Mucin Dynamics and Microbial Populations in Chicken Small Intestine Are Changed by Dietary Probiotic and Antibiotic Growth Promoter Supplementation

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ABSTRACT The mucous layer that covers the intestinal absorptive surface acts as a barrier against bacterial translocation. The chicken gut contains a diverse bacterial population which interacts with the mucous layer. In this report, we studied the effect of changing the intestinal microbial populations on mucin dynamics by feeding 1-d-old chicks a control diet or that diet containing either antibiotic growth promoter (AGP) or a probiotic product for 14 d. Dietary AGP increased the proportions of *Bifidobacterium* species in the duodenum compared with the other groups. In AGP-fed chicks, the villous surface area was increased in the jejunum, goblet cell density was greater in the jejunum and ileum, and mucin glycoprotein levels in the duodenum were lower than in the other groups ($P < 0.05$). Feeding AGP increased the expression of mucin mRNA in the jejunum and ileum compared with controls. The dietary probiotic increased the proportion of *Lactobacillus* species in the ileum compared with the controls ($P < 0.05$) and significantly enlarged the goblet cell "cup" area throughout the small intestine compared with the other groups. Expression of mucin mRNA and the levels of mucin glycoprotein were greater in the jejunum of the probiotic-fed chicks compared with controls ($P < 0.05$). Neither the probiotic nor AGP treatments affected the thickness of the mucous adherent layer. These results indicate that both probiotic and AGP altered processes of mucin biosynthesis and/or degradation mediated via changes in the intestinal bacterial populations. These modifications in mucin dynamics influence gut function and health and may change nutrient uptake. J. Nutr. 135: 187–192, 2005.

KEY WORDS: • *chick* • *small intestine* • *mucin* • *probiotic* • *antibiotic*

In the intestinal lumen, ingested feed moves distally while digestion and absorption occur, and at the same time, a wide variety of microbial species are supported. Absorptive processes occur at the brush border, which encompasses extensions of the epithelial surface. This surface is covered with a mucous gel secreted by epithelial goblet cells that acts as a protective barrier against harmful intraluminal components. The presence of this mucous layer prevents bacterial translocation (1) because to cause damage, gut pathogens must pass through this mucous layer before adherence to and invasion of the epithelial cells. Studies showed various interactions between intestinal mucin and intestinal microflora in vitro. Recently Gusils et al. (2) showed that *Lactobacillus* strains adhere to purified chicken intestinal mucin and, in addition, Jonsson et al. (3) found that the presence of mucin in the growth medium initiates mucin binding properties in several strains of *Lactobacillus*. Other studies indicated that mucin was a site for bacterial adhesion (4), with subsequent competition between pathogenic and beneficial bacteria (5,6). Another aspect of bacterial-mucin interactions is the involvement of the intestinal microbiota in mucin turnover by stimulating mucin gene expression (7–9) on the one hand, and by producing mucin-degrading enzymes (10–12) on the other hand.

The intestinal bacterial microflora may be modified by nutritional manipulations that affect specific substrate availability (13–15). More direct methods such as feeding antimicrobials or the addition of viable bacteria to the food also influence the intestinal microbiota. In animal production, such supplements have been used for many years to improve both performance and health. Antibiotics at subtheraupeutic doses are widely used in animal production to decrease susceptibility to pathogens and disease and to improve meat and egg production. However, continued use of dietary antibiotics was suggested to increase the probability of the development of antibiotic-resistant human pathogens (16). Probiotics are live microbial feed supplements designed to benefit the host animal by improving the intestinal microbial ecology (17); they were found to have positive effects on gut health by immunomodulation and facilitating the elimination of pathogens (18).

Use of antibiotic growth promoters or probiotic diet supplements in poultry production changes the intestinal microenvironment (19–21) and hence may induce changes in mucin dynamics in the chick small intestine. Recent advances in the methodology for bacterial estimation in intestinal contents (22) and in measuring parameters connected with mucin secretion and turnover (23) enable detailed examination of

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these changes. Therefore the purpose of this study was to determine the effects of an antibiotic growth promoter and a probiotic supplement on small intestinal microbial populations and mucin dynamics.

MATERIALS AND METHODS

Animals and diets. Cobb chicks (1 d old) with equal numbers of males and females were blocked into 3 experimental treatments on the basis of body weight, equalizing the body weight and variance among the groups. All groups had free access to food and water. The control group were fed a corn-soybean based commercial diet containing no growth promoter (Matmor Feed Mill, D.N.) (24) formulated to meet or exceed NRC recommendations (25). One group was fed the same diet containing 5 mg/kg of an antibiotic growth promoter (AGP,[2] Avilamycin, Eli Lilly). Another group was fed the same diet with 2 g/kg of a probiotic supplement containing the viable microorganisms *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Enterococcus faecium* (minimum 1.0×10^8 cfu/g) (Pro, PrimaLac® StarLabs) Each dietary treatment was applied to 2 groups of 10 chicks. At 14 d, chicks were killed and intestinal segments were removed. Tissue samples were taken from the midpoint of the duodenum, from the midpoint between the point of entry of the bile duct and Meckel's diverticulum (jejunum) and midway between Meckel's diverticulum and the ileocecal junction (ileum). Tissue samples (2 cm) were taken for histology, washed in PBS, and fixed in 4% (v:v) buffered formalin. Samples for mRNA and protein determination were frozen in liquid nitrogen. Intestinal contents from each segment were collected into sterile PBS, pH 7.5. All procedures were approved by the Animal Care and Welfare Committee of our Institute.

Bacterial genomic DNA extraction. The content of each intestinal segment was homogenized by mixing on a vortex with glass beads (4-mm diameter) for 3 min. Debris was removed by centrifugation at $700 \times g$ for 1 min; the supernatant was collected and centrifuged at $12{,}000 \times g$ for 5 min. The pellet, which contained mainly bacteria, was washed twice with PBS and stored at −20°C until DNA extraction (26,27). For DNA purification, the pellet was resuspended in EDTA and treated with 10 g/L of lysozyme (Sigma Aldrich) for 45 min at 37°C. The bacterial genomic DNA was isolated with Wizard® Genomic DNA purification kit according to the manufacturer's protocol (Promega).

PCR amplification of Lactobacillus and Bifidobacterium 16s rDNA. Primers for *Lactobacillus* were designed according to Wang et al. (28) (forward 5′-CAT CCA GTG CAA ACC TAA GAG, reverse 5′-GAT CCG CTT GCC TTC GCA) and the *Bifidobacterium* primer set was as described by Langendijk et al. (29) (forward 5′-GGG TGG TAA TGC CGG ATG, reverse 5′-CCA CCG TTA CAC CGG CAA). A primer set identifying the 16S rRNA invariant sequences (forward 5′-CGT GCC AGC AGC CGC GGT AAT ACG, reverse 5′-GGG TTG CGC TCG TTG CGG GAC TTA ACC CAA CAT) of all known intestinal bacterial species was designed according to Amit-Romach et al. (27) and used as universal primers for determining the total microflora population.

For PCR amplification of the bacterial targets from intestinal contents, 25 ng of extracted DNA was amplified using Colored Taq DNA polymerase (SileksM). Determination of the linear phase of the amplification was performed with pooled bacterial DNA aliquots removed at 10, 15, 20, 25, 30, 35, 40, and 45 cycles. Amplification of the fragments was as follows: *Lactobacillus*; a 286-bp 16S rDNA fragment was performed for 33 cycles, which consisted of denaturation (95°C, 30 s), annealing (54°C, 1 min), and extension (68°C, 1 min). *Bifidobacterium*; a 510-bp 16S rDNA fragment was amplified at 42 cycles, and a 611-bp 16S rDNA PCR product of the universal primers was amplified under the same conditions in a different tube. PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and quantified using a Gel-Pro Analyzer™ version 3.0 (Media Cybernetics). The relative amount of a particular bacterial species was determined by normalizing the density of the PCR products to the density of the PCR product of the universal primers by densitometric scanning; results are presented as arbitrary units (AUs) as previously described (27).

Total RNA isolation. Total RNA was isolated from the intestinal segments using TRI reagent (10 mL/g tissue) according to the manufacturer's protocol (MRC Molecular Research Center).

Mucin mRNA analysis. RT-PCR was carried out with primers from the fragment of the chicken intestinal mucin gene (GI 45125071) (forward: 5′-TCT TCC GCT ACC CTG GGC TCT GTA A-3′; reverse: 5′-CTC ATG CAG TTC TAG CAA GAT ACT-3′) and with primers from the *Gallus gallus* 18S ribosomal RNA gene with (GI 7262899) (forward: 5′-CGA TGC TCT TAA CTG AGT GT-3′, reverse: 5′-GAG TAT GGT TGC AAA GCT GA-3′). Determination of the linear phase of the amplification RT-PCR was performed with Access RT-PCR System (Promega) as previously described (23). Amplification of the chicken intestinal mucin gene was performed for 41 cycles, which consisted of denaturation (95°C, 30 s), annealing (54°C, 1 min), and extension (72°C, 1 min); 18S was amplified at 30 cycles under the same conditions in a different tube. The 18S (426 bp) and chicken intestinal mucin (317 bp), PCR products were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide, and quantified using Gel-Pro Analyzer version 3.0 (Media Cybernetics). The relative amount of mucin mRNA was determined by normalizing the density of its PCR products to the density of the 18S PCR product; results are presented as AUs.

Western blot analysis. Western blot analysis was performed as described previously (23). Briefly, intestinal tissues were homogenized and centrifuged at $12{,}000 \times g$ at 4°C. Samples were applied to a Sephadex G-150–120 (Sigma Chemical) column and the void volume fraction was collected and subjected to electrophoresis on SDS-polyacrylamide gels [3.5% (wt:v) acrylamide in the stacking and 4.5% (wt:v) acrylamide in the running gel] and subsequently transferred onto nitrocellulose (Schleicher and Schuell). Detection of the mucin glycoprotein was performed with the primary antibody, MUC5AC (Zymed Laboratories) after incubation with peroxidase-conjugated donkey anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories). Immunoblots were developed with Western blotting Luminol reagent (Santa Cruz Biotechnology). The density of the positive bands was quantified using Gel-Pro Analyzer version 3.0 (Media Cybernetics) and the results presented as AUs.

Measurement of the mucous adherent layer thickness. The thickness of the mucous adherent layer was estimated by a modification of Corne's method (30–32) as previously described (23). Briefly, a 1 cm^2 piece of intestinal tissue was incubated in 10 g/L Alcian blue (AB) dye solution in buffer containing 160 mmol/L sucrose and 50 mmol/L sodium acetate, pH 5.8; excess dye was washed and absorbed dye was extracted from the tissue by incubation in 10 g/L docusate sodium salt solution. Samples were cleared by centrifugation at $700 \times g$ and optical density was measured at 620 nm using AB solution as a standard. The amount of absorbed dye is given in μg AB/cm^2 of intestinal tissue.

Morphological examination. Intestinal segments were fixed in 4% (v:v) buffered formaldehyde, dehydrated, cleared and embedded in paraffin. Serial sections were cut at 3 μm, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin. Sections were examined by light microscopy.

Mucin staining. Determination of neutral mucin was by staining 5-μm sections with periodic acid-Schiff reagent (PAS) (33,34). After deparaffinization and rehydration, the slides were incubated in 5 g/L periodic acid for 15 min, then washed and incubated with Schiff's reagent (Sigma Chemical) for 30 min. After being washed in warm water, the slides were dehydrated and mounted. The number of periodic acid-Schiff positive cells along the villi was determined by light microscopy

Morphometric measurements. The area of the goblet cell was calculated from the length and width of the goblet cell "cup" in cross sections of the villi as previously described (23). The density of goblet cells was calculated as the number of goblet cells per unit surface area (mm^2). All measurements were performed with an Olympus light microscope using EPIX® XCAP® software (Epix).

[2] Abbreviations used: AB, Alcian blue; AGP, antibiotic growth promoter; AU, arbitrary unit; PAS, periodic acid-Schiff reagent staining.

Statistical analysis. Data were analyzed by ANOVA using the General Linear Models procedures of SAS (35). Differences between means were tested using Tukey's test. Differences were considered significant at $P < 0.05$ unless otherwise stated.

RESULTS

Bacterial populations. The probiotic dietary supplement increased the relative amounts of *Lactobacillus* species in the ileum by 147% compared with controls ($P < 0.05$) **(Fig. 1)**. The treatments did not affect the relative amounts of *Lactobacillus* species in the jejunum and duodenum.

Feeding AGP increased the relative amounts of *Bifidobacterium* species in the duodenum by 78% compared with the control group and by 260% increase compared with the Pro group ($P < 0.05$). In the distal part of the small intestine, the relative amount of *Bifidobacterium* species was not affected by the treatments **(Fig. 2)**.

Small intestinal morphology. Feeding AGP increased the jejunal villous surface area by 28% compared with the control group and by 17% compared with the Pro-fed chicks ($P < 0.05$) **(Table 1)**. The dietary treatments did not affect the other small intestinal segments. AGP supplementation significantly enhanced goblet cell density in the jejunum by 27 and 25% compared with the control and Pro-fed groups, respectively, and in the ileum by 14 and 13% compared with the control and Pro-fed groups, respectively ($P < 0.05$) (Table 1). AGP did not affect the duodenal goblet cell density. Small intestinal morphology and goblet cell density did not differ between the Pro-fed chicks and the controls (Table 1).

Morphometric measurements of the goblet cell "cup" area revealed that probiotic supplementation enlarged this area throughout the small intestine **(Fig. 3**A). The goblet cell "cup" area was increased by 18% in the duodenum, by 82% in the jejunum, and by 40% in the ileum compared with control chicks ($P < 0.05$) (Fig. 3*B*). Feeding the AGP supplement did not affect the goblet cell "cup" area.

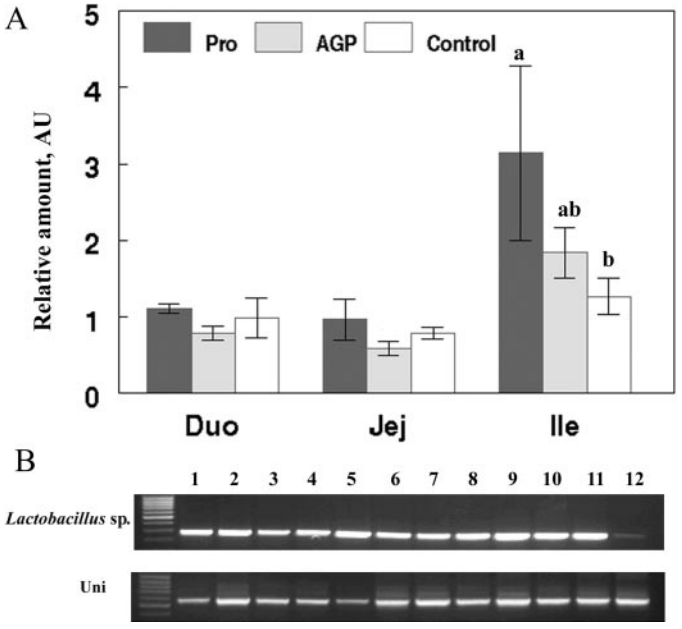

FIGURE 1 The effect of dietary Pro or AGP supplementation on the proportion of *Lactobacillus* species in chicken small intestine. (*A*) Relative proportions of *Lactobacillus* species along the small intestine. Small intestinal segments are: Duo = duodenum, Jej = jejunum, Ile = ileum. Values are means ± SEM, $n = 8$. Means within segments without a common letter differ, $P < 0.05$. (*B*) Representative PCR products of 16S rDNA of *Lactobacillus* species and 16S rDNA of invariant sequences of all known intestinal bacterial species (Uni) in the ileum of Pro (lane1–4), AGP (lane 5–8), and control (lane 9–12) chicks.

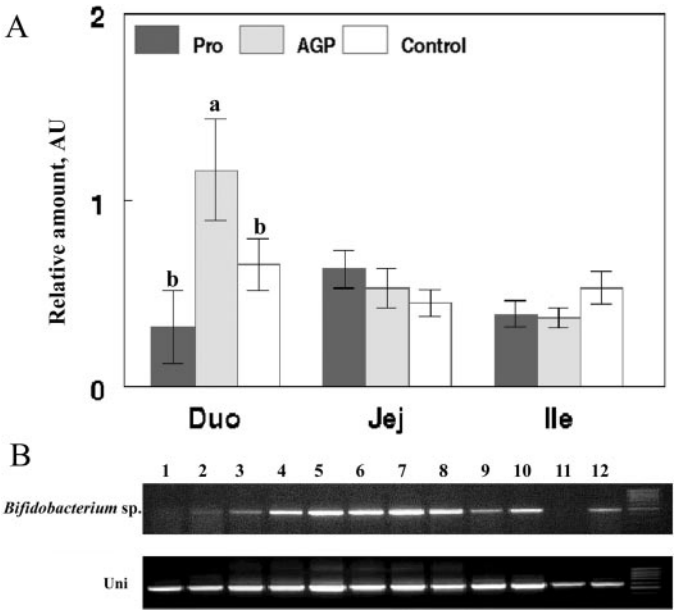

FIGURE 2 The effect of dietary Pro or AGP supplementation on the proportion of *Bifidobacterium* species in chicken small intestine. (*A*) Relative proportions of *Bifidobacterium* species along the small intestine. Small intestinal segments are: Duo = duodenum, Jej = jejunum, Ile = ileum. Values are means ± SEM, $n = 8$. Means within segments without a common letter differ, $P < 0.05$. (*B*) Representative PCR products of 16S rDNA of *Bifidobacterium* species and 16S rDNA of invariant sequences of all known intestinal bacterial species (Uni) in the duodenum of Pro (lane1–4), AGP (lane 5–8) and control (lane 9–12) chicks.

Thickness of the mucous adherent layer. The thickness of the mucous adherent layer was not affected by either the probiotic or the AGP treatment and ranged from 47 to 59 μg AB/cm^2 throughout the small intestine.

Mucin mRNA expression. The expression of the chicken intestinal mucin gene was measured by semiquantitative RT-PCR. Expression of mucin mRNA was enhanced in the AGP group by 236% in the jejunum and 80% in the ileum compared with the controls ($P < 0.05$). Mucin mRNA expression in the duodenum was not affected by AGP treatment. Probiotic supplementation increased the expression of mucin mRNA 160% in the jejunum compared with controls, whereas mucin mRNA expression was not affected in the other small intestinal segments ($P < 0.05$) **(Fig. 4)**.

Mucin glycoprotein level. The total mucin glycoprotein concentration was measured in small intestinal segments, including mucin stored in the goblet cells, mucin in the mucin adherent layer, and mucin present in the intestinal lumen. Supplementing chicks with probiotics increased the mucin glycoprotein concentration 110% in the jejunum compared with the controls ($P < 0.05$). Mucin glycoprotein concentration was 48 and 53% lower in the duodenum of AGP-fed chicks compared with control and Pro-fed chicks, respectively ($P < 0.05$) **(Fig. 5)**.

DISCUSSION

This study indicates that dietary supplements influencing the microbial populations of the small intestine alter mucin

TABLE 1

The effect of dietary Pro or AGP supplementation on the villous surface area and goblet cell density in chick small intestine[1]

	Segment[2]	Pro	AGP	Control
Villous surface area, mm^2	Duo	5119.9 ± 283.0	5715.5 ± 268.5	5799.8 ± 285.0
	Jej	6611.9 ± 300.2[b]	7724.2 ± 291.2[a]	6096.0 ± 260.5[b]
	Ile	8088.5 ± 262.0	8893.8 ± 245.1	7344.5 ± 296.2
Goblet cell density, n/mm^2	Duo	0.514 ± 0.021	0.527 ± 0.018	0.519 ± 0.021
	Jej	0.282 ± 0.022[b]	0.352 ± 0.020[a]	0.274 ± 0.020[b]
	Ile	0.193 ± 0.019[b]	0.219 ± 0.017[a]	0.192 ± 0.017[b]

[1] Values are means ± SEM, $n = 8$. Means within segments without a common letter differ, $P < 0.05$.
[2] Small intestinal segments are: Duo, duodenum, Jej, jejunum, Ile, ileum.

dynamics. Interactions between mucin and bacteria play a role in the integrity of the mucous barrier and thus may influence its protective properties (36).

The small intestinal microflora in young chicks are mainly facultative anaerobes (*Streptococcus*, *Staphylococcus*, *Lactobacillus*, and *Escherichia coli*); however, in the duodenum and ileum, large numbers of anaerobes (9–39% of the small intestine isolates) represented by species such as *Eubacterium*, *Propionibacterium*, and *Clostridium* were also recovered (37).

The probiotic supplement examined in this study was a viable bacterial product consisting of selected subspecies of *Lactobacillus* (*L. acidophilus* and *L. casei*) and *Bifidobacterium* (*B. bifidum*). Persistence of ingested probiotic bacteria in the intestines is a condition for their beneficial effect, and some data in humans indicated that orally administered *Lactobacillus* can survive transit, but efficient colonization was not demonstrated (38). This finding is similar to our study in which inclusion of the probiotic in the diet significantly increased the relative amounts of *Lactobacillus* in the ileum compared with the controls, but did not affect the proportions in the duodenum and jejunum. In a previous study, use of this supplement was observed to alter the proportion of *Lactobacillus* in the chick ceca (39).

The antibiotic growth promoter used here was avilamycin, a mixture of oligosaccharides of the orthosomycin group, which are produced by *Streptomyces viridochromogenes* and are active against gram-positive bacteria. Avilamycin is widely used for growth promotion in swine and poultry at inclusion levels ranging from 5 to 40 mg/kg for swine and from 2.5 to 10 mg/kg for poultry. At growth-promoting levels, avilamycin was shown to reduce the amount of *Clostridium perfringens* in the intestinal tract of chickens (40). In this study, inclusion of avilamycin in the chicken diet for 14 d resulted in relative increases in proportions of *Bifidobacterium* species in the proximal but not in the distal small intestine.

The intestinal microflora interact with mucin on several different levels. Studies showed that intestinal bacterial populations affect mucosal cell proliferation (41). Studies in vitro and in vivo showed that both mucin biosynthesis and secretion may be changed by the presence of bacteria, bacterial lipopolysaccharide, and products of bacterial fermentation. Several authors proposed that some probiotic bacterial strains act on mucin secretion and synthesis via prostaglandin production (42–44). Once mucin is synthesized in the goblet cells

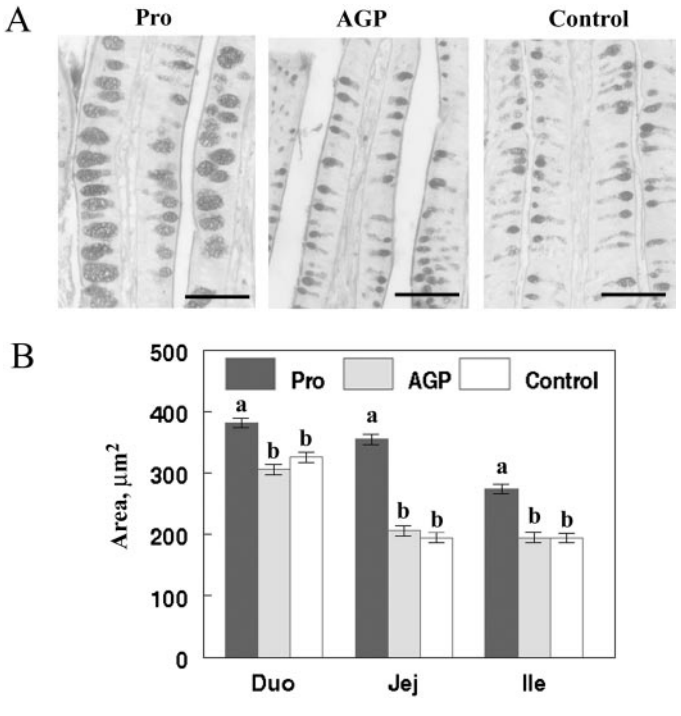

FIGURE 3 The effect of dietary Pro or AGP supplementation on goblet cell size. (*A*) Representative light micrographs of jejunum from the different treatments stained with periodic acid-Schiff reagent. Magnification X400; bar = 50 μm. (*B*) Changes in the area of the goblet cells determined in longitudinal sections in small intestinal segments: Duo = duodenum, Jej = jejunum, Ile = ileum. Values are means ± SEM, $n = 8$. Means within segments without a common letter differ, $P < 0.05$.

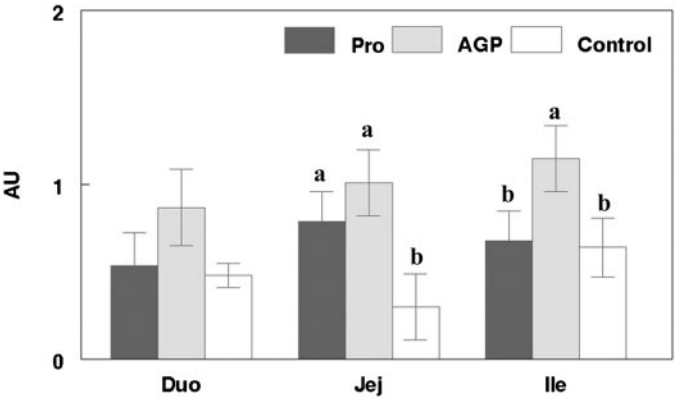

FIGURE 4 The effect of dietary Pro or AGP supplementation on chicken intestinal mucin mRNA expression. Changes in mucin mRNA expression were measured by semiquantitative RT-PCR and expressed relative to expression of 18S rRNA. Small intestinal segments are: Duo = duodenum, Jej = jejunum, Ile = ileum. Values are means ± SEM, $n = 4$. Means within segments without a common letter differ, $P < 0.05$.

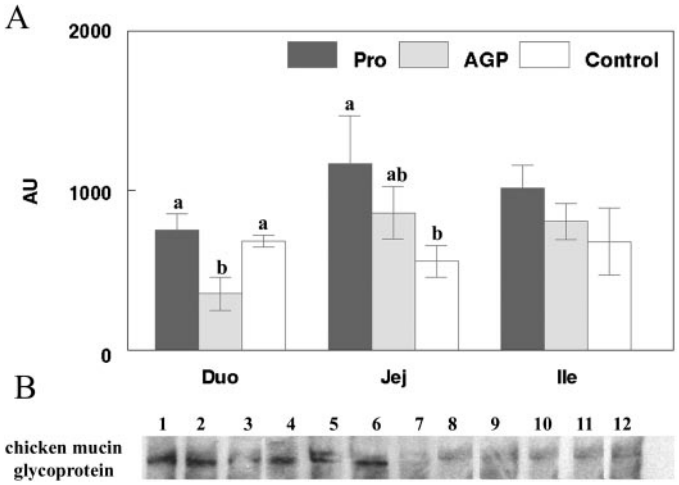

FIGURE 5 The effect of dietary Pro or AGP supplementation on chicken intestinal mucin glycoprotein concentrations. (*A*) Relative concentration of mucin glycoprotein. Small intestinal segments are: Duo = duodenum, Jej = jejunum, Ile = ileum. Values are means ± SEM, $n = 4$. Means within segments without a common letter differ, $P < 0.05$. (*B*) Representative immunoblot from the duodenum of Pro (lane1–4), AGP (lane 5–8), and Control (lane 9–12) chicks.

and secreted to the intestinal surface, it forms an adherent layer that undergoes continuous degradation and renewal (45). In this study, no changes were observed in the thickness of the mucous adherent layer due to the treatments. However, the total amount of mucin proteins in the small intestine, as determined here, included both the mucin synthesized and stored in the goblet cells as well as the mucin that forms the mucous adherent layer. The total amount of mucin glycoprotein present in the small intestine is influenced by both the rate of mucin synthesis and secretion and by the contribution of the microflora to mucin degradation. Because mucins are resistant to proteolytic enzymes of the gastrointestinal tract, the role of microflora in mucous degradation is major. There are many bacterial species that possess mucin-degrading glycosidases and glycosulfatases (11,46–48). Because both the AGP and the probiotic treatments affected the intestinal microbial environment, we determined the effects of these 2 supplements on the intestinal epithelium and on mucin dynamics.

The addition of avilamycin to the diet altered both small intestinal morphology and mucin parameters. Because it is unlikely that avilamycin had a direct effect on the intestinal morphology, the changes observed in the villous surface area and the enhanced goblet cell density in both the jejunum and ileum were likely due to microbial-mucosal interactions. Previous studies also indicated some effects of antibiotics on the thickness of the intestinal wall (49,50). Inclusion of avilamycin in the diet affected mucin biosynthesis by enhancing mucin mRNA expression in the jejunum and ileum. In the duodenum, mucin mRNA was not changed; however, significantly decreased amounts of mucin glycoprotein were found compared with the controls and Pro-fed chicks, suggesting increased rates of microbial mucin degradation. These changes can be explained in part by the presence of a greater proportion of *Bifidobacterium* species, which possess mucin degradation activity (51).

The addition of probiotic supplements to the diet did not change small intestinal morphology; however, it increased goblet cell mucin storage as indicated by the "cup" size in all small intestinal segments compared with the other groups. This would be expected to follow from the increased mucin mRNA expression and the increased amount of mucin glycoprotein that were observed in the jejunum after probiotic supplementation. This probiotic supplement consisted mainly of *Lactobacillus* and *Bifidobacterium* species, which would account for increased mucin synthesis and secretion (43,52,53). However, in this study, the probiotic supplement affected only the relative amounts of the bacterial populations in the ileum in which enhanced proportions of *Lactobacillus* species were observed compared with the controls. A similar lack of effect of probiotic supplements on small intestinal bacterial populations was reported previously (19); however, the microbial-mediated changes observed here may have been caused by a shift in the proportions within *Lactobacillus* species. Minimal survival and/or mucosal colonization may be the reason for mild effects of probiotics on the small intestinal morphology and mucin dynamics observed in this study.

In conclusion, the addition of both probiotic and AGP additives to the diet altered small intestinal bacterial populations and affected the intestinal epithelium and mucin dynamics. These changes may influence gut function and health and may affect nutrient uptake. However, further understanding of the mechanisms of the relations between microbial populations and host are required to clarify the role of microorganisms in gut physiology.

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