Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks

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ABSTRACT Grapes have high amounts of phenolic compounds, which can modulate the gut activity as well as modify the structure and function of the gastrointestinal tract. The microbiological activity of avoparcin, grape pomace concentrate, and grape seed extract was evaluated in an in vitro study. An in vivo experiment was also conducted to study the effect of the inclusion of grape pomace concentrate and grape seed extract in the diet of broiler chicks on performance, intestinal microflora (by cultured and terminal restriction fragment length polymorphism methodology), and gut morphology at 21 d of age. Dietary treatments included an antibiotic-free diet (CON), a positive control (AVP; 50 mg/kg of avoparcin), and antibiotic-free diets containing grape pomace concentrate (GPC; 60 g/kg) or grape seed extract (GSE; 7.2 g/kg). Performance was not affected by dietary treatment except in the case of birds fed the GSE diet, which showed decreased weight gain. In the ileal content, birds fed CON and GSE diets had the highest populations of *Lactobacillus*. Compared with the CON diet, the AVP, GPC, and GSE diets increased the populations of *Enterococcus* and decreased the counts of *Clostridium* in the ileal content. In the cecal digesta, birds fed GPC and GSE diets had higher populations of *Escherichia coli*, *Lactobacillus*, *Enterococcus*, and *Clostridium* than birds in any other treatment group. Animals fed GPC and GSE diets showed a higher biodiversity degree than those fed control diets. The frequency of detection of several potential phenol-degrading bacteria as well as unidentified and uncultured organisms was increased in animals fed GPC and GSE diets. Birds fed the CON diet had longer villi and deeper crypt depth than birds in any other treatment group. The highest villi height:crypt depth ratio corresponded to birds fed GPC and AVP diets and the lowest to those fed CON and GSE diets. In conclusion, dietary polyphenol-rich grape products modify the gut morphology and intestinal microflora and increase the biodiversity degree of intestinal bacteria in broiler chicks.

Key words: grape by-product, intestinal microflora, gut morphology, chick

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INTRODUCTION

The manipulation of gut functions and microbial habitat of domestic animals with feed additives has been recognized as an important tool for improving growth performance and feed efficiency. The increasing antimicrobial resistance of pathogens isolated from humans and animals combined with the ban of the use of antibiotics as feed additives has accelerated and led to investigations of alternative options for more efficient antimicrobials in animal production. The removal of antibiotic growth promoters from feed is expected to result in reduced feed efficiency and increased incidence of intestinal disorders as a result of proliferation of gut pathogens (Hughes et al., 2005). As an alternative, several researchers have reported the antimicrobial effects of various plant extracts against certain pathogens (Tepe et al., 2004; Papadopoulou et al., 2005). Phenolic compounds are currently receiving much attention because of their putative health effects related to their antioxidant, anticarcinogenic, antiinflammatory, and antimicrobial activities. Some of these effects could be attributed to their bioactive metabolites and also to the modulation of the intestinal bacterial population (Selma et al., 2009). Specifically, some phenolic compounds such as resveratrol, hydroxytyrosol, quercetin, and several phenolic acids have been reported to inhibit various pathogenic microorganisms (Aziz et al., 1998).

Grape (*Vitis vinifera*) is one of the world's largest fruit crops (FAO-STAT, 2007). Grape pomace is the residue left after juice extraction by pressing grapes

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in the wine industry. This by-product (constituted by seeds, skin, and stem) is used every year either as animal feed (with low nutritional value) or for ethanol production by fermentation and distillation. Grape seed extract is a heterogeneous mixture of polyphenols (anthocyanidins, catechins, and their derivatives) obtained from solvent extraction.

Recent investigations have stressed the importance of by-products from wine processing as plant materials that are particularly rich in polyphenols and have a wide range of biological activities. Extracts obtained from grape seeds and pomace contain large quantities of monomeric phenolic compounds such as (+)-catechins, (−)-epicathechin and (−)-epicatechin-3-O-gallate, and dimeric, trimeric, and tetrameric proanthocyanidins (Saito et al., 1998). The biological activity of these polyphenols depends on their bioavailability and can be exerted at both local (intestinal tract) and systemic levels (Hervert-Hernández et al., 2009). It is generally accepted that the availability of phenolic compounds is rather low. This bioavailability can be even lower when the feed polyphenols have a large molecular weight, as is the case of hydrolyzable and condensed tannins (Jimenez-Ramsey et al., 1994; Manach et al., 2004). Available data on the absorption and digestibility of polyphenols suggest relatively low bioavailability of polymeric proanthocyanidins in rats (Donovan et al., 2002) and chickens (Brenes et al., 2008). Substantial levels of unabsorbed phenolics remain in the gut. They and their metabolites may play a key role in the maintenance of intestinal environment by modulation of the microbiota (Selma et al., 2009).

Several phenolic compounds have been recognized as potential antibacterial compounds able to repress pathogenic bacteria in the gut. Evidence of the antimicrobial effect of grape seed extract has been observed in vitro (Rodríguez-Vaquero et al., 2007; Gañán et al., 2009; Hervert-Hernández et al., 2009) and in vivo in rats (Dolara et al., 2005) and chickens (McDougald et al., 2008). Moreover, the structure of the intestinal mucosa can reveal some information on gut health. Data are scarce concerning the possible effect of grape seed by-products on chicken intestinal epithelium. Several authors have shown changes in the intestinal morphology by the addition of grape by-products in rats (López-Oliva et al., 2006), piglets (Sehm et al., 2007), and humans (Laurent et al., 2005).

To date enough evidence exists to support antimicrobial activity of polyphenols. However, research on the possible stimulatory role of phenolic compounds on intestinal microbiota is scarce and no experimental studies have been published on the effects of grape seed polyphenols in chickens. The majority of the studies investigating host–microflora–diet relationship in chickens were analyzed by culture-based methods, but the isolation methods have several limitations such as being time and labor intensive. Recent advances in ribosomal and RNA- and DNA-based molecular techniques make it possible to identify different bacterial populations in environmental samples without culturing. This methodology has been used to determine variation in human feces (Harmsen et al., 2000), bovine rumen (Nelson et al., 1998), and chicken cecum (Gong et al., 2002). The aim of the present study was to use both culture-based methods and terminal RFLP (**T-RFLP**) analysis to examine changes in gut microbial communities in response to the addition of grape pomace concentrate and grape seed extract to a wheat-based diet in broiler chicks. Likewise, to verify the effect of these grape by-products, intestinal morphology was also evaluated.

MATERIALS AND METHODS

Birds and Diets

A total of one hundred 1-d-old male broiler Cobb chicks were housed in electrically heated starter batteries in an environmentally controlled room. The chicks were allocated to 20 pens, each pen containing 5 chicks, to receive 4 dietary treatments with 5 replicates of each treatment for 21 d. Diets in mash form and water were provided for ad libitum consumption. All diets were formulated to contain similar levels of ME, CP, and crude fiber and to meet or exceed the minimum NRC (1994) requirements for broiler chickens. Experimental procedures were approved by the University Complutense of Madrid Animal Care and Ethics Committee in compliance with the Ministry of Agriculture, Fishery and Food for the Care and Use of Animals for Scientific Purposes. Ingredients and nutrient composition of diets are shown in Tables 1 and 2. Dietary treatments included an antibiotic-free diet (**CON**), a positive control (**AVP**; 50 mg/kg of avoparcin), and antibiotic-free diets containing grape pomace concentrate (**GPC**; 60 g/kg) or grape seed extract (**GSE**; 7.2 g/kg). The concentrations of extractable polyphenols of grape pomace concentrate and grape seed extract in the diets were similar (2.9 and 2.7 g/kg, respectively).

Chemical Analysis

Dry matter (method 930.15), CP (method 976.05), crude fiber (method 978.10), and ash (method 942.05) were analyzed according to the methods of Association of Official Analytical Chemists (1995). Crude fat was determined by extraction in petroleum ether following

Table 1. Proximate composition of grape pomace concentrate[1]

Item	g/kg of DM
Protein	138.5 ± 1.2
Soluble sugars	20.7 ± 0.3
Fat	9.87 ± 0.17
Fiber	151.8 ± 0.72
Ash	24.1 ± 0.3
Extractable polyphenols	48.7 ± 0.07
Hydrolyzable polyphenols	26.6 ± 0.05
Condensed tannins	150.9 ± 0.05

[1]Data are the mean of 4 determinations ± SD.

Table 2. Ingredients and nutrient composition of experimental diets

Item[1]	Control	Avoparcin	GPC[2]	GSE[3]
Ingredient				
Wheat (11.6% CP)	474.7	473.7	412.7	458.8
Soybean (44% CP)	378.8	378.8	376.5	382.8
Sunflower oil	88.4	88.4	108.0	92.7
Cellulose	16.2	16.2	—	16.5
GPC	—	—	60.0	—
GSE	—	—	—	7.2
Monocalcium phosphate	15.7	15.7	18.6	15.8
Calcium carbonate	16.4	16.4	14.3	16.4
NaCl	3.0	3.0	3.0	3.0
Vitamin–mineral premix[4]	5.0	5.0	5.0	5.0
DL-Methionine	1.8	1.8	1.9	1.8
Avoparcin	—	1.0	—	—
Analyzed composition[5]				
CP	200.9	202.3	201.4	200.8
Crude fat	105.7	104.4	103.8	104.6
Extractable polyphenols	1.6	1.5	5.4	4.3
Hydrolyzable polyphenols	13.8	13.3	16.7	13.5
Condensed tannins	—	—	8.7	—
Calculated composition				
AME[6] (kcal/kg)	3,000	3,000	3,000	3,000
Crude fiber	40.0	40.0	40.0	40.0
Methionine + cystine	7.1	7.1	7.1	7.1
Ca	8.8	8.8	8.8	8.8
Available P	3.6	3.6	3.6	3.6

[1]Grams per kilogram as fed unless noted.

[2]GPC: grape pomace concentrate. Contained 48.70 g/kg of extractable polyphenols, 26.60 g/kg of hydrolyzable polyphenols, and 150.90 g/kg of condensed tannins. The concentration of extractable polyphenols of GPC in the diet was 2.9 g/kg.

[3]GSE: grape seed extract. Contained more than 80% of total polyphenols of which 60% was procyanidols and 0.75% of anthocyanidins (composition as stated by the manufacturer, Norfeed Sud, Angers, France). The concentration of extractable polyphenols of GSE in the diet was 2.7 g/kg.

[4]Vitamin and mineral mix supplied the following per kilogram of diet: vitamin A, 8,250 IU; cholecalciferol, 1,000 IU; vitamin E, 11 IU; vitamin K, 1.1 mg; vitamin B_{12}, 12.5 μg; riboflavin, 5.5 mg; Ca pantothenate, 11 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, 0.75 mg; biotin, 0.25 mg; ethoxyquin, 125 mg; DL-methionine, 500 mg; Amprol (Huvepharma Inc., Peachtree City, GA), 1 g; Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg; NaCl, 2,500 mg.

[5]Data are the mean of 3 determinations.

[6]Calculated value (FEDNA, 2003).

acidification with a 4 *N* HCl solution (Wiseman et al., 1992). Analysis of soluble sugars was carried out by using anthrone and thiourea as a reagent following the conditions described by Southgate (1976). Extractable polyphenols were determined in methanol, acetone, and water extracts obtained from grape pomace and diet by the Folin-Ciocalteu procedure (Montreau, 1972) using gallic acid as a standard. Residues from the extract were treated with 5 mL/L of HCl-butanol for 3 h at 100°C (Reed et al., 1982). Nonextracted polyphenols were calculated from the absorbance at 550 nm of the anthocyanidin solutions. Condensed tannins from Mediterranean carob pod (*Ceratonia siliqua* L.) supplied by Nestlé S.A. (Vevey, Switzerland) were treated under the same conditions to obtain standard curves.

Microbiological Analysis

In Vitro Study. The activity of the grape pomace concentrate and grape seed extract compounds and avoparcin were assessed against different isolates of species of gram-positive (*Enterococcus faecium* BA05/00630–02D and *Clostridium perfringens* BA05/00439–5B) and gram-negative (*Escherichia coli* ICM07/00306–2 and *Salmonella enteritidis* VE06/02211SK2) bacteria by comparing the inhibition zone diameters determined by the paper disk diffusion bioassay. The bacterial isolates included in the antimicrobial activity assays were obtained from the Animal Health Surveillance Center of the Universidad Complutense of Madrid, Spain. For inoculum preparation, 4 colonies of an overnight Columbia blood agar plate (bioMérieux España S.A., Madrid, Spain) were inoculated in 5 mL of Müeller-Hinton broth (Oxoid, Basingstoke, UK). Tubes were incubated at 37°C for 5 h until they reached a visible turbidity. Inocula were further adjusted to a 0.5 McFarland standard using sterile distilled water and were finally diluted to 1:20 (*Ent. faecium*, *E. coli*, and *S. enteritidis)* and 1:10 (*C. perfringens)* and then swabbed onto a Mueller-Hinton plate (bioMérieux España S.A.). Test compounds were dissolved in water to produce a 1 mg/mL stock solution. A concentration of 30 μg/disk of each compound was loaded on standard disks and were placed on the Mueller-Hinton agar plate, earlier inoculated with bacterial suspension. Following incubation for 24 h at 37°C, the inhibition zone around each disk

was recorded. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by title compounds.

In Vivo Study. At 21 d of age, broiler chicks were killed by cervical dislocation and their intestinal tracts were removed. Samples (5/diet; each pooled from 2 chicks) of fresh digesta (0.1 to 0.2g) from the ileum and ceca were collected aseptically in preweighed 20-mL sterilized plastic tubes. The samples were weighed and diluted in peptone water to an initial 10^{-1} dilution. Microbial populations were determined by serial dilution (10^{-1} to 10^{-7}) of samples in PBS before inoculation onto Petri dishes of sterile agar. The selective media for *Campylobacter* was prepared using charcoal cefoperazone desoxycholate agar (Oxoid). *Lactobacillus* was grown on de Man, Rogosa, and Sharpe agar (Difco Laboratories, Detroit, MI). *Escherichia coli* was grown on Coli ID agar (bioMerieux España S.A.). Agars used to grow *Clostridium* and *Enterococcus* were sulfite polymyxin sulfadiazine (Difco Laboratories) and Enterococcus agar (Difco Laboratories), respectively. The plates were incubated at 37°C anaerobically (73% N, 20% CO_2, 7% H_2) for *Clostridium* and *Lactobacillus*, aerobically for *E. coli* and *Enterococcus*, and under microaerobic environment (5% O_2, 10% CO_2, 85% N_2) at 42°C for *Campylobacter*. Plates were counted between 24 and 48 h after inoculation. Colony-forming units were defined as being distinct colonies measuring at least 1 mm in diameter.

T-RFLP

DNA Extraction. Samples pooled from 2 birds (n = 6 samples/treatment) of volume equivalent to 400 mg of cecal digesta were preserved in ethanol and precipitated by centrifugation (13,000 × *g* for 5 min at room temperature). The DNA in the precipitate was extracted and purified using the commercial QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK). The recommended lyses temperature was increased to 90°C and an incubation step with lysozyme was added afterward (10 mg/mL, 37°C, 30 min) to improve bacterial cell rupture. The DNA was stored at –80°C until analysis.

T-RFLP Analysis. Terminal RFLP analysis of bacterial community was performed following the procedure described by Hojberg et al. (2005). Briefly, a 1,497-bp fragment of the 16S rRNA gene was amplified using a 6-carboxyfluorescein-labeled forward primer [S-D-Bact-0008-a-S-20 (5′-6-FAM-AGAGTTTGATCMTGGCTCAG-3′)] and a reverse primer [PH1552 (5′-AAGGAGGTGATCCAGCCGCA-3′)]. Duplicate PCR analyses were performed for each sample. Fluorescent-labeled PCR product was purified on QIAquick PCR purification kit columns (Qiagen) and eluted in a final volume of 30 μL of Milli-Q water (Millipore, Billerica, MA). Then, the resultant PCR product was subjected to a restriction with *Hha*I (20,000 U/μL; New England Biolabs, Ipswich, MA). Fluorescent-labeled terminal restriction fragments (**TRF**) were analyzed by capillary electrophoresis on an automatic sequence analyzer (ABI 3100 Genetic Analyzer, PE Biosystems, Warrington, UK) in Gene-Scan mode with a 25 U detection threshold. Determination of the TRF sizes in the range 50 to 700 bp were performed with the size standard GS-1000-ROX (PE Biosystems).

Analysis of T-RFLP Data. Sample data consisted of size (bp) and peak area for each TRF. To standardize the DNA loaded on the capillary, the sum of all TRF peak areas in the pattern was used to normalize the peak detection threshold in each sample. Following the method of Kitts (2001), a new threshold value was obtained by multiplying a pattern's relative DNA ratio (the ratio of total peak area in the pattern to the total area in the sample with the smallest total peak area) by 323 area units (the area of the smallest peak at the 25 U detection threshold in the sample with the smallest total peak area). For each sample, peaks with a lower area were deleted from the data set. New total area was obtained by the sum of all the remaining peak areas in each pattern. Richness was considered as the number of peaks in each sample after standardization. For pair-wise comparisons of the profiles, a Dice coefficient was calculated and dendograms were constructed using Fingerprinting II software (Informatix, Bio-Rad, Hercules, CA) and an unweighted pair-group method with averaging algorithm. To deduce the potential bacterial composition of the samples, we used the Microbial Community Analysis tool (http://mica.ibest.uidaho.edu) based on the RDP release 9.60 16S rRNA gene database (Shyu et al., 2007)

Gut Morphology

On d 21, 2 chicks from each replicate (n = 5/diet) were killed following a 12-h fast to limit intestinal throughput. The whole length of the small intestine was removed and a length of approximately 5 cm of the jejunum was cut from the midpoint between the point of bile duct entry and Meckel's diverticulum. The samples were gently flushed twice with physiological saline (1% NaCl) to remove intestinal contents and placed in 10% formalin in 0.1 *M* phosphate buffer (pH = 7.0) for fixation. The samples were processed for 24 h in a tissue processor with ethanol as dehydrant and samples were embedded in paraffin. Sections (5 μm) were made from the tissue and were stained with hematoxylin-eosin and a combination of the periodic acid-Schiff method (PAS staining). Histological sections were examined with an Axioplan-2 optical microscope (Carl Zeiss Jena GmbH, Oberkochen, Germany) coupled with a refrigerated QImaging Retiga 4000R digital camera (QImaging, Surrey, Canada) with charge-coupled device detector. The images were analyzed using image software (MetaMorp Imaging System, Molecular Devices Ltd., Sunnyvale, CA). The variables measured were villus height, crypt depth, villus height:crypt depth ratio, and thickness of the muscularis layer. A total of 10 intact, well-oriented

villus–crypt units were selected for each intestinal cross-section (2 cross-sections/sample and 20 cross-sections/treatment, for a total of 100 measurements/treatment). Villus height (μm) was measured from the tip of the villus to the villus crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. For the purpose of statistical analysis, the average of these values was used.

Statistical Analysis

Data were analyzed as a one-way ANOVA using the GLM procedure of SAS (SAS Institute, 2003), and single degree of freedom linear contrasts were used to separate treatments. Pen served as the experimental unit for performance parameters and bird as the experimental unit for histology and microbiology parameters. Treatment means were separated using Bonferroni's multiple comparison test. Statistical significance was declared at a probability of $P < 0.05$. All microbiological concentrations were subject to base-10 logarithm transformation before analysis. Mean comparison of frequency of detection was made using a chi-squared test.

RESULTS

Performance

Performance was not affected by dietary treatment except in the case of birds fed the GSE diet, which showed decreased weight gain and feed efficiency (Table 3). Growth performance was depressed in chicks fed the GSE diet compared with those fed the GPC diet.

In Vitro Microbiological Analysis

Differences were detected in the antimicrobial susceptibility activity of both products, or between the isolates tested (data not shown). In this sense, grape seed extract showed moderate antimicrobial response activity against *C. perfringens* at a concentration of 30 μg/disk (inhibition zone diameter: >10 mm). This product showed relatively less activity against *Ent. faecium* (inhibition zone diameter: 7 mm) and had no response against gram-negative bacteria (*E. coli* and *S. enteritidis*). By contrast, grape pomace concentrate had no detectable antibacterial activity against any of the bacterial species.

Ileal and Cecal Microflora

In the ileal digesta, the inclusion of avoparcin in the chicken diets reduced ($P < 0.001$) the concentration of *Lactobacillus* and *Clostridium* and increased ($P < 0.01$) the concentration of *Enterococcus* compared with the control diet (Table 4). Also, *Enterococcus* was not significantly increased by the GPC diet compared with the CON diet. The viable counts of *Enterococcus* were significantly increased ($P < 0.05$) whereas those of *Clostridium* were significantly reduced ($P < 0.001$) in broilers fed GPC and GSE diets compared with those fed the CON diet. In the case of *Lactobacillus*, a significant decrease ($P < 0.05$) was observed in chicks fed the GPC diet compared with those fed the CON diet. The numbers of *Lactobacillus* and *Clostridium* were increased in birds fed the GSE diet compared with those fed AVP ($P < 0.05$) and GPC ($P < 0.001$) diets. No differences were found in the ileal population of *E. coli* among all dietary treatments at 21 d of age.

In the cecal digesta the inclusion of avoparcin in the chicken diets increased ($P < 0.001$) the concentration of *E. coli*, *Lactobacillus*, and *Enterococcus* compared with the control diet. The viable counts of *E. coli* ($P < 0.001$), *Lactobacillus* ($P < 0.001$), *Enterococcus* ($P < 0.001$), and *Clostridium* ($P < 0.05$) were significantly increased in birds fed GPC and GSE diets compared with those fed the control diet. The numbers of *Lactobacillus* and *Clostridium* were significantly increased, whereas those of *E. coli* were significantly reduced (only in the case of GSE diet) in birds fed GPC and GSE diets compared with those fed the AVP diet. Compared with birds fed GPC diet, viable counts of *E. coli* and *Enterococcus* in the GSE diet were significantly ($P < 0.01$) lower. Populations of *Campylobacter* could not be enumerated because their concentrations were too low.

Composition of the Cecal Microbial Community

Birds fed GPC and GSE diets showed a higher ($P < 0.05$) degree of biodiversity, measured as the number of

Table 3. Performance[1] of broiler chicks (0–21 d) fed diets containing grape pomace concentrate (GPC), grape seed extract (GSE), and avoparcin (AVP)

	Treatment					*P*-value			
Item	Control (CON)	AVP	GPC	GSE	Pooled SEM	CON vs. AVP	CON vs. GPC + GSE	AVP vs. GPC + GSE	GPC vs. GSE
Weight gain (g)	553[a]	557[a]	542[a]	486[b]	36	NS[2]	NS	NS	0.05
Feed consumption (g)	835	797	775	733	51	NS	NS	NS	NS
Feed efficiency (g:g)	1.51[a]	1.43[b]	1.43[b]	1.51[a]	0.06	0.05	NS	NS	0.05

[a,b]Means in rows with no common superscript differ significantly ($P < 0.05$).

[1]Values are the means of 5 pens of 5 chicks each per diet.

[2]NS = $P > 0.05$.

TRF obtained per treatment, than those fed the CON diet (Table 5). Analysis of electropherograms revealed that a total of 72 different TRF were produced ranging in size from 56 to 677 bp. Of the TRF obtained in our study, 52% were compatible with different identified bacteria, whereas 21 and 27% were classified into the uncultured and unidentified categories, respectively. Half of those identified TRF belonged to the Firmicutes division, with Clostridia and Bacilli the classes detected, followed by Proteobacteria (32%), Actinobacteria (10%), and Bacteroidetes (8%) divisions. The inclusion of avoparcin in the diet increased the frequency of detection compatible with the presence of *Pseudomonas* spp. ($P < 0.01$) but did not affect other identified bacteria. By the supplementation with avoparcin, an increase in the frequency of detection of unidentified organisms belonged to TRF sized 128 and 498 bp ($P < 0.05$), and a decrease in that of 258 bp ($P < 0.05$) were observed. When we compared the cecal microbiota of birds fed the CON diet with those fed GPC or GSE diets, an increase in the frequency of detection of *Nocardioides* spp. ($P < 0.001$), *Bacillus/Paenibacillus* spp., *Desulfitobacterium hafniense*, and *Pseudomonas/Acinetobacter* spp. ($P < 0.05$), as well as uncultured (539 bp; $P < 0.05$) and unidentified (75, 128, 275, and 247 bp; $P < 0.05$) bacteria, was observed in animals fed GPC and GSE diets. With the exception of *Pseudomonas* spp., an increase ($P < 0.05$) in the frequency of detection of the previous identified bacteria and in the frequency of uncultured (78, 109, and 539 bp; $P < 0.05$) and unidentified (75, 275, and 247 bp; $P < 0.05$) bacteria was observed in birds fed GPC and GSE diets compared with those fed the AVP diet. Birds fed the GPC diet showed a lower ($P < 0.05$) frequency of detection of *Pseudomonas/Acinetobacter* spp. and uncultured (265, 126, 500, and 109 bp) bacteria and a higher ($P < 0.05$) frequency of detection of uncultured (179 and 116 bp) and unidentified (258 and 159 bp) bacteria than those fed the GSE diet. No distinct clusters according to the different diets were obtained. Two major clusters, both containing birds fed AVP and CON diets, are shown in Figure 1. Birds fed the GPC diet were grouped in the first cluster also containing GSE-, AVP-, and CON-fed chicks (2, 2, and 3, respectively). In the second cluster there was a higher number of GSE-fed birds and another group of AVP- and CON-fed chicks (4 and 3, respectively), with no GPC-fed birds.

Gut Morphology

At 21 d of age, villus height and crypt depth were significantly reduced ($P < 0.05$) and muscularis thickness was increased ($P < 0.001$) in birds fed the AVP diet compared with those fed the CON diet (Table 6). Villus height was significantly reduced ($P < 0.05$) in chickens fed GSE and AVP diets compared with those fed the CON diet. Crypt depth was reduced ($P < 0.05$) in chickens fed AVP, GPC, and GSE diets compared with those fed the CON diet. The ratio of the villus height to crypt depth at the jejunum was significantly increased ($P < 0.05$) in chickens fed GPC and AVP diets compared with those fed CON and GSE diets. Birds fed the GPC diet had higher villus height:crypt depth ratio than those fed the GSE diet. Muscularis thickness was increased in birds fed AVP, GPC, and GSE diets in comparison with those fed the CON diet.

DISCUSSION

Growth Performance

Few references in the literature exist in relation to feeding grape by-products to chickens. In the present study the inclusion of grape pomace concentrate did not change the weight gain compared with birds fed the CON and AVP diets. However, feed efficiency was im-

Table 4. Effect of inclusion of grape pomace concentrate (GPC), grape seed extract (GSE), and avoparcin (AVP) on colony-forming units of anaerobic (*Lactobacillus* and *Clostridium*) and aerobic (*Escherichia coli* and *Enterococcus*) bacterial species[1] per gram of ileal and cecal content of 21-d-old broiler chicks[2]

	Treatment					*P*-value			
Item	Control (CON)	AVP	GPC	GSE	Pooled SEM	CON vs. AVP	CON vs. GPC + GSE	AVP vs. GPC + GSE	GPC vs. GSE
Ileum									
E. coli	4.21	4.31	4.25	4.09	0.25	NS[3]	NS	NS	NS
Lactobacillus	8.35^{a}	7.58^{b}	7.11^{c}	8.20^{a}	0.20	0.001	0.01	NS	0.001
Enterococcus	3.96^{b}	4.51^{a}	4.23^{ab}	4.38^{a}	0.31	0.01	0.05	NS	NS
Clostridium	7.21^{a}	6.12^{c}	6.21^{c}	6.80^{b}	0.22	0.001	0.001	0.05	0.001
Cecum									
E. coli	6.60^{c}	7.67^{a}	7.79^{a}	7.31^{b}	0.21	0.001	0.001	NS	0.01
Lactobacillus	8.48^{c}	8.91^{b}	9.06^{a}	9.16^{a}	0.12	0.001	0.001	0.001	NS
Enterococcus	6.16^{c}	6.94^{ab}	7.10^{a}	6.80^{b}	0.16	0.001	0.001	NS	0.01
Clostridium	7.58^{ab}	7.36^{b}	7.90^{a}	7.90^{a}	0.30	NS	0.05	0.01	NS

$^{a–c}$Means in rows with no common superscript differ significantly ($P < 0.05$).

[1]Log_{10} cfu/g.

[2]Values are the means of 5 samples from 2 birds each per diet.

[3]NS = $P > 0.05$.

Table 5. Effect of inclusion of grape pomace concentrate (GPC), grape seed extract (GSE), and avoparcin (AVP) on the frequency of detection[1] (%) and the degree of biodiversity[2] of bacteria at cecum of 21-d-old broiler chicks[3]

				Dietary treatment				*P*-value[5]			
Bacterial group	Class	Genus or species	TRF[4] (bp)	Control (CON)	AVP	GPC	GSE	1	2	3	4
Frequency of detection											
Actinobacteria	Actinobacteria	*Nocardioides*	677	0.0	0.0	66.7	100.0	NS[6]	0.001	0.001	NS
		Bifidobacterium	180	16.7	50.0	16.7	33.3	NS	NS	NS	NS
		Micrococcus	174	33.0	50.0	0.0	33.0	NS	NS	NS	NS
		Streptomyces rimosus	125	33.0	0.0	16.7	0.0	NS	NS	NS	NS
Bacteroidetes	Bacteroidetes	*Flavobacterium*	226	50.0	66.7	33.3	50.0	NS	NS	NS	NS
		Proteiniphilum	101	33.3	33.3	33.3	0.0	NS	NS	NS	NS
		Cytophaga	92	0.0	33.3	0.0	16.7	NS	NS	NS	NS
Firmicutes	Bacilli	*Lactobacillus*	68, 97, 115, 175, 223, 254, 544	50.0	83.3	83.3	83.3	NS	NS	NS	NS
		Bacillus, *Paenibacillus*	244	16.7	0.0	66.7	83.3	NS	0.05	0.01	NS
		Enterococcus	218, 591	16.7	16.7	0.0	33.3	NS	NS	NS	NS
	Clostridia	*Clostridium*	66, 189, 220, 229, 231, 236, 237	66.7	100.0	66.7	100.0	NS	NS	NS	NS
		Desulfitobacterium hafniense	118	33.3	0.0	100.0	66.7	NS	0.05	0.001	NS
		Butyrivibrio fibrisolvens	190	33.3	16.7	16.7	16.7	NS	NS	NS	NS
Proteobacteria	α-Proteobacteria	*Sphingomonas*	81	50.0	50.0	16.7	33.3	NS	NS	NS	NS
		Flexibacter	84	33.3	0.0	16.7	16.7	NS	NS	NS	NS
		Gluconobacter	176	16.7	33.0	0.0	0.0	NS	NS	NS	NS
	β-Proteobacteria	*Thiomonas*	67	33.3	33.3	16.7	33.3	NS	NS	NS	NS
	γ-Proteobacteria	*Pseudomonas*/*Acinetobacter*	207, 208	16.7	100.0	50.0	100.0	0.01	0.05	NS	0.05
		Citrobacter, *Enterobacter*, *Yersinia*, *Salmonella*	373	33.3	50.0	16.7	66.7	NS	NS	NS	NS
		Escherichia	371, 372, 374	16.7	33.3	33.3	33.3	NS	NS	NS	NS
	δ-Proteobacteria	*Desulfovibrio*	93, 95	33.3	50.0	33.3	50.0	NS	NS	NS	NS
Uncultured			187	50.0	83.3	66.7	66.7	NS	NS	NS	NS
			110	66.7	83.3	33.3	66.7	NS	NS	NS	NS
			78	50.0	16.7	83.3	50.0	NS	NS	0.05	NS
			265	50.0	50.0	16.7	83.3	NS	NS	NS	0.05
			126	33.3	33.3	33.3	100	NS	NS	NS	0.05
			179	16.7	33.3	83.3	0.0	NS	NS	NS	0.01
			500	33.3	50.0	0.0	50.0	NS	NS	NS	0.05
			104	33.3	50.0	16.7	50.0	NS	NS	NS	NS
			109	16.7	0.0	16.7	83.3	NS	NS	0.05	0.05
			80	0.0	33.3	16.7	66.7	NS	NS	NS	NS
			539	0.0	0.0	50.0	50.0	NS	0.05	0.05	NS
			137	0.0	16.7	33.3	50.0	NS	NS	NS	NS
			148	50.0	16.7	16.7	0.0	NS	0.05	NS	NS
			397	16.7	16.7	16.7	33.3	NS	NS	NS	NS
			116	0.0	0.0	50.0	0.0	NS	NS	NS	0.05
Unidentified			56	83.3	83.3	66.7	83.3	NS	NS	NS	NS
			62	33.3	83.3	33.3	66.7	NS	NS	NS	NS
			59	50.0	50.0	50.0	66.7	NS	NS	NS	NS
			77	83.3	50.0	66.7	16.7	NS	NS	NS	NS
			75	33.3	16.7	83.3	100.0	NS	0.001	0.001	NS
			494	33.3	66.7	33.3	83.3	NS	NS	NS	NS
			121	50.0	33.3	83.3	33.3	NS	NS	NS	NS

Continued

Table 5 (Continued). Effect of inclusion of grape pomace concentrate (GPC), grape seed extract (GSE), and avoparcin (AVP) on the frequency of detection[1] (%) and the degree of biodiversity[2] of bacteria at cecum of 21-d-old broiler chicks[3]

				Dietary treatment				*P*-value[5]			
Bacterial group	Class	Genus or species	TRF[4] (bp)	Control (CON)	AVP	GPC	GSE	1	2	3	4
			128	0.0	66.7	33.3	83.3	0.05	0.05	NS	NS
			275	16.7	0.0	66.7	83.3	NS	0.05	0.01	NS
			138	16.7	50.0	33.3	50.0	NS	NS	NS	NS
			498	0.0	66.7	16.7	66.7	0.05	NS	NS	NS
			391	16.7	50.0	33.3	33.3	NS	NS	NS	NS
			127	33.3	50.0	50.0	33.3	NS	NS	NS	NS
			120	16.7	50.0	16.7	33.3	NS	NS	NS	NS
			133	33.3	50.0	16.7	0.0	NS	NS	0.05	NS
			247	0.0	0.0	50.0	50.0	NS	0.05	0.05	NS
			258	50.0	0.0	50.0	0.0	0.05	NS	NS	0.05
			139	50.0	16.7	0.0	33.3	NS	NS	NS	NS
			159	16.7	0.0	50.0	0.0	NS	NS	NS	0.05
Degree of biodiversity				35.6	37.5	38.8	43.8	NS	0.05	NS	NS

[1]The frequency of detection of a bacterium: percentage of chicks that presented a TRF compatible with the theoretical TRF for a certain bacterium.

[2]Biodiversity: number of fragments obtained from each chick; SEM = 2.04.

[3]Results were obtained from the Microbial Community Analysis tool (http://mica.ibest.uidaho.edu).

[4]TRF: terminal restriction fragment.

[5]Values are the means of 6 chicks per diet. Contrast: 1) CON vs. AVP; 2) CON vs. GPC + GSE; 3) AVP vs. GPC + GSE; 4) GPC vs. GSE.

[6]NS = $P > 0.05$.

Table 6. Effect of inclusion of grape pomace concentrate (GPC), grape seed extract (GSE), and avoparcin (AVP) on the morphology of the intestinal mucosa in the small intestine (jejunum) in 21-d-old broiler chickens[1]

	Treatment					*P*-value			
Item	Control (CON)	AVP	GPC	GSE	Pooled SEM	CON vs. AVP	CON vs. GPC + GSE	AVP vs. GPC + GSE	GPC vs. GSE
Villus height (μm)	1,104[a]	1,019[b]	1,041[ab]	1,007[b]	24	0.05	0.05	NS[2]	NS
Crypt depth (μm)	71.5[a]	54.5[c]	56.2[bc]	61.2[b]	1.8	0.01	0.001	NS	NS
Villus height:crypt depth	15.5[b]	18.8[a]	18.6[a]	16.5[b]	0.65	0.01	0.05	NS	0.05
Muscularis thickness (μm)	99.2[b]	117.5[a]	115.2[a]	109.6[a]	11	0.001	0.01	NS	NS

[a-c]Means in rows with no common superscript differ significantly ($P < 0.05$).

[1]Values are the means of 100 measurements per treatment (2 chicks per cage × 5 cages per diet × 2 cross-sections per sample × 5 measurements per cross-section).

[2]NS = $P > 0.05$.

proved by the addition of grape pomace concentrate in comparison with birds fed CON and GSE diets. Similar results have been reported by Goñi et al. (2007) and Brenes et al. (2008) with a dietary inclusion up to 60 g/kg of grape pomace concentrate. Moreover, the growth depression obtained by the use of GSE (7.2 g/kg) diet in the current experiment was similar to the data reported by Hughes et al. (2005). However, Brenes et al. (2010) reported that the inclusion of a lower concentration of grape seed extract, up to 3.6 g/kg, in chicken diets did not change the growth performance. The poor response obtained in the current experiment could be explained by a higher concentration of this by-product in the diet. The growth depression observed in birds fed the GSE diet compared with those fed the GPC diet could be attributed to the presence of a pure form of polyphenols in the GSE diet despite containing similar concentrations of extractable polyphenols.

In Vitro and In Vivo (Ileal and Cecal) Microbiological Analysis

Limited studies are available to assess the possible application of polyphenols as alternatives to antibiotics in broiler chicken production. In the current study, grape seed extract showed moderate in vitro antimicro-

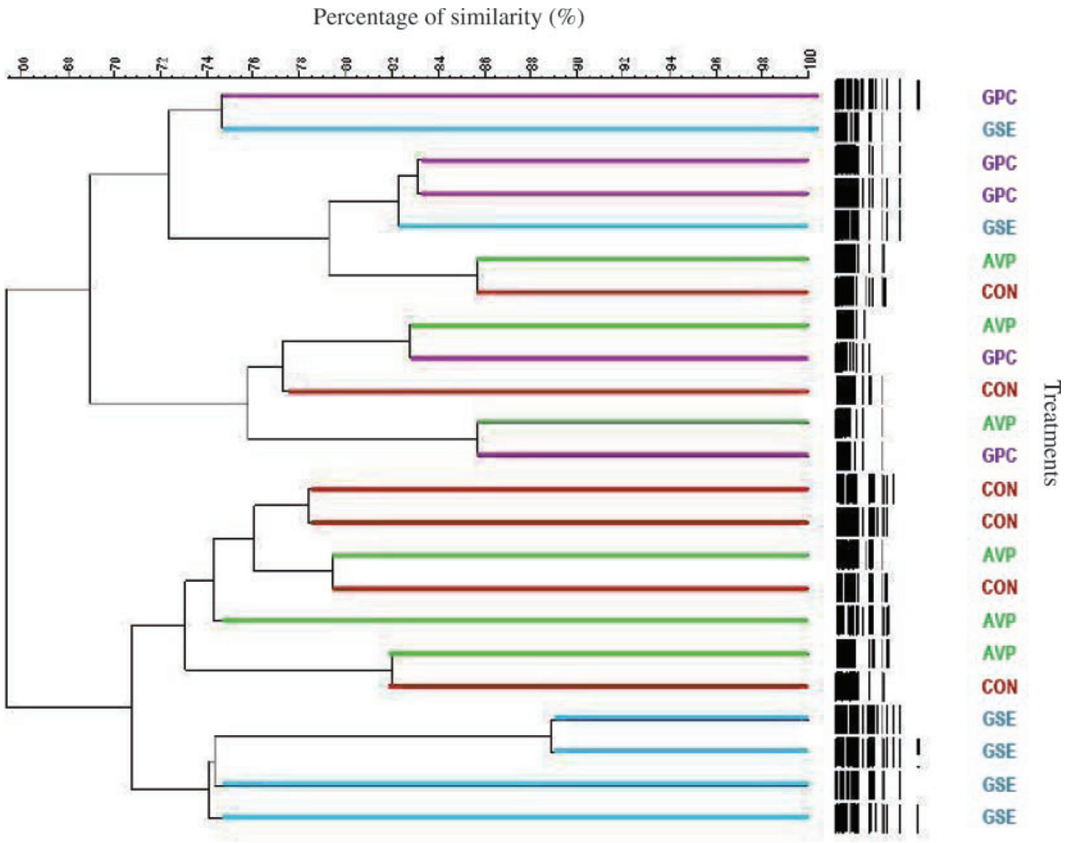

Figure 1. Percentage of similarity of terminal RFLP banding patterns in cecum samples from chicks fed experimental diets: grape pomace concentrate (GPC), grape seed extract (GSE), control (CON), and avoparcin (AVP). Data are means of 6 samples (each pooled from 2 birds) corresponding to 12 chicks for each treatment. Color version available in the online PDF.

bial response activity against *C. perfringens* and *Ent. faecium* and had no response against *Lactobacillus* and gram-negative bacteria (*E. coli* and *Salmonella*; data not shown). Baydar et al. (2006) also showed antibacterial activity of the grape seed extract against different bacteria, including *E. faecalis*, *E. coli*, and *S. enteritidis*. Rodríguez-Vaquero et al. (2007) and Papadopoulou et al. (2005) demonstrated the antimicrobial properties of phenolic compounds from different wines and phenolic extracts of wines against *E. coli*. In contrast, Hervert-Hernández et al. (2009) showed a stimulatory role of grape pomace polyphenols on *L. acidophilus* growth in agar diffusion test.

Likewise, in the present experiment, feeding birds diets containing grape pomace concentrate and grape seed extract was effective in increasing the ileal populations of beneficial bacteria such as *Enterococcus* and in decreasing the counts of potential pathogens such as *Clostridium*. However, in the cecal digesta, birds fed GPC and GSE diets had a higher population of *E. coli*, *Lactobacillus*, *Enterococcus*, and *Clostridium* than those in any other treatment group, maintaining the balance between populations of beneficial and pathogenic bacteria. This study confirmed that polyphenolics cause a shift in the bacterial population in the intestinal tract. Dolara et al. (2005) reported, in an in vivo study, that the percentage of *Clostridium* was reduced and *Lactobacillus* was increased in the colon content of rats fed red wine polyphenols.

The exact mechanism by which polyphenols exhibit antimicrobial activity is not clear. They could have bacteriostatic or bactericidal actions or act to inhibit adhesion of infection-causing bacteria within cells of the intestinal tract. In the present experiment, the different lactobacilli stimulant activity of grape pomace concentrate and grape seed extract in cecum could be attributed to the composition and the phenolic profile of these by-products and could depend directly on extractable polyphenol contents. A possible explanation for the stimulatory effect of polyphenolic compounds on bacterial growth is that some microorganisms are able to use these compounds as nutritional substrates. In the particular case of lactobacilli, these bacteria posses the ability to metabolize phenolic compounds supplying energy to cells and positively affecting the bacterial metabolism (García-Ruíz et al., 2008). Isolation of tannin-degrading lactobacilli from humans capable of degrading hydrolyzable tannins in human gut microflora has been reported (Osawa et al., 2000).

Composition of the Cecal Microbial Community

To our knowledge, no reports exist on the effects of inclusion of grape pomace concentrate and grape seed extract on intestinal microbiota composition studied by T-RFLP in any species, including poultry. To deduce the potential bacterial composition of the samples, the frequency of detection of a bacterium, defined as percentage of birds that presented a TRF compatible with a certain bacterium, was calculated. It should be noted that a single TRF may represent more than one organism, and results, therefore, are presented as potential compatible bacteria. It is generally accepted in many species, including chickens (Bjerrum et al., 2006), that a high proportion of a gastrointestinal microbial ecosystem belongs to new species or even new genera that remain to be identified. Because of the importance of this fraction, it is also relevant to reflect the effect of diet on unidentified and uncultured organisms. In this sense, our study showed that only half of all TRF obtained were compatible with different identified bacteria, which agrees with previous studies. Approximately 50% of identified TRF were related to the Firmicutes division, followed by Proteobacteria (32%), Actinobacteria (10%), and Bacteroidetes (8%) divisions. Among the Firmicutes division, the majority of TRF were assigned to *Clostridium* and *Lactobacillus* genera, confirming the dominance of these genera in the cecum as obtained by cultivation procedures in this study. Same findings were reported in previous culture-independents studies by Gong et al. (2002) and Bjerrum et al. (2006).

Dendrograms (Figure 1) showed that the addition of avoparcin in the diet did not promote great changes on the overall intestinal microbial ecosystem. It is generally accepted that antibiotics reduce the number of bacteria in the gut, but results obtained in the literature are not always consistent and probably depend on the type and doses of antibiotic administered, the cleanliness of the environment, and the health status of the animals involved. In this sense, although we observed a reduction of *Lactobacillus* and *Clostridium* counts with the supplementation with avoparcin, no major effects were obtained either on the overall microbial structure or on the frequency of detection of this genus in T-RFLP study.

When using TRF to analyze variation in bacterial community with the use of GPC and GSE diets, we observed differences in the number (biodiversity degree) and in the size (potential species composition) of TRF. Birds fed GPC and GSE diets showed a higher biodiversity degree than those fed control diets. A higher biodiversity degree has been related to a more stable ecosystem and with a reduced susceptibility to colonization by opportunistic pathogens (Deplancke et al., 2002). The analysis of frequencies of detection revealed that several bacteria, and also unidentified and uncultured species, were responsible for the overall differences obtained in gut bacterial community composition. Among identified bacteria, the inclusion of grape pomace concentrate and grape seed extract increased the frequency of detection compatible with several genera belonging to Actinobacteria (*Nocardioides* spp.), Bacilli (*Bacillus*/*Paenibacillus* spp.), Clostridia (*Desulfitobacterium* spp.), and α-Proteobacteria (*Pseudomonas*/*Acinetobacter* spp.). Some of these (*Bacillus* and *Paenibacil-*

lus) have been isolated in bird cecum and possess the ability to produce proteins that kill competitors (bacteriocins) providing protection against *Campylobacter jejuni* (Svetoch et al., 2005) and may help to explain the antimicrobial effects of grape phenolic compound reported by others (Gañán et al., 2009). In our study, *Campylobacter* spp. were not observed either in culture or in T-RFLP studies. Despite the effect observed in identified bacteria, most of the diet changes were related to unidentified (122, 128, 129, 150, 247, and 275 bp) and uncultured (78, 148, and 539 bp) organisms. These results confirm the importance of these fractions in studies conducted to investigate the effect of the diet on the intestinal microbial community. Interestingly, fragments 677, 539, and 247 bp, compatible with the presence of *Nocardioides* spp. and with an uncultured and unidentified bacterium, respectively, were exclusively found in animals fed grape by-products. These results suggest that the intake of polyphenols sources as grape pomace concentrate and grape seed extract might favor the growth of several species. Despite many in vitro studies having demonstrated the antimicrobial properties of polyphenols, many fungi, bacteria, and yeasts are quite resistant to such compounds and are able to grow and develop on them (Arunachalam et al., 2003). In this sense, several studies (Dolara et al., 2005; Tzounis et al., 2008) reported that grape-derived foodstuffs might act as prebiotics favoring beneficial bacteria as *Bifidobacterium* spp. and *Lactobacillus* spp. Many polyphenol-degrading bacteria, such as *Pseudomonas* spp., *Acinetobacter* spp., *Rhizobium* spp., *Bacillus spp.*, *Eubacterium* spp., *Nocardioides* spp., and *Desulfovibrio* spp., have been isolated from different ecosystems, including the gastrointestinal tract (Arunachalam et al., 2003).

Gut Morphology

Mucosa status and their microscopic structure can be good indicators of the response of the intestinal tract to active substances in feeds. Few reports have documented the effect of dietary polyphenols or related phenolics on the localized intestinal growth and function in broiler chickens and the contribution to changes in performance. In a study done with chicks and rats given diets high in freeze-dried tannins extracted from fava beans (*Vicia faba)*, Ortiz et al. (1994) found histological lesions in the ileum, suggesting a loss in absorptive capacity. Results obtained by Sell et al. (1985) using high and low tannin sorghum in rats and laying hens revealed milder negative effects. Because long villi are correlated with improved gut health, CON and GPC diets offer a comparative advantage over AVP and GSE diets in improving the gut health status of the birds in the current experiment. It is assumed that an increased villus height is paralleled by an increased digestive and absorptive function of the intestine as a result of increased absorptive surface area, expression of brush border enzymes, nutrient transport systems (Caspary, 1992), and an increased body weight gain (Zijlstra et al., 1996). Sehm et al. (2007) reported that the inclusion of red wine pomace had an inhibitory effect on the jejunum villi growth. In agreement with our results, Laurent et al. (2005) observed a decrease in the size of the microvilli by the addition of grape seed extract in human intestinal Caco-2 cells and Miles et al. (2006) and Baurhoo et al. (2007) reported that antibiotic-fed broilers had shorter villi in the ileum and duodenum compared with those fed a control diet.

Regarding other measures of gut integrity, crypt depth was reduced in birds fed the AVP diet compared with those fed the CON diet. However, Ferket et al. (2002) reported that crypt depth was not affected by the addition of an antibiotic-free diet in turkeys. Crypt depth was also slightly reduced in birds fed GPC and GSE diets. Sell et al. (1985) observed a slight reduction in the crypt depth of the duodenal tissue in rats, chicks, and laying hens fed the high tannin sorghum. In contrast, a stimulating effect on crypt colon size in piglets fed red wine pomace was reported by Sehm et al. (2007).

In the present study, an increase in villus height:crypt depth ratio at the jejunum in birds fed GPC and AVP diets was also found. A lengthening of the villus and a short crypt can lead to better nutrient absorption, decreased secretion in the gastrointestinal tract, increased disease resistance, and greater overall performance. In this sense, the low villi height:crypt depth ratio could have caused the poorer growth in GSE-fed chicks. Moreover, muscularis thickness was increased in birds fed AVP, GPC, and GSE diets. This is difficult to explain because the inclusion of feed antibiotics in diets of chicken reportedly leads to a decreased thickness of walls and a reduced weight (Miles et al., 2006).

Based on the results of the present study, it can be stated that dietary polyphenol-rich grape products were effective in increasing the ileal populations of beneficial bacteria in the ileum as well as increasing villus height:crypt depth ratio at the jejunum. These facts may have an important influence on the physiology and biochemistry of the gut. Advances in the knowledge of the interactions between bioactive feed compounds and specific intestinal bacteria could contribute to a better understanding of both positive and negative interactions in vivo and to the identification of new functional microorganisms inhabiting the intestinal tract. Future chicken studies will provide further insight into the potential of these bioactive substances to act as prebiotics in the intestinal tract.

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REFERENCES

Arunachalam, M., M. M. Raj, N. Mohan, and A. Mahadevan. 2003. Biodegradation of catechin. Proc. Indian Natl. Sci. Acad. 69:353–370.

Association of Official Analytical Chemists. 1995. Official Methods of Analysis. 16th ed. AOAC Int., Arlington, VA.

Aziz, N. H., S. E. Farag, L. A. A. Mousa, and M. A. Abo-Zaid. 1998. Comparative antibacterial and antifungal effects of some phenolic compounds. Microbios 93:43–54.

Baurhoo, B., L. Phillip, and C. A. Ruiz-Feria. 2007. Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. Poult. Sci. 86:1070–1078.

Baydar, N. G., O. Sagdic, G. Özkan, and S. Cetin. 2006. Determination of antibacterial effects and total phenolic contents of grape (*Vitis vinifera* L.) seed extracts. Int. J. Food Sci. Technol. 41:799–804.

Bjerrum, L., R. M. Engberg, T. D. Leser, B. B. Jensen, K. Finster, and K. Pedersen. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult. Sci. 85:1151–1164.

Brenes, A., A. Viveros, I. Goñi, C. Centeno, F. Saura-Calixto, and I. Arija. 2010. Effect of grape seed extract on growth performance, protein and polyphenol digestibilities, and antioxidant activity in chickens. Span. J. Agric. Res. 8:326–333.

Brenes, A., A. Viveros, I. Goñi, C. Centeno, S. G. Sayago-Ayerdi, I. Arija, and F. Saura-Calixto. 2008. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poult. Sci. 87:307–316.

Caspary, W. F. 1992. Physiology and pathophysiology of intestinal absorption. Am. J. Clin. Nutr. 55:299S–308S.

Deplancke, B., O. Vidal, D. Ganessunker, S. M. Donovan, R. I. Mackie, and H. R. Gaskins. 2002. Selective growth of mucolytic bacteria including *Clostridium perfringens* in neonatal piglet model of total parenteral nutrition. Am. J. Clin. Nutr. 76:1117–1125.

Dolara, P., C. Luceri, C. De Filippo, A. P. Femia, L. Giovannelli, G. Caderni, C. Cecchini, S. Silvi, C. Orpianesi, and A. Crecí. 2005. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat. Res. Fund. Molec. Mech. Mutag. 591:237–246.

Donovan, J. L., C. Manach, L. Rios, C. Morand, A. Scalbert, and C. Rémésy. 2002. Procyanidins are not bioavailable in rats fed a single meal containing a grape seed extract or the procyanidin dimer B3. Br. J. Nutr. 87:299–306.

FAO-STAT. 2007. FAO statistical data base. Accessed Feb. 2010. http://www.fao.org.

FEDNA. 2003. Tablas FEDNA de Composición y Valor Nutritivo de Alimentos para la Fabricación de Piensos Compuestos. Fundación Española para el Desarrollo de la Nutrición Animal, Madrid, Spain.

Ferket, P. R., C. W. Parks, and J. L. Grimes. 2002. Mannan oligosaccharides versus antibiotics for turkeys. Nutritional biotechnology in the feed and food industries. Pages 43–64 in Proc. Alltech's 18th Ann. Symp. Nottingham University Press, Nottingham, UK.

Gañán, M., A. J. Martínez-Rodríguez, and A. V. Carrascosa. 2009. Antimicrobial activity of phenolic compounds of wines against *Campylobacter jejuni*. Food Contr. 20:739–742.

García-Ruíz, A., B. Bartolomé, A. J. Martínez-Rodríguez, E. Puello, P. J. Martín- Álvarez, and M. V. Moreno-Arribas. 2008. Potential of phenolic compounds for controlling lactic acid bacteria growth in wine. Food Contr. 19:835–841.

Gong, J., R. J. Forster, H. Yu, J. R. Chambers, P. M. Sabour, R. Wheatcroft, and S. Chen. 2002. Diversity and phylogenetic analysis of bacteria in mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiol. Lett. 208:1–7.

Goñi, I., A. Brenes, C. Centeno, A. Viveros, F. Saura-Calixto, A. Rebolé, I. Arija, and R. Estevez. 2007. Effect of dietary grape pomace and vitamin E on growth performance, nutrient digestibility, and susceptibility to meat lipid oxidation in chickens. Poult. Sci. 86:508–516.

Harmsen, H. J., G. R. Gibson, P. Elfferich, G. C. Raangs, A. C. Wildeboer-Veloo, A. Argaiz, M. B. Roberfroid, and G. W. Welling. 2000. Comparison of viable cell counts and fluorescence in situ hybridization using specific rRNA-based probes for the quantification of human fecal bacteria. FEMS Microbiol. Lett. 183:125–129.

Hervert-Hernández, D., C. Pintado, R. Rotger, and I. Goñi. 2009. Stimulatory effect of grape pomace polyphenols on *Lactobacillus acidophilus* growth. Int. J. Food Microbiol. 136:119–122.

Højberg, O., N. Canibe, H. D. Poulsen, M. S. Hedemann, and B. B. Jensen. 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. Appl. Environ. Microbiol. 71:2267–2277.

Hughes, R. J., J. D. Brooker, and C. Smyl. 2005. Growth rate of broiler chickens given condensed tannins extracted from grape seed. Pages 56–68 in Aust. Poult. Sci. Symp. Poultry Research Foundation, University of Sidney, Sidney, Australia.

Jimenez-Ramsey, J. C., L. Rogler, T. L. Housley, L. G. Butler, and R. G. Elkin. 1994. Absorption and distribution of ^{14}C-labeled condensed tannins and related sorghum phenolics in chickens. J. Agric. Food Chem. 42:963–967.

Kitts, C. L. 2001. Terminal restriction fragment patterns: A tool for comparing microbial communities and assessing community dynamics. Curr. Issues Intest. Microbiol. 2:17–25.

Laurent, C., P. Besancon, and B. Caporiccio. 2005. Ethanol and polyphenolic free wine matrix stimulate the differentiation of human intestinal Caco-2 cells. Influence of their association with a procyanidin-rich grape seed extract. J. Agric. Food Chem. 53:5541–5548.

López-Oliva, M. E., A. Agis-Torres, P. García-Palencia, I. Goñi, and E. Muñoz-Martínez. 2006. Induction of epithelial hypoplasia in rat cecal and distal colonia mucosa by grape antioxidant dietary fiber. Nutr. Res. 26:651–658.

Manach, C., A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez. 2004. Polyphenols: Food sources and bioavailability. Am. J. Clin. Nutr. 79:727–747.

McDougald, L. R., C. Hofacre, G. Mathis, L. Fuller, J. L. Hargrove, P. Greenspan, and D. K. Hartle. 2008. Enhancement of resistance to coccidiosis and necrotic enteritis in broiler chickens by dietary muscadine pomace. Avian Dis. 52:646–651.

Miles, R. D., G. D. Butcher, P. R. Henry, and R. C. Littell. 2006. Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters and qualitative morphology. Poult. Sci. 85:476–485.

Montreau, F. R. 1972. Sur le dosage des composés phénoliques totaux dans les vins par la methode Folin-Ciocalteau. Conniass. Vigne Vin 24:397–404.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Nelson, K. E., M. L. Thonney, T. K. Woolston, S. H. Zinder, and A. N. Pell. 1998. Phenotypic and phylogenetic characterization of ruminal tannin-tolerant bacteria. Appl. Environ. Microbiol. 64:3824–3830.

Ortiz, L. T., C. Alzueta, J. Trevino, and M. Castaño. 1994. Effects of faba bean tannins on the growth and histological structure of the intestinal tract and liver of chicks and rats. Br. Poult. Sci. 35:743–754.

Osawa, R., K. Kuroiso, S. Goto, and A. Shimizu. 2000. Isolation of tannin-degrading lactobacilli from humans and fermented foods. Appl. Environ. Microbiol. 66:3093–3097.

Papadopoulou, C., K. Soulti, and G. Roussis. 2005. Potential antimicrobial activity of red and white wine phenolic extracts against strains of *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. Food Technol. Biotechnol. 43:41–46.

Reed, J. D., R. E. McDowell, P. J. Van Soest, and P. J. Horvath. 1982. Condensed tannins: A factor limiting the use of cassava forage. J. Sci. Food Agric. 33:213–220.

Rodríguez-Vaquero, M. J., M. R. Alberto, and M. C. Manca de Nadra. 2007. Antibacterial effect of phenolic compounds from different wines. Food Contr. 18:93–101.

Saito, M., H. Hosoyama, T. Ariga, S. Kataoka, and N. Yamaji. 1998. Antiulcer activity of grape seed extract and procyanidins. J. Agric. Food Chem. 46:1460–1464.

SAS Institute. 2003. SAS Stat User's Guide. Version 8. SAS Institute Inc., Cary, NC.

Sehm, J., H. Lindermayer, C. Dummer, D. Treutter, and M. W. Pfaffl. 2007. The influence of polyphenol rich apple pomace or red-wine pomace diet on the gut morphology in weaning piglets. J. Anim. Physiol. Anim. Nutr. (Berl.) 91:289–296.

Sell, D. R., W. M. Reed, C. L. Chrisman, and J. C. Rogler. 1985. Mucin excretion and morphology of the intestinal tract as influenced by sorghum tannins. Nutr. Rep. Int. 31:1369–1374.

Selma, M. V., J. C. Espin, and F. A. Tomás-Barberán. 2009. Interaction between phenolics and gut microbiota: Role in human health. J. Agric. Food Chem. 57:6485–6501.

Shyu, C., T. Soule, S. J. Bent, J. A. Foster, and L. J. Forney. 2007. MiCA: A web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. Microb. Ecol. 53:562–570.

Southgate, D. A. T. 1976. Selected methods. Pages 108–109 in Determination of Food Carbohydrates. Appl. Sci. Publishers Ltd., London, UK.

Svetoch, E. A., N. J. Stern, B. V. Eruslanov, Y. N. Kovalev, L. I. Volodina, V. V. Perelygin, E. V. Misevich, I. P. Mitsevich, V. D. Pokhilenko, V. N. Borzenkov, V. P. Levchuk, O. E. Svetoch, and T. Y. Kudriavttseva. 2005. Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins. J. Food Prot. 68:11–17.

Tepe, B., D. Daferera, M. Sokmen, M. Polission, and A. Sokmen. 2004. In vitro antibacterial and antioxidants activities of essential oils and various extracts of *Thymus eigii.* J. Agric. Food Chem. 52:1132–1137.

Tzounis, X., J. Vulevic, G. G. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. K. Uribe, and J. P. E. Spencer. 2008. Flavanol monomer-induced changes to the human faecal microflora. Br. J. Nutr. 99:782–792.

Wiseman, J., B. K. Edmundo, and N. Shepperson. 1992. The apparent metabolizable energy of sunflower oil and sunflower acid oil for broiler chickens. Anim. Feed Sci. Technol. 36:41–51.

Zijlstra, R. T., K. Y. Whang, R. A. Easter, and J. Odle. 1996. Effect of feeding a milk replacer to early-weaned pigs on growth, body composition, and small intestinal morphology, compared with suckled littermates. J. Anim. Sci. 74:2948–2959.