Dietary β-galactomannans have beneficial effects on the intestinal morphology of chickens challenged with Salmonella enterica serovar Enteritidis

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ABSTRACT: Salmonella enterica serovar Enteritidis is one of the leading causes of food-borne salmonellosis in humans. Poultry is the single largest reservoir, and the consumption of incorrectly processed chicken meat and egg products is the major source of infection. Since 2006, the use of antibiotics as growth promoters has been banned in the European Union, and the dietary inclusion of β-galactomannans (βGM) has become a promising strategy to control and prevent intestinal infections. The aim of this study was to investigate the effect of various βGM-rich products on intestinal morphology in chickens challenged with Salmonella Enteritidis. To assess this effect, a total of 280 male Ross 308 chickens were studied (40 animals per treatment housed in 5 cages). There were 7 treatments, including controls: uninoculated birds fed the basal diet (negative control) and inoculated birds fed the basal diet (positive control) or the basal diet supplemented with Salmosan (1 g/kg), Duraió gum (1 g/kg), Cassia gum (1 g/kg), the cell walls of Saccharomyces cerevisiae (0.5 g/kg), or the antibiotic colistine (0.8 g/kg). The birds were fed these diets from the d 1 to 23, except the animals in the colistine group, which were fed the diet containing the antibiotic only from d 5 to 11. The inoculated animals were orally infected on d 7 with 10⁸ cfu of Salmonella Enteritidis. Bird performance per replicate was determined for the whole study period (23 d), and the distal ileum and cecal tonsil of 5 animals per treatment (1 animal per replicate) were observed at different magnification levels (scanning electron, light, and laser confocal microscopy). In the images corresponding to the treatments containing βGM we observed more mucus, an effect that can be associated with the observation of more goblet cells. Moreover, the images also show fewer M cells, which are characteristic of infected animals. Regarding the morphometric parameters, the animals that received Duraió and Cassia gums show greater (P = 0.001 and P = 0.016, respectively) villus length compared with the animals in the positive control, thus indicating the capacity of these products to increase epithelial surface area. However, no effect (P > 0.05) on microvillus dimensions was detected. In conclusion, the results obtained indicating the beneficial effects of these βGM on intestinal morphology give more evidence of the positive effects of these supplements in poultry nutrition.

Key words: chicken, goblet cells, M cells, mannan oligosaccharides, Salmonella, villus length
Enteritidis infection and often develop systemic disease with varying degrees of mortality, whereas most adult animals typically remain asymptomatic (Lister, 1988; Velge et al., 2005; Golden et al., 2008).

One of the European Union’s objectives is the reduction of food-borne human diseases. Since 2006, the use of antibiotics as growth promoters (AGP) has been banned in the European Union (regulation [EC] no. 1831/2003) because of the risk of transmission of antibiotic resistance to human pathogens (WHO, 1997). For this reason, natural feed additives such as mannan oligosaccharides (MOS) are promising alternatives to AGP (Zanello et al., 2009; Gaggìa et al., 2010). It has been indicated that MOS benefit intestinal function by enhancing the mucosal immune system (Gaggìa et al., 2010). In addition, Salmonella can adhere to MOS by mannose-specific lectins in type 1 fimbriae, thus competing for adhesion to glycoproteins of the intestinal epithelium, which are also rich in mannose (Spring et al., 2000).

The objective of this study was to determine the effect of various MOS products rich in β-galactomannans (βGM) on the morphology of the intestinal epithelium in chickens challenged by Salmonella Enteritidis. The βGM tested were developed at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA; Generalitat de Catalunya, Constantí, Spain) from Ceratonia siliqua L. and Cassia obtusifolia L. seeds. The results obtained may contribute to the design of dietary strategies to improve chicken intestinal homeostasis and thus the quality of poultry products destined for human consumption.

MATERIALS AND METHODS

The experimental protocols were approved by the Ethical Committee for Animal Experimentation of IRTA, in accordance with current regulations on the use and handling of experimental animals (Decree 214/97, Generalitat de Catalunya), and were performed following the European Union principals for animal care and experimentation.

Animals and Diets

A total of 280 male Ross 308 chickens (Gallus gallus domesticus L. from Granja Crusvi, Montblanc, Spain) were raised at IRTA’s Research Station in Valls for 23 d at standard temperature (d 0 to 2: 32°C to 34°C, d 3 to 7: 27°C to 30°C, d 8 to 14: 25°C to 27°C, and d 15 to 23: 24°C to 27°C), humidity, and light:dark conditions (d 0 to 4: 23:1 h, d 5 to 10: 20:4 h, and d 11 to 23: 18:6 h). The experimental design was as follows: 40 animals per treatment were housed in 5 cages (5 replicates of 8 birds) and were fed ad libitum from hatching until d 23 with balanced diets (IRTA; Table 1). The basal diet did not include AGP, coccidiostats, or exogenous enzymes, except those specified in the treatments.

The animals were orally inoculated on d 7 with 1 mL of PBS (0.1 mol/L, pH 7.2) suspension containing 10⁸ cfu of Salmonella enterica serovar Enteritidis (phage type 4, nalidixic acid–resistant strain; Centre de Recerca en Sanitat Animal, Bellaterra, Spain). To prepare the inoculum, the bacteria were grown at 37°C in broth culture triptych soy agar (Difco, Barcelona, Spain) supplemented with 200 μg/mL nalidixic acid.
(Sigma-Aldrich, St. Louis, MO) for 18 h. The bacterial suspension was prepared in PBS (0.1 mol/L, pH 7.2) to obtain an optical density of around 0.82 at 450 nm. The presence of *Salmonella* Enteritidis in the ceca was confirmed by PCR following the previously described ISO methodology (UNE-EN ISO6579:2003/A1:2007).

There were 7 treatments, including controls: 1) uninoculated birds fed the basal diet (defined as the negative control treatment, NC), 2) inoculated birds fed the basal diet (defined as the positive control treatment, PC), 3) inoculated birds fed the basal diet plus Salmosan (SA; 1 g/kg, patent WO2009/144070A2, licensed by Industrial Tècnica Pecuaria [ITPSA], Barcelona, Spain), 4) inoculated birds fed the basal diet plus Duraíó gum (DU; 1 g/kg, 90% Duraíó gum and 10% Sipernat 2200 from Evonik Industries, Essen, Germany, plus 0.4% “on top” β-mannanase, ITPSA), 5) inoculated birds fed the basal diet plus Cassia gum (CA; 1 g/kg, 90% Cassia gum and 10% sipernet plus 0.4% “on top” β-mannanase, ITPSA), 6) inoculated birds fed the basal diet plus the cell walls of *Saccharomyces cerevisiae* (SC; 0.5 g/kg, Lynside, Lesaffre Feed Additives, Marcq-en-Barœul, France), and 7) inoculated birds fed the basal diet plus colistine (CO; 0.8 g/kg colistine 4%). The birds were fed these diets for the 23 d of the experiment, except the animals in the CO treatment, which were fed the diet containing the antibiotic only from d 5 to 11. The effect of *Salmonella* Enteritidis infection was assessed by comparing animals in the PC and NC treatments; the effect of feeding treatments was assessed by comparing inoculated birds fed the products with inoculated birds fed the basal diet (PC). Bird performance was determined for the whole study period (23 d) for the 5 replicates of 8 birds per treatment.

**Sample Processing**

Five animals per treatment (1 per replicate) were euthanized by cervical dislocation. The ileum (the portion connected by mesenteric tissue to the ceca) and the proximal region of the ceca containing the cecal tonsil were removed and washed in PBS (0.1 mol/L, pH 7.4). For electron microscopy, 9 intestinal portions of approximately 4 mm$^2$ in surface area were obtained from the distal region of the ileum (just before the ileocecal junction): 6 for transmission electron microscopy (TRA) and 3 for scanning electron microscopy (SCA). Moreover, 4 complete transverse intestinal portions of approximately 10 mm in length were taken from the area proximally adjacent to the SCA and TRA samples for light (LIG) and confocal (CON) microscopy. Only 3 intestinal portions for SCA were taken from the cecal tonsil. Scanning and transmission electron specimens were fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L PBS (pH 7.4, 4°C) for 2 h. The samples for LIG and CON were fixed with 3.0% paraformaldehyde in 0.1 mol/L PBS for 2 h at room temperature and stored overnight at 4°C before being further processed.

**Scanning Electron Microscopy**

After fixation, the samples were washed in PBS (0.1 mol/L, pH 7.4), postfixed in 1% OsO$_4$ and 0.8% potassium ferrocyanide in 0.2 mol/L PBS (pH 7.4), and dehydrated in graded series of ethanol. Finally, the samples were maintained in ethanol for critical point drying with liquid CO$_2$ and coated with gold. Specimens were examined with a Zeiss DSM 940A electron microscope (Jena, Germany) operating at 15 kV. Sample processing and observation was performed at the Centres Científics i Tecnològics of the Universitat de Barcelona.

**Transmission Electron Microscopy**

After fixation, the samples were washed in PBS (0.1 mol/L, pH 7.4) and postfixed in OsO$_4$ (1%, in 0.2 mol/L PBS, pH 7.4, 4°C), dehydrated with acetone, and further embedded in Spurr’s embedding medium. Ultrathin sections (60 nm, Ultracut, Reichert-Jung, Wetzlar, Germany) were obtained from the areas of interest (the upper third of the villi, avoiding the extrusion zone) and were stained with uranyl acetate and lead citrate. Specimens were examined with a JEOL JEM 1010 electron microscope (Tokyo, Japan) operating at 80 kV. Sample processing and observation were performed at the Centres Científics i Tecnològics of the Universitat de Barcelona.

Microvillus length and diam. were measured from longitudinal and transversal sections, respectively, in micrographs of the same magnification for each variable. The images were processed using IMAT software (Centres Científics i Tecnològics, Universitat de Barcelona). The results of these morphometric parameters were obtained from at least 10 microvilli in each micrograph. In the case of microvillus length, a total of 105 to 131 micrographs per treatment were measured, and for microvillus diam., a total of 43 to 95 micrographs were measured. First, the average value for each micrograph was calculated, and from these data, the mean value for each replicate was obtained.

**Light Microscopy**

After fixation, the samples were cryoprotected in an ice-cold sucrose gradient in 0.1 mol/L PBS (5% for 2 h, 10% for 2 h, and 30% overnight), mounted at optimal cutting temperature (OCT, Aname, Barcelona, Spain) with adequate orientation to obtain transverse...
sections of the intestine, and stored at −20°C. Four groups of 7 serial transverse cryostatic sections (20 µm, CM 3050 S, Leica, Wetzlar, Germany) were obtained at intervals of 300 µm to guarantee the observation of different villi. Images were captured with an Olympus BX41 microscope (Tokyo, Japan). The morphometric variables determined in these images were villus length, measured using the lamina propria as the base (Uni et al., 1995), and muscularis thickness, using the serosa as the base (Chichlowski et al., 2007). These variables were measured in micrographs of the same magnification, processed with IMAT software. In the case of villus length, first, the average value for each micrograph was calculated, and from those data, the mean value for each replicate was obtained. As for muscularis thickness, because only 1 measurement can be obtained in each micrograph, the mean value for each replicate was obtained from those data. The same number of micrographs for both variables were analyzed per treatment (75 to 120).

Confocal Microscopy

The samples were fixed and mounted as for LIG. Cryosections that were 10-µm-thick were collected on Superfrost Plus slides (Aname), washed twice for 5 min in 10 mmol/L PBS containing 20 mmol/L glycine (PBS-glycine), and permeabilized with 1% Triton X-100 for 10 min at room temperature. The slides were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). Tissues were incubated with primary antibody against zonula occludens-1 (mouse anti-ZO-1, 1:100, Life Technologies, Invitrogen, Barcelona, Spain) for 1 h and washed twice in PBS-glycine before incubating with Hoechst (1:4,000, Life Technologies) for 1 h and washed twice in PBS-glycine for 5 min at room temperature. The samples were then incubated for 1 h at room temperature with the secondary antibody (Alexa Fluor 647 donkey anti-mouse, 1:300, Life Technologies). Finally, the slides were incubated with secondary antibody (Alexa Fluor 647 donkey anti-mouse, 1:300, Life Technologies) and mounted with Prolon Gold (Life Technologies). The samples were observed in the Leica TCS-SP5 microscope of the Serveis Científics i Tecnològics of the Universitat de Barcelona.

Statistical Analysis

Results are given as means with their SD or SEM. The study had a randomized complete block (based on replicate location within the room) design with 5 replicates of 8 birds per treatment and 5 replicates of 1 bird per treatment for the morphometric study. The cage was used as the experimental unit. Significant differences were detected by 1-way ANOVA followed by Student’s t test using PASW Statistics 18 software (formerly SPSS Statistics, IBM, Chicago, IL). \( P < 0.05 \) was considered to denote significance.

RESULTS

The bird performance data are shown in Table 2. The growth values are lower than Ross 308 standards but according to growing conditions in cages instead of floor pens. No differences \( (P > 0.05) \) were detected among treatments. Inoculation of Salmonella Enteritidis did not affect (PC vs. NC group; \( P > 0.05 \)) performance.

The images reported in Fig. 1 were selected as representative images of the samples observed per treatment. Villus shape and distribution in the ileum and cecal tonsil did not differ among treatments (Fig. 1, column A for the ileum; images not shown for the cecal tonsil). The most striking feature at this magnification level is that the epithelium of the animals that received the diets supplemented with βGM (SA, DU, CA, and SC; Fig. 1, SA-A to SC-A) is covered in greater amounts of mucus than those of animals in NC and PC treatments (Fig. 1, column A for the ileum; images not shown for the cecal tonsil). The most striking feature at this magnification level is that the epithelium of the animals that received the diets supplemented with βGM (SA, DU, CA, and SC; Fig. 1, SA-A to SC-A) is covered in greater amounts of mucus than those of animals in NC and PC treatments (Fig. 1, column A for the ileum; images not shown for the cecal tonsil). At higher magnification, the animals in the PC treatment show rod-shaped bacteria (Fig. 1, PC-B for the ileum), morphologically compatible with Enterobacteriaceae such as Salmonella Enteritidis (Chadfield and Hinton, 2004), adhered to the mucus and to the epithelium. In these animals, the images also reveal the presence of numerous M cells in the epithelium in comparison to animals in the NC treatment (Fig. 1, PC-C vs. NC-B for the cecal tonsil); in some cases the M cells were found forming lamellipodia covered by bacteria (Fig. 1, PC-D for the cecal tonsil and ileum). At this magnification level, the animals that received the

### Table 2. Bird performance for the whole period (23 d)\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>BW, g</th>
<th>ADG, g</th>
<th>ADFI, g</th>
<th>G/F, kg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>844 ± 86.5</td>
<td>34.7 ± 3.76</td>
<td>50.4 ± 5.12</td>
<td>1.457 ± 0.0371</td>
</tr>
<tr>
<td>PC</td>
<td>874 ± 64.4</td>
<td>36.0 ± 2.78</td>
<td>51.5 ± 3.90</td>
<td>1.432 ± 0.0159</td>
</tr>
<tr>
<td>SA</td>
<td>914 ± 131.4</td>
<td>37.7 ± 5.71</td>
<td>54.9 ± 8.44</td>
<td>1.459 ± 0.0798</td>
</tr>
<tr>
<td>DU</td>
<td>809 ± 35.8</td>
<td>33.1 ± 1.56</td>
<td>49.7 ± 3.61</td>
<td>1.498 ± 0.0467</td>
</tr>
<tr>
<td>CA</td>
<td>884 ± 37.5</td>
<td>36.4 ± 1.63</td>
<td>52.3 ± 2.46</td>
<td>1.437 ± 0.0156</td>
</tr>
<tr>
<td>SC</td>
<td>903 ± 77.3</td>
<td>37.2 ± 3.36</td>
<td>53.7 ± 4.54</td>
<td>1.442 ± 0.0439</td>
</tr>
<tr>
<td>CO</td>
<td>807 ± 133.1</td>
<td>33.0 ± 5.79</td>
<td>51.1 ± 6.60</td>
<td>1.562 ± 0.1919</td>
</tr>
</tbody>
</table>

\(^1\)Results are expressed as mean ± SD of 5 replicates of 8 birds per treatment; initial BW was 46.9 g.

\(^2\)NC, uninoculated birds fed the basal diet; PC, inoculated birds fed the basal diet; SA, inoculated birds fed the basal diet plus 1 g/kg Salmosan (Industrial Técnica Pecuaria, Barcelona, Spain); DU, inoculated birds fed the basal diet plus 1 g/kg Duraió gum; CA, inoculated birds fed the basal diet plus 1 g/kg Cassia gum; SC, inoculated birds fed the basal diet plus 0.5 g/kg cell walls of Saccharomyces cerevisiae; and CO, inoculated birds fed the basal diet plus 0.8 g/kg colistin.
diets supplemented with βGM show fewer M cells than the animals in the NC treatment (Fig. 1, SA-D, CA-D, and SC-D vs. NC-D for the ileum and DU-D vs. NC-B for the cecal tonsil). In addition, in animals in the SC treatment, the formation of lesions consisting of the loss of villus mass (lamina propria and epithelium) was observed (Fig. 1, SC-D).

The images show less mucus production (Fig. 1, CO-A for the ileum) and fewer bacteria attached to the mucous blanket and to the epithelium (Fig. 1, CO-B for the ileum) in chickens fed with CO than in animals fed βGM diets. Moreover, the formation of numerous lesions consisting of the loss of epithelial cells was observed in both the ileum and cecal tonsil (Fig. 1, CO-C and CO-D, respectively).

The structural integrity of the intestinal epithelium is maintained by distinct adhesion systems for which tight junctions, apically located, are the rate-limiting step for paracellular permeability. Tight junctions are multiprotein complexes composed of transmembrane proteins associated with the cytoskeleton and with cytosolic proteins (ZO-1, ZO-2, ZO-3, AF6, and cingulin) involved in cell signaling and vesicle trafficking (Shin et al., 2006). The disorganization of tight-junction proteins and the consequent disruption of the epithelial barrier function allow the passage of harmful water-soluble molecules and microbes present in the gastrointestinal lumen and contribute to water loss in intestinal inflammatory processes such as in bacterial infections (Marchiando et al., 2010). The localization of the tight-junction protein ZO-1, as an indicator of the state of epithelial barrier function, was studied in the ileum using CON. In animals in the NC treatment, the images reveal the typical regular dotted fluorescence pattern corresponding to the localization of ZO-1 at the tight junction without a cytoplasmatic signal (Fig. 2, first panel). In contrast, in the chickens in the PC treatment, a small amount of fluorescence was observed in the cytosol (Fig. 2, second panel, Cy), which was reduced in animals fed βGM and CO (Fig. 2, third to seventh panels).

Regarding the morphometric study (Fig. 3A and 3B), the statistical analysis of the data for the villus length reveals an increase ($P = 0.001$ and $P = 0.003$, respectively) in the ileum of chickens fed DU and CA treatments compared with animals in the NC and PC treatments ($P = 0.001$ and $P = 0.016$). The mean values were greater ($P = 0.001$) for the animals in the DU treatment with respect to the CA treatment. In contrast, the results obtained for muscularis thickness reveal that animals fed CA and CO show a reduction ($P = 0.041$ and $P = 0.004$, respectively) of this variable with respect to birds in the PC treatment. No effects ($P > 0.05$) on the microvillus length or diam. were detected (Table 3).
DISCUSSION

The effect of the dietary inclusion of βGM on intestinal morphology in Salmonella Enteritidis–challenged chickens was studied and compared with the results obtained with the antibiotic colistine and cell walls of Saccharomyces cerevisiae (Zanello et al., 2009). Moreover, villus and microvillus dimensions were quantified as morphometric parameters indicative of epithelial surface area for nutrient absorption. The study was performed in the ileum because it is a segment of the small intestine with a high capacity for nutrient absorption (Ferrer et al., 1994) and in the cecal tonsil, which is the largest secondary lymphoid organ of the chicken gastrointestinal tract and the preferred place for Salmonella Enteritidis invasion (Chappell et al., 2009).

Salmonella Enteritidis possesses mannose-specific lecithin in type 1 fimbriae that adhere to glycoproteins of the intestinal epithelium (Spring et al., 2000) and allow the passage mainly, but not exclusively, through M cells (microfold cells; Santos and Bäumler, 2004). In the chicken, M cells are present mainly in the cecal tonsil (Kitagawa et al., 2000). However, our images reveal, as in mammals and according to other authors (Frost et al., 1997; Meyerholz et al., 2002), that these cells can also be found dispersed in ileal villi. The observation of more M cells in the ileum and cecal tonsil of animals in the PC treatment in comparison to animals in the NC treatment is a common feature that has already been described for intestinal infections by Salmonella and other bacterial species (Frost et al., 1997; Jepson and Clark, 1998; Jang et al., 2004). Structurally, M cells are characterized by the presence of short, irregular microvilli on the apical side (Gebert et al., 1999). For this reason, in SCA images these cells have a depressed apical surface area and higher electronic density than surrounding enterocytes. Our images obtained with TRA confirm these ultrastructural features. In SCA images of the animals fed βGM we observed fewer M cells, which suggests a reduction in the Salmonella Enteritidis interaction with the intestinal epithelium.

In the invasive process of Salmonella, Salmonella Pathogenicity Island-1 (SPI-1) injects several effector proteins into the cytosol of the host (M cells or enterocytes), which promote changes in the cytoskeleton (actin rearrangement), resulting in the formation of lamellipodia and bacterial internalization by macropinocytosis (Frost et al., 1997). Our results confirm these data because the formation of lamellipodia was observed by SCA both in the ileum and in the cecal tonsil. These images are similar to those obtained for intestinal infection with Salmonella Typhimurium in other animal species (Frost et al., 1997; Meyerholz et al., 2002). In contrast, the formation of lamellipodia in animals fed βGM has not been observed.

Scanning and transmission electron microscopy images show that compared with NC and PC diets, diets containing βGM increase mucus production as a result of the increased presence of goblet cells. This effect, here described for Salmosan and Duraió and Cassia gums, has already been assigned to Saccharomyces cerevisiae (Baurhoo et al., 2007; Chichlowski et al., 2007; Leforestier et al., 2009; Chee et al., 2010). Moreover, the images reveal a high bacterial load in the mucous blanket, both in the ileum and in the cecal tonsil, and a reduction of bacteria attached to the epithelium in animals fed βGM comparison with animals fed the NC and PC treatments. In fact, bacterial adherence to the mucus layer through either lectins or low-affinity bonds has previously been described (Barnett et al., 2012). Thus, the stimulation of mucus production by βGM may lead to the formation of a large surface for Salmonella Enteritidis adhesion and may produce a physical protective barrier for the epithelium. In chickens infected with Salmonella Typhimurium, Zhang et al. (2012) detected a reduction in the expression of MUC2, the major mucin gene, which would facilitate bacterial adherence to epithelial cells. Therefore, in addition to the direct effect of binding to mannose-specific lectins of Salmonella and thus blocking bacterial adhesion (Spring et al., 2000; Fernandez et al., 2002; Badia et al., 2012a), an indirect effect can also be attributed to the βGM assayed because of their
capacity to increase mucus production. Another indirect effect attributed to these dietary additives is that they may provide substrates for the metabolism and growth of normal intestinal microflora, thus inhibiting pathogen colonization by competitive exclusion (Vandeplas et al., 2010). Baurhoo et al. (2007) and Yang et al. (2008) found that βGM increase the presence of Lactobacillus spp. and Bifidobacterium spp. in the chicken intestine. Baurhoo et al. (2007) concluded that the beneficial effects of these bacteria, among others, can be attributed to their capacity to stimulate mucus secretion. Moreover, our results suggest that compared with the NC and PC treatments, diets containing Salmosan, Duraio gum, or Cassia gum increase the presence of filamentous bacteria, although in the present study we cannot demonstrate their role in Salmonella Enteritidis infection.

The animals in the NC treatment show the same villus morphology and distribution in the ileum and cecal tonsil as that previously described in chickens at a similar developmental stage (Glick et al., 1978; Ferrer et al., 1991; Chichlowski et al., 2007). At this magnification level, no differences were observed with respect to the other treatments. Regarding the morphometric parameters, the mean values of villus length for birds in the NC treatment are in the range of results previously obtained in the ileum of 3- to 7-wk-old chickens (Baurhoo et al., 2007; Yang et al., 2008; Chee et al., 2010). The statistical analysis shows greater villus development in chickens fed Duraió and Cassia gums, which are richer in βGM than Salmosan (Badia et al., 2012b). This effect has also been described for Saccharomyces cerevisiae, although in some cases the differences were only statistically significant for chickens older than those considered here (Solis de los Santos et al., 2005; Baurhoo et al., 2007; Chichlowski et al., 2007; Morales-López et al., 2009). Perhaps this is the reason why we did not find this effect in the animals fed SC. Solis de los Santos et al. (2005) and Baurhoo et al. (2007) considered that villus development is not a direct effect of βGM on mucosal development, but rather, it is an indirect effect exerted by an increase in the population of Lactobacillus and Bifidobacterium. In fact, Chichlowski et al. (2007) found an increase in villus length in chickens fed a diet supplemented with these probiotics. One of the beneficial effects of including Lactobacillus and Bifidobacterium in the diet has been ascribed to their capacity to improve nutrient absorption by increasing epithelial surface area (Hajati and Rezaei, 2010).

Table 3. Microvillus dimensions in the ileum of chickens in the different treatments

<table>
<thead>
<tr>
<th>Item</th>
<th>Microvillus length, µm</th>
<th>Microvillus diam., µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>1.23 ± 0.08</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>PC</td>
<td>1.18 ± 0.23</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>SA</td>
<td>0.99 ± 0.11</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>DU</td>
<td>1.02 ± 0.07</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>CA</td>
<td>1.09 ± 0.17</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>SC</td>
<td>1.13 ± 0.17</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>CO</td>
<td>1.11 ± 0.05</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>P-value</td>
<td>0.886</td>
<td>0.898</td>
</tr>
</tbody>
</table>

1 Results are expressed as mean ± SEM of 5 replicates of 1 bird per treatment.
2 NC, uninoculated birds fed the basal diet; PC, inoculated birds fed the basal diet; SA, inoculated birds fed the basal diet plus 1 g/kg Salmosan (Industrial Técnica Pecuaria, Barcelona, Spain); DU, inoculated birds fed the basal diet plus 1 g/kg Duraio gum; CA, inoculated birds fed the basal diet plus 1 g/kg Cassia gum; SC, inoculated birds fed the basal diet plus 0.5 g/kg cell walls of Saccharomyces cerevisiae; and CO, inoculated birds fed the basal diet plus 0.8 g/kg colistine.

As for the muscularis thickness of the intestinal wall, values for chickens in the NC treatment are in the range of previous data that were also obtained in the ileum (Miles et al., 2006; Chichlowski et al., 2007). Our results reveal that this variable was reduced in...
animals in the CA and CO groups with respect to the other treatments. The effect of the antibiotic has already been described (Ferket et al., 2002; Yang et al., 2008) and has been attributed to a lessening of the inflammatory process due to a reduction in the number of bacteria (Ferket et al., 2002; Miles et al., 2006), an effect that has been observed in the present study. Nevertheless, treatment with this antibiotic induces the formation of epithelial lesions in the ileum and in the cecal tonsil, an effect that has also been described by Chichlowski et al. (2007).

In conditions of intestinal inflammation, the proteins of the tight junction are lost from its apical location, and they appear in the cytoplasm. In this sense, Awad et al. (2012) recently found an acute decrease in ion permeability in the intestine of chickens infected in vitro with Salmonella Enteritidis. They interpreted these results as a counteracting response that induces the closure of the Cl− and K+ channels and the reduction of paracellular permeability to protect the chicken intestine from water loss. They conclude that this mechanism may underlie the lack of overt secretory diarrhea in infected chickens in contrast to mammals. Accordingly, our CON images of ZO-1 localization do not reveal any effect on tight-junction structure, which is in line with results indicating the subclinical evolution of chickens infected with Salmonella Enteritidis. Nevertheless, the presence of cytosolic fluorescence in animals in the PC treatment, indicating a slight de-localization of ZO-1 away from the tight junction and the prevention of this effect by βGM and CO, suggests an improvement of epithelial barrier function.

In summary, the images of Salmonella-infected animals fed βGM show an increase in the presence of goblet cells compared with the images of animals in the NC and PC treatments and, consequently, higher mucus production in the ileum and in the cecal tonsil. This mucus constitutes a large surface for Salmonella adhesion, forming a physical barrier that blocks the access of the bacteria to the epithelium. Moreover, the presence of M cells, which is characteristic of infected animals, was not as evident in animals fed βGM as it was in animals in the PC treatment. In addition, the inclusion of βGM in the diet (mainly in the form of Duratío and Cassia gums) induces an increase in villus length in animals fed βGM diets compared with animals in the PC treatment, thus increasing epithelial surface area. In conclusion, the results indicating the beneficial effects of these βGM on intestinal morphology give more evidence of the positive effects of these supplements in poultry nutrition.

**LITERATURE CITED**


