8-SR0-v8.0724~~ (OriginLab Corporation,  
235 Northampton, MA, USA). A value of  $P < 0.05$  was considered statistically significant.

## 31 RESULTS

### 339 WGA cytotoxicity

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

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7 250 that induce the death of half of the cells in the suspension (lethal dose 50, LD<sub>50</sub>) at each  
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9 251 incubation time (Purich and Allison, 2000). Results for LD<sub>50</sub> were 56.7 and 8.4 µg/mL  
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11 252 for cells incubated during 1 and 2 hours, respectively. Maximum death percentages  
12  
13 253 produced by the lectin were 90.6 % and 90.2 %, respectively.  
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15 254

### 17 255 **Characterization of surface carbohydrates and WGA removal**

18  
19 256 The characterization of surface carbohydrates of the strains was carried out through the  
20  
21 257 use of FITC-labeled lectins. The binding of lectins to the cells surface was confirmed by  
22  
23 258 fluorescence microscopy and the relative abundance of lectin-linked carbohydrates was  
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25 259 registered by fluorospectrophotometry as arbitrary units of fluorescence (AU). Figure  
26  
27 260 2A represents the AU obtained when *Arachis hypogaea* agglutinin (PNA), *Dolichos*  
28  
29 261 *biflorus* agglutinin (DBA) or *Ulex europaeus* agglutinin (UEA-I) were incubated with  
30  
31 262 the studied strains; Figure 2B shows the results of incubation with *Phaseolus vulgaris*  
32  
33 263 agglutinin (PHA-P) or WGA. The only strain tested of *L. salivarius* ligated significant  
34  
35 264 amount of PNA and WGA; one of the strains of *L. vaginalis* ligated PNA and UEA-I  
36  
37 265 while *E. faecium* only captured WGA. Nine strains of *L. reuteri* removed WGA, three  
38  
39 266 of them captured also PNA and two strains removed PHA-P. The lectins DBA and  
40  
41 267 UEA-I linked to only one of the strains of *L. reuteri* each. The fluorescence intensity  
42  
43 268 measured evidenced that WGA was captured by lactobacilli and *E. faecium* LET 301 in  
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45 269 significantly higher amount than the other lectins tested.

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47 270  **Figure 2 somewhere near here**

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### 48 49 271 **Exopolysaccharide production**

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51 272 Only *L. reuteri* LET 206, *L. reuteri* LET 209 and *E. faecium* LET 301 out of 14 strains  
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53 273 produced exopolysaccharide, as evidenced by the observation of bright areas  
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274 surrounding bacteria due to colloidal carbon exclusion by exopolysaccharide (data not  
275 shown).

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### 277 **Protection of cells by adherent and non-adherent bacteria**

278 Lectin removal by lactic acid bacteria was evaluated as a tool to protect epithelial cells  
279 facing cytotoxic lectins. Taking into account that the ability to remove WGA was  
280 observed in adherent and non-adherent strains, the protective effect of bacteria on WGA  
281 cytotoxicity was evaluated following different protocols depending on the strains  
282 studied. Non-adherent bacteria were studied simulating the conditions of free bacteria in  
283 the intestinal lumen (Fig. 3). Dead cells due to the toxic effect of WGA were  
284 determined and represented as percentages of total cells in the suspensions. These  
285 values were compared with the percentage obtained in samples of IEC incubated with  
286 supernatants of lectin-bacteria interaction for each strain under study. As expected, cell  
287 death was significantly reduced when IEC were exposed to supernatants of the lectin-  
288 bacteria interactions. A remarkable protective effect was observed for *L. salivarius* LET  
289 201, which reduced cell death in almost 54 % while the lowest effect was that of *L.*  
290 *reuteri* LET 209 with a death reduction of almost 10%. An unexpected result was  
291 observed for *L. reuteri* LET 208, which reduced cells death in almost 42%, as the  
292 previously assessed amount of lectin removed by this strain was lower than ~~in~~ that for *L.*  
293 *reuteri* LET 209 (Fig. 2). The effective ~~amounts~~ amount of lectin in supernatants of  
294 WGA-bacteria interactions and in the WGA solutions used in each assay were  
295 determined from the percentages of cellular death measured in the cytotoxicity assays  
296 using the %<sub>max</sub> and LD<sub>50</sub> values previously obtained (subsection 3.1). The lectin  
297 removed by each strain was assessed as the difference between these values. –The  
298 suspension of  $1 \times 10^8$  cells of *L. salivarius* LET 201 removed  $46.9 \pm 0.4$  µg of WGA,

299 while *L. reuteri* LET 209 removed  $26.0 \pm 0.6$   $\mu\text{g}$  of WGA in the conditions used in the  
300 trial.

here near here

301 The effect of ~~binding the~~ lectin ~~capture~~ by adherent bacteria was first assessed in  
302 a protocol that simulates the conditions of free bacteria in the intestinal lumen (Fig.  
303 4A3). *L. reuteri* LET 210 exerted the highest protection lowering ~~45.5 % of the~~ cell  
304 ~~death~~ ~~death~~ approximately 45 %. The less efficient strain was *L. reuteri* LET 204 which  
305 reduced in ~~almost 22.1~~ % the percentage of dead cells. The lectin removal assessed by  
306 the percentage of ~~death~~ ~~dead~~ cells was  $45.6 \pm 0.3$   $\mu\text{g}$  of lectin for *L. reuteri* LET 210 and  
307  $37.2 \pm 0.5$   $\mu\text{g}$  of lectin for the strain LET 204.

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Figure 4a  
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308 The same strains were assayed for the ability to protect the epithelial cells of  
309 lectin toxicity during the bacteria-IEC interaction. As observed in Figure 4B4, strain *L.*  
310 *reuteri* LET 210 exerted scarce protection of the cells when it was in intimate relation to  
311 them; cell death was reduced in approximately 5 % and represented the removal of  $15.2$   
312  $\pm 2.5$   $\mu\text{g}$  of lectin. In the contrary, *L. reuteri* LET 204 reduced in ~~almost~~ 17 % the cells  
313 death by removal of  $33.2 \pm 0.3$   $\mu\text{g}$  of lectin. Moderate protection was achieved with *L.*  
314 *reuteri* LET 211, *L. reuteri* LET 207 and *E. faecium* LET 301. *L. reuteri* LET 206 was  
315 the only strain that increased slightly the cell death when IEC with adhered bacteria  
316 were exposed to WGA. The damage was also observed during the bacteria-IEC  
317 interaction without exposure to WGA (data not shown).

Figure 4b somewhere near here

Figure 4 somewhere near here

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318  
319 As shown in Figures 3 and 4, comparisons of the protective effect of the strains  
320 were performed for each protocol used. The statistical analysis of the reduction of cell  
321 death obtained by each strain in the protocol that simulates free bacteria in the intestinal  
322 lumen, demonstrated that the highest protection was exerted by *L. salivarius* LET 201, a  
323 non-adherent strain, followed by *L. reuteri* LET 210, an adherent one (Fig. 3). The

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

### 327 **Effect of WGA on the bacterial adhesion to IEC**

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

332 **Figure 5 somewhere near here**

## 333 DISCUSSION

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

339 ~~Plant lectins are associated to practical applications such as detection of~~  
340 ~~carbohydrates on cells surface, mapping neuronal pathways, glycoproteins biosynthesis,~~  
341 ~~among others (Singh and Sarathi, 2012), but they are also related to toxicity and human~~  
342 ~~diseases. The toxicity of a few lectins has been studied (Wu and Sun, 2012), and most~~  
343 ~~of these studies have been done using tumoral cell lines. Several researches have shown~~  
344 ~~that these proteins may inhibit tumor growth, especially by causing cytotoxicity and~~  
345 ~~apoptosis and thus could be considered as potential therapeutic agents (Mody et al.,~~  
346 ~~1995), although their contribution in this field is still under study.~~

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

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

357 In this study, WGA showed cytotoxicity towards IEC of fourteen-day-old chicks  
358 ~~two-week-old~~, which was dependent on the time of exposition and concentration used.  
359 The percentage of cell death caused by the incubation of WGA with IEC ~~allowed was~~  
360 used to establish the assessment values of ~~the~~ LD<sub>50</sub> for 1 and 2 h of exposure (56.7 and  
361 8.4 µg/mL, respectively). These ~~values~~ were comparable to the ~~effect of WGA reported~~  
362 on other cells types. Indeed, the LD<sub>50</sub> for WGA on the human leukemic cell lines Molt4,  
363 K562 and PBMC was 5.0 µg/mL for the first and higher than 72.0 for the last two, after  
364 24 h of incubation (Ohba, Bakalova and Muraki, 2003).

365 ~~Lactic acid bacteria are Gram (+) microorganisms, thus they show a~~  
366 ~~peptidoglycan rich cell wall which accounts for almost 40% of its weight (Shockman~~  
367 ~~and Barrett, 1983). This structure contains one or more accessory polymers, such as~~  
368 ~~teichoic and teichuronic acids, and other neutral or acidic polysaccharides (Munson and~~  
369 ~~Glaser, 1981, Rogers *et al.*, 1980, Ward, 1981). Repetitive units of N-acetyl-muramic~~  
370 ~~acid and N-acetyl-glucosamine joint through β-1,4-glycosidic links are the main~~  
371 ~~constituent of this polymer (Rogers, Perkins and Ward, 1980, Schleifer and Kandler,~~  
372 ~~1972, Tipper and Wright, 1979). The production of extracellular polymers seems to be~~  
373 ~~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  $\alpha$ -D-mannose and/or  $\alpha$ -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- $\beta$ -1,4-GlcNAc, NeuNAc and  $\alpha$ -L-Fucose as surface carbohydrates. Only one strain of *L. vaginalis* expressed Gal- $\beta$ -1,3-GalNAc and minor amount of  $\alpha$ -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- $\beta$ -1,4-GlcNAc and/or NeuNAc as surface carbohydrates. Minor amount of Gal- $\beta$ -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- $\beta$ -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

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7 424 species. In our ~~investigations~~study, the strains binding WGA evidenced higher  
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9 425 fluorescence intensity than ~~interacting with~~ after capture of other lectins. Moreover, all  
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11 426 the strains of *L. reuteri* ~~species~~ were able to ~~capture~~bind WGA but the amount of lectin  
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13 427 attached to each strain was significantly different.

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15 428 Adherent and non-adherent strains of lactic acid bacteria could avoid the  
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17 429 interaction eukaryotic cells-WGA by different mechanisms depending on the location of  
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19 430 these bacteria within the intestine. The protection against cytotoxicity was assayed  
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21 431 considering that the ~~lectin~~-capture of WGA by non-adherent bacteria could take place in  
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23 432 the intestinal lumen, thus reducing the amount of free ~~WGA~~lectin able to interact with  
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25 433 IEC. The protection mediated by adherent bacteria, was assayed allowing the adhesion  
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27 434 of bacteria to IEC prior to the addition of WGA to interfere in the interaction between  
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29 435 the lectin and eukaryotic cells. Results showed that binding of WGA depended on the  
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31 436 strain assayed and the conditions used to study this property. –Among non-adherent  
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33 437 bacteria, *L. salivarius* LET 201 exerted the highest protection. *L. reuteri* LET 208, was  
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35 438 more efficient for the binding of lectin in RPMI medium at  $41.5 \pm 0.5$  °C than in lectin  
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37 439 buffer at 25 °C when compared with the strain *L. reuteri* LET 209, suggesting that  
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39 440 environmental factors could be involved in the interaction of WGA with some strains.  
40  
41 441 On the other hand, the adherent strain *L. reuteri* LET 210 was the less efficient when  
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43 442 IEC, bacteria and WGA were incubated together in the same medium, simulating the  
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45 443 environment near to the intestinal epithelium. This finding suggested that despite the  
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47 444 binding of bacteria to IEC surfaces, in some cases the determinants involved in the  
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49 445 interaction IEC-WGA remain exposed in the cellular surface. The failure in blocking  
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51 446 the access to these determinants due to the lower size of bacteria related to eukaryotic  
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53 447 cells or the binding of bacteria to IEC by surface molecules other than WGA receptors  
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55 448 could be the reasons of these results. Conversely, many adherent strains succeeded in  
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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  $1 \times 10^8$  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

#### 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.~~

478

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## 486 DISCLOSURE STATEMENT

487 No potential conflict of interest was reported by the authors.

488

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**FITC-labeled dietary lectins**

Lectin	Source	Specificity
Con A	<i>Canavalia ensiformis</i> (jack bean)	$\alpha$ -D-mannose, $\alpha$ -D-glucose
DBA	<i>Dolichos biflorus</i> (horse gram)	GalNAc- $\alpha$ -1,3-GalNAc
PHA-P	<i>Phaseolus vulgaris</i> (kidney bean)	Gal- $\beta$ -1,4-GalNAc- $\beta$ -1,2-Man
PNA	<i>Arachis hypogaea</i> (peanut)	Gal- $\beta$ -1,3-GalNAc
UEA-I	<i>Ulex europaeus</i> (furze)	$\alpha$ -L-Fucose
WGA	<i>Wheat germ agglutinin</i> (wheat)	GlcNAc- $\beta$ -1,4-GlcNAc, NeuNAc

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12 **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

14 assays ± standard deviations (SD).

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18 **Figure 2.** Relative abundance of lectin-linked carbohydrates on the surface of bacteria. Formatted: Font: Times New Roman, 12 pt

19  
20 (A) Binding of PNA (■), DBA (□) and UEA-I (▣). (B) Binding of PHA-P (□) and

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22 WGA (▣) by the strains *L. salivarius* LET201, *L. vaginalis* LET 202 and 203, *L.*

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24 *reuteri* LET 204, LET 205, LET 206, LET 207, LET 208, LET 209, LET 210, LET 211,

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26 LET 212 and LET 213; *E. faecium* LET 301. Results are expressed as mean values ±

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28 SD of three independent assays ± SD for each strain and lectin used.

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31 **Figure 3.** Protection of WGA cytotoxicity by non-adherent removal of lectin by strains- Formatted: Font: Times New Roman, 12 pt

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33 of lactobacilli and *Enterococcus*. Percentage of dead cells after IEC incubation with

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35 WGA ( ) and after their incubation with supernatant of bacteria-WGA interaction ( → ),

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37 and the reduction in cell death ( ) due to the lectin removal are represented as mean

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39 values of three independent assays ± SD. Significant differences between results for

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41 each strain and its control are indicated with an asterisk ( $p \leq$  Values of reduction in cell

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43 death with no common superscript letter differ significantly at  $P < 0.05$ ).

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45 **Figure 4.** Protection of WGA cytotoxicity by adherent strains. (A) Percentage of dead Formatted: Font: Times New Roman, 12 pt

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47 cells after attachment of lactobacilli and *Enterococcus* to IEC incubation with WGA ( → )

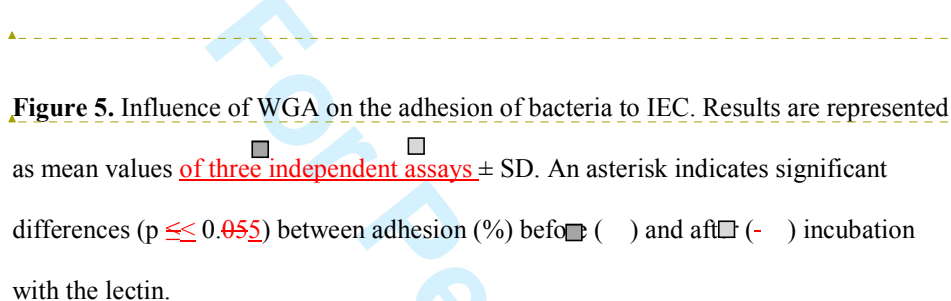
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49 and after their incubation with supernatant of bacteria-WGA interaction ( — ). (B) Formatted: Indent: First line: 0"

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51 Percentage of dead cells after IEC incubation with WGA ( → ) and after incubation of

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53 bacteria-bound IEC with WGA ( → ). Results), and the reduction in cell death ( ) are

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613 represented as mean values of three independent assays  $\pm$  SD. Significant differences  
 614 between results for each strain and its control are indicated ( $p \leq$  Values of reduction in  
 615 cell death with no common superscript letter differ significantly at  $P < 0.05$ ).

616  **Figure 5.** Influence of WGA on the adhesion of bacteria to IEC. Results are represented  
 617 as mean values of three independent assays  $\pm$  SD. An asterisk indicates significant  
 618 differences ( $p \leq 0.05$ ) between adhesion (%) before (□) and after (-) incubation  
 619 with the lectin.

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