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Effect of Red Orange and Lemon Extract-Enriched Diet in Suckling Lambs' Fecal Microbiota

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Abstract: Red orange and lemon extract (RLE) is an anthocyanins-rich dietary supplement that may influence gastrointestinal bacterial community in ruminants. The aim of the present study was to investigate the RLE effects on gut microbiota composition in lambs. Twenty-eight lambs were randomly divided into a control group (CON; n = 14) and an anthocyanin group (ANT; n = 14) and fed the same diet; additionally, only the ANT received 90 mg/kg live weight of RLE at day. After lamb slaughter (40 ± 1 days), fecal samples were collected from the rectum and stored at −20 °C until analysis. Analysis of fecal microbiome was carried out by metabarcoding analysis of 16S rRNA. After reads denoising, sequences were aligned against SILVA rRNA sequence database using MALT, and taxonomic binning was performed with MEGAN. A significant increase in Firmicutes and Bacteroidetes and a decrease in Proteobacteria and Actinobacteria was observed in ANT compared to CON. Moreover, an interesting increase of *Lactobacillus* and *Bifidobacterium* genera and a decrease in *Escherichia coli* and *Salmonella* species were detected in ANT compared to CON. Results recommend that anthocyanin supplementation in lamb diet is able to modulate positively gut microbiota and may inhibit the growth of some potential pathogenic microorganisms.

Keywords: gut microbiota; lambs; anthocyanins

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1. Introduction

Mammals' gut microbiota affects a wide range of host physiological functions, including digestion, stress adaption and immune response regulation [1,2]. Thus, gut microbial imbalance may cause health disorders in the host [3,4]. Investigation of ruminant gut microbiota represents a main aspect considering its responsibility for feed digestion but also its importance in physiological and immune functions [5]. In ruminants, the gastrointestinal tract harbors a high-density and -diversity microbiota composed of species and genera belonging to bacteria, archaea, fungi, ciliate protozoa and viruses (approximately 10^{11} microbial cells per gram of digesta) [6]. The ruminants' gut microbiota is composed of trillions of microorganisms, and is the most complicated microecosystem [7]. Its composition is driven by various factors, like host genetic variations, weaning, diets, and stochastic events [8,9]. It plays a key role in pathogenic Gram-negative bacteria mucosal immunity, nutrient absorption and intestinal epithelium differentiation [7]. Among these, weaning plays a pivotal role in the establishment of a functional bacterial community in young animals. In the postnatal life, from birth to weaning, lambs are defined as functional

monogastric, since they are born without a functional rumen, and the symbiotic ruminal microbial community is not established until the first months of life [10,11]. In pre-weaned lamb, milk is the main nourishment, which bypasses the rumen, due to the activation of the esophageal groove, and undergoes digestion in the abomasum and intestine [11]. The gut microbial community in 30-days lambs was previously shown to be heterogenous, with *Lactobacillaceae* (phylum Firmicutes) being predominant. Some other Firmicutes families exhibiting a remarkable abundance in the gut were Lachnospiraceae, Clostridiaceae, and Ruminococcaceae [12]. At weaning, lambs undergo a drastic change in nutrient intake from milk to a plant-based carbohydrates diet, and this transition phase may lead to sequential changes within microbial population of intestine [1,13]. It is known that dietary interventions in pre- and post-weaned lambs significantly affect the microbial community structure and functions, with likely consequences on physiological and practical implications for animal health [14,15]. These may include the use of plant matrices or plant-derived bioactive compounds as feed supplements, and several efforts have been addressed during the last decade to exploit agro-industrial by-products in animal diets. Researchers addressed their interest to phenolic compounds, for their ability to enhance the growth of specific beneficial bacteria and to exclude certain pathogenic microorganisms at gut level. Dietary anthocyanins may improve intestinal barrier functionality, enhance host/bacteria interaction, and promote the proliferation of beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. [16]. These bacterial groups, actively involved in anthocyanin catabolism, exert antimicrobial effects towards pathogens microorganisms, and are usually associated to health benefit for the host [17,18]. According to Cheng et al. [19], phenolic compounds from blueberry pomace may contribute to reshaping the structure and diversity of fecal microbiota community. Moreover, phenolics-rich blueberries extracts had inhibitory effects on some Gram-positive (*Listeria monocytogenes*, *Bacillus subtilis*, and *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica* ser. *Typhimurium*) [20]. In pigs supplemented with grape seeds extract, ecological changes in gut microbial population, with a dramatic increase in *Lachnospiraceae*, *Clostridales*, *Lactobacillus* and *Ruminococcacceae*, was recorded [21]. Cranberry and olive by-products were also shown to modulate the composition of gut microbiota and improve its integrity in broiler chicken [22,23].

Within this framework, our study proposed a metagenomic approach to investigate the effect of anthocyanin-rich red orange and lemon extracts (RLE) on lamb gut microbiota. Red orange (*Citrus sinensis* L.) and lemon (*Citrus limon* L.) peels are food industry by-products resulting from mechanical pulp removal and juice extraction [24]. Citrus by-products have a broad spectrum of application in pharmaceutical, nutraceutical, and food industries [25]. Their inherent chemical composition and the high content in phenolic compounds make citrus extracts suitable as a supplement in ruminant diets [26,27]. To the best of our knowledge, information about the effects of RLE on weaning lambs gut microbial ecology is lacking.

2. Materials and Methods

2.1. Animal Management and Feeding

The experiment was authorized by the Animal Welfare Organization of the University of Naples Federico II (PG/2019/0028161 of 03/19/2019).

The experimental procedures were carried out at the experimental farm of the Council for Agricultural Research and Economics, Research Centre of Animal Production and Aquaculture (CREA-ZA, Bella, PZ, Italy). Twenty-eight Merino male lambs were natural suckled and, from 25 days of life until slaughtering (40 $\pm$ 1 days), they were fed with ad libitum alfalfa hay (188 g/kg crude protein DM, 322 g/kg crude fiber DM) and starter (205 g/kg crude protein DM, 18 g/kg fat DM, 250 g/kg crude fiber DM). Feed chemical composition was determined according to the methods described by Maggiolino et al. [28]. At the start of the present trial, all animals were in good health; no disease was diagnosed during and at the end of the treatments.

After natural colostrum administration, within 2 h from birth, all lambs were randomly subdivided into two treatment groups: one group received the RLE (ANT; $n = 14$), and the control group (CON; $n = 14$) did not receive the additive. During their life, until slaughter (40 ± 1 days), the lambs were permanently housed indoors. The daily intake was calculated daily from the unconsumed feed before the next feeding. It was of 65 g/day and 68 g/day of starter and of 18 g/day and 20 g/day of hay, respectively, for the ANT and CON groups. Each lamb received 90 mg/kg of live weight of RLE once daily. The supplement was orally administered to each lamb of the ANT group. It was mixed with water to obtain a cream [28], which was then administered directly in the mouth using a large syringe. All animals were weighed every 2 days at the same time (7:00 a.m.) and the correct amount of RLE that had to be administered to each ANT lamb was quantified.

2.2. Composition of Red Orange and Lemon Extract

The additive (dry powder) was obtained by a patented extraction process (Italian Patent No. 102017000057761) from red orange and lemon processing wastes. It was created at CREA—Research Centre for Olive, Fruit and Citrus Crops (CREA-OFA, Acireale, Italy), for research purposes only. The separation of anthocyanins content was performed as described by Maggiolino et al. [29]. The RLE chemical composition is shown in Table 1.

Table 1. Red orange and lemon extract (RLE) chemical composition.

Compound	$[M]^+$ (m/z)	MS^n (*m*/*z*)	Anthocyanin	Relative Composition (%) [(a)]
1	611	449/287	cyanidin 3,5-diglucoside	1.29
2	465	303	delphinidin 3-glucoside	2.67
3	611	287	cyanidin 3-sophoroside	0.41
4	449	287	cyanidin 3-glucoside	39.97
5	595	287	cyanidin 3-rutinoside	1.30
6	479	317	petunidin 3-glucoside	1.59
7	551	465/303	delphinidin 3-(6″-malonyl)glucoside	1.43
8	463	301	peonidin 3-glucoside	2.98
9	565	479/317	petunidin 3-(6″-malonyl)glucoside	1.45
10	535	449/287	cyanidin 3-(6″-malonyl)glucoside	21.76
11	-	271	pelargonidin derivative	1.44
12	549	463/301	peonidin 3-(6″-malonyl)glucoside	13.80
13	-	287	cyanidin derivative	2.39
14	-	301	peonitin derivative	1.82
			Total anthocyanins (g CGE/100 g)	2.66 ± 0.01

$[M]^+$ (*m*/*z*): mass peak; MS^n (*m*/*z*): MS fragmentation model; [(a)] Relative composition of anthocyanins calculated from peak areas recorded at 520 nm. The total anthocyanin content was expressed as mg of cyanidin 3-glucoside equivalents (CGE) 100 mL^{-1} and mg CGE 100 g^{-1} dry weight (DW) for the juice samples.

2.3. Fecal Sampling and Microbiome Analysis

All lambs were slaughtered in a slaughterhouse approved by the European Community. Fecal samples were collected soon after stunning and jugulation, before evisceration procedures, directly from the rectal ampoule using digital rectal retrieval. All samples were stored immediately at −80 °C until analysis.

Fecal samples were subjected to DNA extraction and 16S rRNA sequencing. For 16S rRNA gene amplicon sequencing, the bacterial V4 regions were amplified from extracted DNA by using the universal primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTAAT-3′). The PCR reactions were carried out in triplicates using Platinum SuperFi DNA Polymerase (Invitrogen, Carlsbad, CA, USA) with the following conditions: 98 °C for 30 min, 25 cycles of 98 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s and a final extension of 72 °C for 5 min. Amplified libraries were verified on 2% agarose gel and PCR products were purified using Agencourt AMPure XP magnetic beads (Agencourt Bioscience, Beverly, MA, USA). Purified libraries were quantified with Qubit

dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) and pooled at a final concentration of 100 pM. Libraries were sequenced on an Ion S5 Sequencing System (Thermo-Fisher Scientific, Waltham, MA, USA). Quality filtered reads were aligned using MALT (v0.4.1) [30], against the SILVA rRNA sequence database (SSURef_NR99, release 128). Taxonomic binning was performed by using MEGAN software [31]. Core microbiome was calculated at genus level by MEGAN (sample threshold = 80%; class threshold = 1%).

2.4. Statistical Analysis

Statistical comparison of the relative abundance of taxonomic categories was performed using STAMP software [32] by two-sided G-test (*w*/Yates' + Fisher's) with asymptomatic confidence intervals (0.95) and the Benjamini–Hochberg False Discovery Rate (FDR) method (q-value < 0.05). Rank abundance and alpha and beta diversity indexes were estimated using Microbiome Analyst [33]. Moreover, principal coordinate analysis (PCoA) was calculated using the Bray–Curtis index. Hypothesis testing was conducted by the analysis of molecular variance (AMOVA) test (p < 0.05) [34].

3. Results

A total of 11,757,088 quality-filtered sequences with an average length of 291 bp were obtained from the 28 fecal samples. The abundance-based coverage estimators (ACE) and the alpha diversity index Chao1 indicated that the richness of the samples was increased by anthocyanins supplementation. A similar behavior was observed for Shannon and Simpson indexes, although less marked between the two groups (Figure S1). Taxonomic assignment identified a total of 11 different phyla among all fecal samples (Figure 1). Overall, the lamb fecal microbiota was dominated by the Proteobacteria and Firmicutes phyla, followed by Actinobacteria and Bacteroidetes.

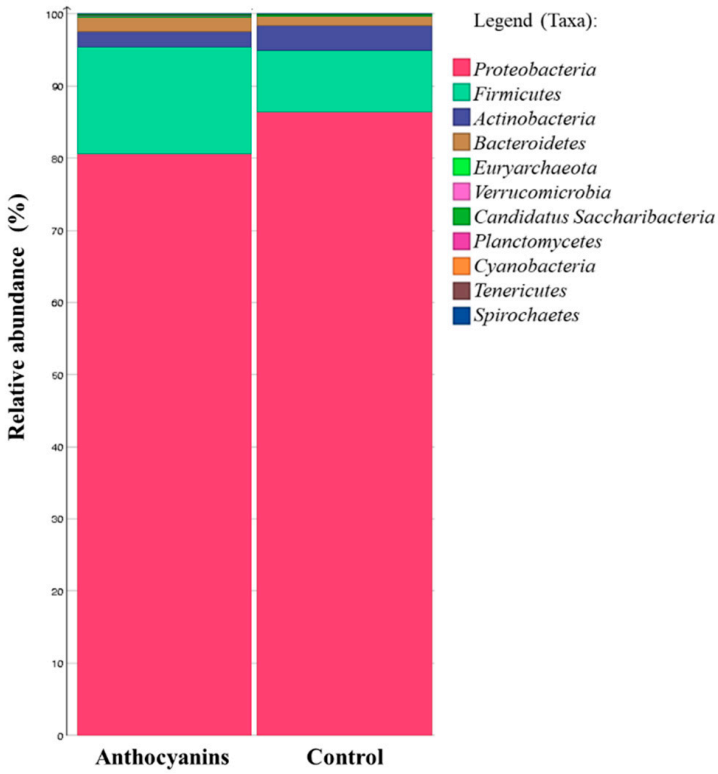

Figure 1. Relative abundance and taxonomic assignments at phylum level of the control group diet and the anthocyanin group diet supplemented with the red orange and lemon extract (RLE).

Significant differences between groups at phylum level are reported in Figure 2.

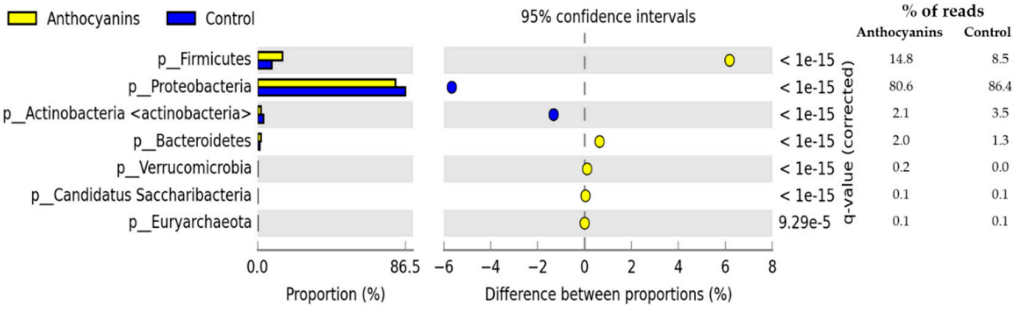

Figure 2. Differences in bacterial abundance at phylum level between the control and anthocyanin groups. The left side of the graph shows the abundance ratios of different taxa. The middle graph shows the difference in bacterial abundance with the 95% confidence interval with the respective q-values ($q < 0.05$). The right table shows the reads percentage assigned for each taxonomic group.

The ANT group showed a reduced ($q < 0.01$) presence of Proteobacteria and Actinobacteria and increased ($q < 0.01$) presence of Firmicutes, Bacteroidetes, Verrucomicrobia, Candidatus Saccharibacteria and Euryarchaeota quantity. Differences at family level are reported in Figure 3.

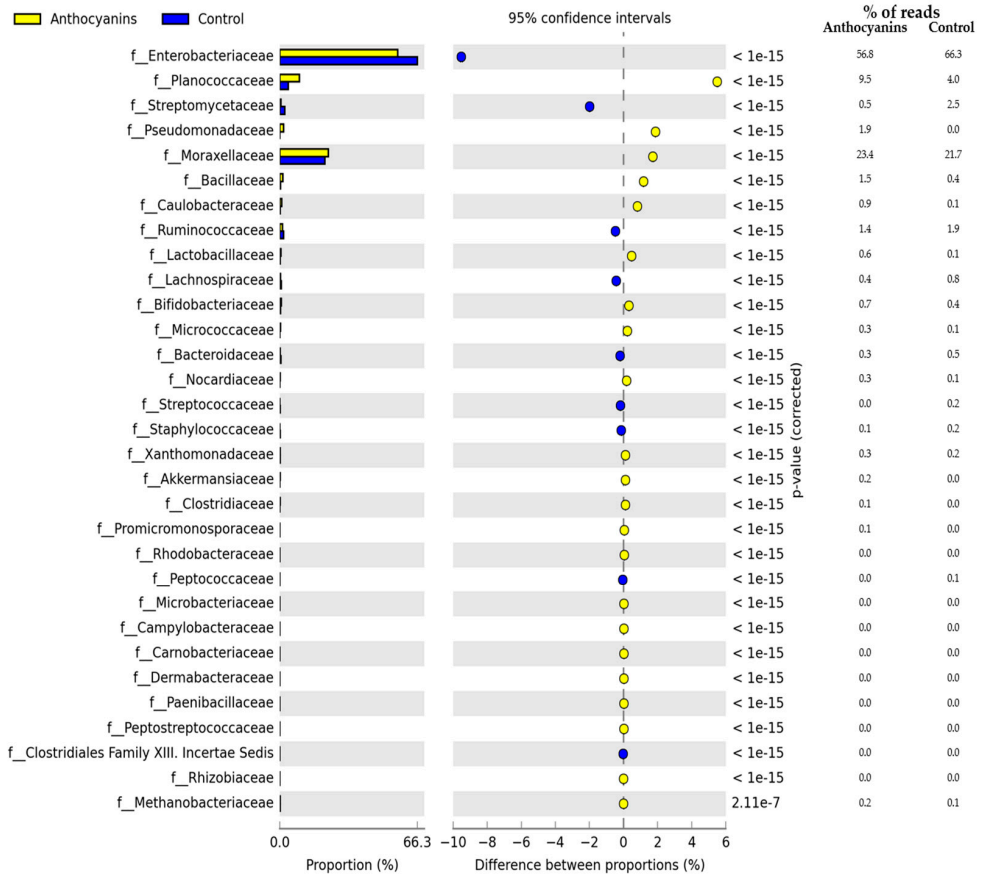

Figure 3. Differences in bacterial abundance at family level between the control and anthocyanin groups. The left side of the graph shows the abundance ratios of different taxa. The middle graph shows the difference in bacterial abundance with the 95% confidence interval with the relative q-values ($q < 0.05$). The right table shows the reads percentage assigned for each taxonomic group.

Lambs fed with anthocyanin dietary supplementation showed higher ($q < 0.01$) quantity of Planococcaceae, Pseudomonadaceae, Moraxellaceae, Bacillaceae, Caulobacteraceae, Lactobacillaceae and Bifidobacteriaceae families and lower ($q < 0.01$) quantity of Enterobacteriaceae, Sptreptomycetaceae, Ruminococcaceae and Lachnospiraceae. Moreover, the an-

thocyanin addition slightly increased Micrococcaceae, Nocardiaceae, Xanthomonadaceae, Akkermansiaceae, Clostridiaceae, Promicromonosporaceae, Rhodobacteriaceae, Microbacteriaceae, Campylobacteriaceae, Carnobacteriaceae, Dermabacteraceae, Paenibacellaceae, Preptostreptococcaceae, Rhizobiaceae and Methanobacteriaceae families, reducing the Bacteroidaceae, Streptococcaceae, Staphylococcaceae, Peptococcaceae and Clostridiales families ($q < 0.01$). Figure 4 shows statistical differences between experimental groups at genus level.

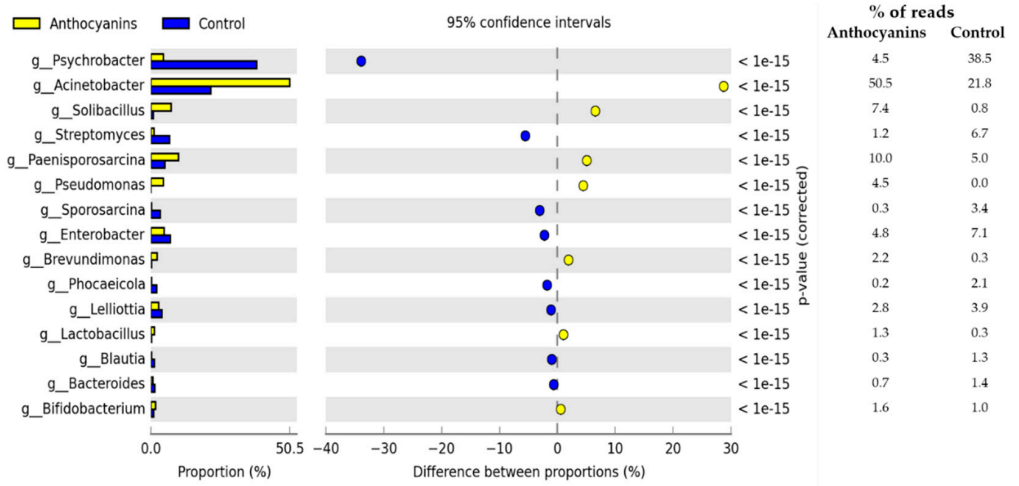

Figure 4. Differences in bacterial abundance at genus level between the control and anthocyanin groups. The left side of the graph shows the abundance ratios of different taxa. The middle graph shows the difference in bacterial abundance with the 95% confidence interval with the *q*-values ($q < 0.05$). The right table shows the reads percentage assigned for each taxonomic group.

Feces of lambs fed with anthocyanins were richer ($q < 0.01$) in microorganisms belonging to the *Acinetobacter, Solibacillus, Paenisporosarcina, Pseudomonas, Brevundimonas, Lactobacillus* and *Bifidobacterium* genera, and poorer ($q < 0.01$) of those belonging to *Psychrobacter, Streptomyces, Sporosarcina, Enterobacter, Phocaeicola, Lelliottia, Blautia* and *Bacteroides* genera. However, considering the common core microbiota of the control and anthocyanin groups was composed by *Acinetobacter* (50%), *Psychrobacter* (27%), *Paenisporosarcina* (10%), *Enterobacter* (8%) and *Lelliottia* (4%), as shown in Figure S2. Taking into account the unique genera identified in the two experimental groups, 14 and 23 unique taxa were detected in the control and anthocyanin groups, respectively (Table S1).

Principal coordinate analysis at species level showed that the microbial community profiles were almost similar, although some individuals had a distinct profile (Figure 5).

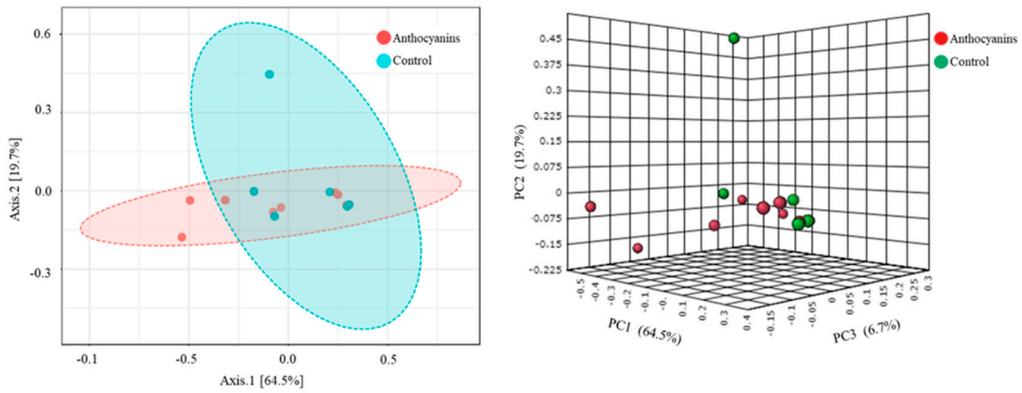

Figure 5. Principal coordinate analysis (PCoA) as 2D (left) and 3D (right) plots of bacterial species assigned for the anthocyanin and control groups. PCoA was calculated using the Bray-Curtis index to compute dissimilarities among different samples. Hypothesis testing was conducted by analysis of molecular variance (AMOVA) test ($p < 0.05$).

Differences at species level were reported in Figure 6.

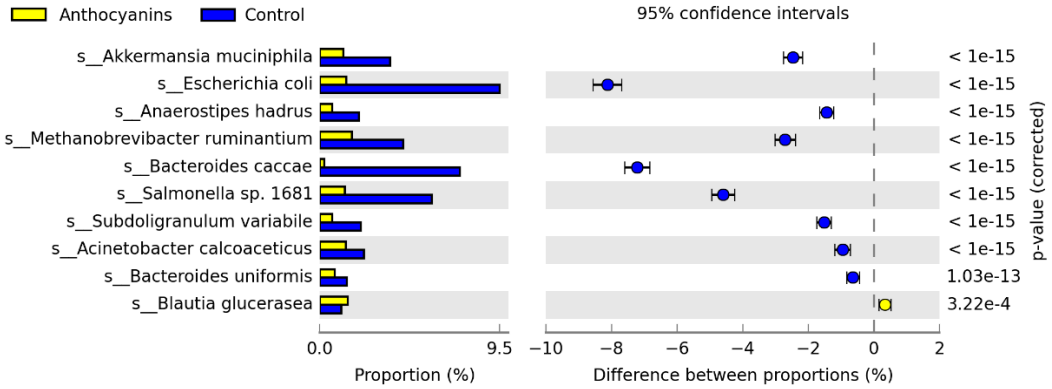

Figure 6. Top ten most abundant bacterial species and relative differences between proportions in bacterial abundance with the 95% confidence interval ($q < 0.01$).

Considering the total number of reads assigned at species level, AG recorded higher ($q < 0.05$) presence of *Methanobrevibacter ruminantium* and *Bacteroides uniformis*, but lower ($q < 0.05$) of *Escherichia coli, Bacteroides caccae* and *Salmonella* sp. 1681.

4. Discussion

Gut microbiota composition, especially in young ruminant, is very susceptible to modifications [14,35]. In the present study, a total of 11 different *phyla* were identified among all fecal samples. Regardless of the treatment, Proteobacteria and Firmicutes were the most abundant phyla (about 80% and 15% of the reads respectively), representing over 90% of the total reads, followed by Actinobacteria and Bacteroidetes. These phyla are predominant in mammal small intestine [36] and in chicken cecum intestine [37], but their proportion is influenced by multiple factors such as species, diet, age and genotype [38,39]. Similarly, Li et al. [7] reported that these phyla are the most abundant in goats gut microbiota, representing over 90% of the taxonomic groups identified; differently, some authors in lambs [40] and calves [41,42] reported that Firmicutes and Actinobacteria represented the most abundant phyla, covering about 90% of the groups identified. Particularly for ruminant, Firmicutes, and in particular *Lactobacillus* genus, play a pivotal role in fiber and cellulose degradation [43] and contribute to maintain microflora balance by counteracting pathogenic invasions [44]. On the contrary, Bacteroidetes degrade sugars and proteins, and are positively related to immune system effectiveness [45,46]. Differently, Proteobacteria mainly consists of Gram-negative bacteria and some of them are responsible for important pathologies, such as diarrhea and malabsorption, affecting the gastrointestinal system [47]; however, the amount of Proteobacteria in small ruminants, as in goat kids, decreased with increasing age [38]. The relative abundance of Proteobacteria and Firmicutes was 86.4% and 8.5% in the control group and 80.6% and 14.8% in anthocyanin group, respectively. Comparable results were obtained from a study conducted on male goats, fed adding condensed tannins to the diet, where the predominant *phyla* were Proteobacteria and Firmicutes, followed by Bacteroidetes [48]. Regardless of the age and feeding system, Proteobacteria and Firmicutes were detected as the dominant *phyla* also in goat kids [38]. Anthocyanin supplementation significantly decreased the percentage of Proteobacteria (−5.8%) and Actinobacteria (−1.4%), and increased, favourably, Firmicutes (+6.3%) and Bacteroidetes (+0.7%) compared to the control group, which could have positive effects on intestinal health and growth rates in lambs.

There was a significant reduction in the Enterobacteriaceae population (−9.5%) in the fecal microbiota of the lambs supplemented with anthocyanins compared to the control group. Although it is not fully elucidated the anthocyanins mechanism of action in the gut, it could be that these substances act by inhibiting some microorganism replication, favoring the replication of some other, probably inducing more availability of some substances

that may act as prebiotics [49]. Enterobacteriaceae is a large family of Gram-negative bacteria that includes many commensal bacteria but also many spread pathogens such as *Salmonella, E. coli, Shigella, Yersinia, Proteus* [50], which could result in diarrhea, gastritis, vomiting, gastrointestinal ulcers and often death of the animal [47,51]. Although the Bifidobacteriaceae and Lactobacillaceae families are poorly represented in all samples, anthocyanin supplementation resulted in an increase in the presence of bacteria belonging to the Lactobacillaceae (+0.5%) and Bifidobacterium (+0.3%) family compared to the control group.

A total of 27 genera were identified in the fecal samples. Important differences in composition were observed for the genus level between the groups. The most represented genera were *Psychrobacter* (36.5% and 4.5% for the control and anthocyanin group, respectively) and *Acinetobacter* (21.8% and 50.5% for the control and anthocyanin group, respectively), representing together more than 50% for both experimental groups. Interestingly, the decrease in *Enterobacter* and the increase in *Bifidobacterium* and *Lactobacillus* were observed in the anthocyanin group. In fact, both *Bifidobacterium* and *Lactobacillus* are well-known beneficial microbiota [52], also representing spread probiotic matrices sold on the market [53]. *Bifidobacterium* has been reported to stimulate the early development of gut regulating its microbial homeostasis, inhibiting pathogens and modulating local and systemic immune responses, improving vitamins production and increasing the bioconversion of dietary components to active compounds [54]. *Lactobacillus* has been linked to immune system improvement in young calves [55]. Some studies reported that these bacteria in young ruminants are able to increase weight gain and to decrease feed conversion ratio [56,57], as reported for kids fed with anthocyanins administration by Salzano et al. [58]. *Lactobacillus* and *Bifidobacterium* have an antagonist activity on some potential pathogens like *Salmonella, E. coli* and *Clostridium perfrigens* [59]. *Escherichia* is a commensal bacterium in the intestinal microbiota, although some strains are harmless, while other ones can provoke intestinal diseases, diarrhea and inflammatory events, that represented the main cause of morbidity and mortality in new-born ruminants [56,60]. The *Escherichia coli* species and *Salmonella spp.* bacteria were significantly lower in the anthocyanin group, showing a positive effect of additive. This is an important result for the intestinal health of lambs, digestibility and for the growth rate of young animals, showing a positive effect of anthocyanins administration. Furthermore, a reduction in *E. coli* could significantly reduce the risk of transmission of food poisoning from animals to humans [61]. The low percentage of *E. coli* in the anthocyanin group could be explained by the antimicrobial activity of anthocyanins and phenolic compounds [62], but the inhibition mechanism is not yet fully known [49]. Some studies reported the *Bifidobacterium* and *Lactobacillus* effect in hindering the *Escherichia* attachment to the epithelial cell surface, reducing indices of intestinal disease [63,64].

5. Conclusions

The administration of a red orange and lemon extract in suckling lambs induced changes in their gut microbiota, with a favorable increase of bacteria belonging to the Firmicutes phylum and *Bifidobacterium* and *Lactobacillus* genera and a decrease in potentially pathogenic bacteria such as *E. coli* and *Salmonella spp*. This should be considered as a potential way for reducing the use of antimicrobial substances, as well as improving animals' health and welfare status.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11070572/s1, Figure S1: Alpha diversity indexes (ACE, Chao1, Shannon, Simpson) of bacterial species assigned for anthocyanins and control group, Figure S2: Relative abundance and taxonomic assignments at genus level of the core microbiota common to the control and anthocyanins group, Table S1: List of unique taxa detected in control and anthocyanins group.

Author Contributions: Conceptualization, M.F., A.M. and P.D.P.; methodology, M.F., M.F.S., S.D. and F.I.; software, M.F.; validation, P.D.P. and G.M.; formal analysis, M.F. and S.D.; investigation, A.M., M.F. and P.D.P.; resources, P.D.P.; data curation, M.F. and M.F.S.; writing—original draft preparation, A.M., M.F.S. and M.F.; writing—review and editing, P.D.P. and G.M.; visualization, P.D.P. and G.M.; supervision, S.D. project administration, P.D.P. and A.M.; funding acquisition, P.D.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Animal Welfare Organization of the University of Naples Federico II (PG/2019/0028161 of 03/19/2019).

Data Availability Statement: The sequence data are available at NCBI SRA under BioProject ID: PRJNA726662.

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Conflicts of Interest: The authors declare no conflict of interest.

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