

# Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens

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**ABSTRACT** This study evaluated the effect of sodium butyrate (SB) on performance, expression of immune-related genes in the cecal tonsils, and cecal microbiota of broiler chickens when dietary energy and amino acids concentrations were reduced. Day-old male Ross 708 broiler chicks were fed dietary treatments in a 3 × 2 factorial design (8 pens per treatment) with 3 dietary formulations (control diet; reduction of 2.3% of amino acids and 60 kcal/kg; and reduction of 4.6% of amino acids and 120 kcal/kg) with or without the inclusion of 0.1% of SB. Feed intake (FI), body weight gain (BW gain), and feed conversion ratio (FCR) were recorded until 28 d of age. From 14 to 28 d, there was an interaction of nutrient density by SB ( $P = 0.003$ ) wherein BW gain of birds fed SB was impaired less by the energy/amino acids reduction than unsupplemented birds. A similar result was obtained from 1 to 28 d ( $P = 0.004$ ). No interaction ( $P < 0.05$ ) between nutrient density by SB was observed for FCR. Nutritional density of the diets and SB modified the struc-

ture, composition, and predicted function of the cecal microbiota. The nutritionally reduced diet altered the imputed function performed by the microbiota and the SB supplementation reduced these variations, keeping the microbial function similar to that observed in chickens fed a control diet. The frequency of bacterial species presenting the butyryl-CoA: acetate CoA-transferase gene increased in the microbiota of chickens fed a nutritionally reduced diet without SB supplementation, and was not changed by nutrient density of the diet when supplemented with SB (interaction;  $P = 0.01$ ). SB modulated the expression of immune related genes in the cecal tonsils; wherein SB upregulated the expression of A20 in broilers fed control diets ( $P < 0.05$ ) and increased IL-6 expression ( $P < 0.05$ ). These results show that SB had positive effects on the productive performance of broilers fed nutritionally reduced diets, partially by modulating the cecal microbiota and exerting immune-modulatory effects.

**Key words:** broiler, immune system, intestinal microbiota, sodium butyrate

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## INTRODUCTION

Intestinal bacteria produce short-chain fatty acids (**SCFA**), which are derived from cecal fermentation of compounds that cannot be digested by the animals, such as cellulose, fiber, starch, and sugar (Guilloteau et al., 2010). Butyrate, a SCFA, can be used as an energy source by intestinal cells and can also positively influence intestinal cell proliferation, differentiation, maturation, and could positively alter the intestinal barrier, among other functions (Onrust et al., 2015).

While butyrate is naturally produced by fermentation in the cecum, the production of SCFA in the small intestine is limited (Levy et al., 2015). Synthetic sources

of butyrate have been the focus of numerous studies in poultry (Leeson et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Sunkara et al., 2011; Qaisrani et al., 2015). However, it has been described that uncoated butyrate could be absorbed before reaching the distal portions of the small intestine (van der Wielen et al., 2002). Dietary supplementation with a protected source of butyrate may delay the release of the substance along the gastrointestinal tract, thereby having plausible functional effects on the lower gastrointestinal tract (**GIT**). Besides the location of the GIT where butyrate is released, the dose of butyrate is also a factor that should be considered when aiming to decrease the variability of results across studies; for example, Hu and Guo (2007) used up to 2,000 mg/kg of butyrate, while Timbermont et al. (2010) used a maximum of 330 mg/kg. Barcelo et al. (2000) showed that 100 mM of butyrate may be toxic for colonic goblet cells, and

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decrease the secretion of mucus, demonstrating that high concentrations of butyrate may also have deleterious effects.

Butyrate may regulate the production of inflammatory cytokines by modulating the intestinal immune cells, such as lymphocytes and macrophages (Guilloteau et al., 2010). Despite this knowledge, the mechanism by which butyrate exerts its anti-inflammatory effects remains to be determined (Chang et al., 2014). However, butyrate seems to have an anti-inflammatory effect mediated by signaling pathways (Meijer et al., 2010), such as the modulation of pro-inflammatory cytokines via impairment in NF- $\kappa$ B activation (Guilloteau et al., 2010). On the other hand, some studies have shown the effects of butyrate in controlling pathogens in poultry, such as *Salmonella*, *Clostridium perfringens*, and modulating the *Lactobacillus* population (Van Immerseel et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Namkung et al., 2011). Furthermore, a question remains on what effect butyrate may elicit on the cecal microbiota, and what translational effects it elicits on the host.

To our knowledge, there is no study examining the effects of protected sources of dietary butyrate on the cecal microbiota of broiler chickens and its association with the intestinal immune system. Therefore, dietary sodium butyrate was hypothesized to improve the performance of broilers fed a nutritionally reduced diet, by modulating the expression of immune-related genes and modifying the cecal microbiota of broiler chickens. The objective of this study was to evaluate the effect of a coated sodium butyrate-based additive on performance, expression of immune-related genes in the cecal tonsils, and the composition and predicted function of the cecal microbiota of broiler chickens when dietary energy and amino acids concentrations were reduced.

## MATERIALS AND METHODS

### Housing, Birds, and Treatments

The animal care and use protocol was reviewed and approved by the Purdue University Animal and Use Committee. One-day-old male Ross 708 broiler chicks (2,208) were used in the experiment. Chicks were weighed individually and allocated by pen such that mean pen body weight were not different (48 pens with 46 birds/pen and 8 replicates/treatment), in a completely randomized design. The nutritional program consisted of two diets; a starter diet (0 to 14 d) and a grower diet (15 to 28 d) fed from 1 to 28 d of age. Birds had *ad libitum* access to water and feed in mash form during the entire experimental period. For each phase, a basal diet was formulated with corn and soybean meal to meet or exceed the nutrient specifications (with the exception of dietary treatment reductions in energy and amino acids) of chicks to meet or exceed NRC (1994;

Table 1). The grower feed samples were analyzed for nutrient content (Table 1); however, the starter feed samples were not properly stored and the nutrient content could not be determined. Amino acid profile (method 982.30; AOAC International, 2006) was determined at the University of Missouri Experiment Station Field Laboratory (Columbia, MO). Crude fiber, and crude protein (methods 962.09, and 990.03, respectively; AOAC International, 2006) were determined by Dairy One (Ithaca, NY).

The dietary treatments were arranged in a  $3 \times 2$  factorial design (6 treatments) with 3 dietary formulations (control diet, a diet formulated with a reduction of 2.3% of amino acids and 60 kcal, and a diet formulated with a reduction of 4.6% of amino acids and 120 kcal) with or without the inclusion of 0.1% of sodium butyrate (Norel S.A., Madrid, Spain). The sodium butyrate (**SB**) additive, which contained 70% sodium butyrate and 30% sodium salts of palm fatty acid distillate, was supplemented to replace Sulkafloc (purified cellulose) that was used to dilute the energy and amino acid content of the diets.

### Sample Collection and Analysis Performed

Birds and feed were weighed weekly by pen and the mortality recorded daily. Average feed intake (**FI**) and body weight gain (**BW** gain) were corrected for mortality when calculating feed conversion ratio (**FCR**) for each pen. At 28 d of age, one bird per pen, totaling eight birds per treatment, was selected, euthanized by  $\text{CO}_2$  exposure, and the cecal tonsils and cecum content were collected and frozen at  $-80^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , respectively, for subsequent analysis. Based on the productive performance results, the gene expression and cecal microbiota analyses were performed only in four treatments (control diet and diet formulated with a reduction of 2.3% of amino acids and 60 kcal with or without the supplementation of SB; i.e., a  $2 \times 2$  factorial design).

### DNA Extraction of the Cecal Microbiota

The DNA isolation was conducted following the manufacturer recommendations (PowerViral Environmental RNA/DNA Isolation Kit—Mo Bio; Qiagen, Carlsbad, CA). Briefly, bacterial cells were lysed using beads, phenol:chloroform:isoamyl alcohol and solution 1 of the Mo Bio DNA extraction kit by vortexing at maximum speed for 10 min in the Mo Bio Vortex Adapter (Qiagen). After this step, the upper aqueous layer was transferred to a clean collection tube, the solution 2 was added, incubated at  $4^{\circ}\text{C}$  for 5 min, centrifuged, and the supernatant transferred to a new tube. The solutions 3 and 4 were added and the lysate was filtered. The filter was then washed and the DNA recovered. The presence of DNA was verified by agarose gel electrophoresis (1.5%).

**Table 1.** Composition of the experimental diets and calculated and determined nutrient composition.

Ingredient, %	1 to 14 d			15 to 28 d		
	Control	Reduced	Reduced 2	Control	Reduced	Reduced 2
Corn	57.12	55.96	54.81	58.72	57.40	55.84
Soybean meal, 47.5% CP	35.70	34.87	34.11	33.04	32.49	31.96
Soybean oil	2.300	2.300	2.300	3.614	3.600	3.670
Monocalcium phosphate	1.801	1.814	1.826	1.258	1.268	1.279
Limestone	1.369	1.371	1.372	1.502	1.503	1.503
Sodium chloride	0.457	0.458	0.459	0.407	0.408	0.409
L-lysine HCl	0.321	0.313	0.303	0.174	0.161	0.150
DL-methionine	0.400	0.390	0.380	0.313	0.300	0.290
L-threonine	0.091	0.088	0.085	0.025	0.020	0.016
Vitamin Premix- broilers <sup>1</sup>	0.350	0.350	0.350	0.350	0.350	0.350
Sulka-Floc <sup>2</sup>	0.100	2.083	4.000	0.100	2.004	0.500
<b>Calculated nutrient and energy content</b>						
ME Kcal/Kg	3,050	2,990	2,930	3,150	3,090	3,030
CP, %	22.17	21.67	21.20	20.78	20.40	20.00
Lysine, %	1.430	1.397	1.364	1.240	1.211	1.183
Thr, %	0.940	0.918	0.897	0.830	0.811	0.792
Met+Cys, %	0.070	0.045	0.021	0.950	0.928	0.906
nPP, %	0.500	0.500	0.500	0.380	0.380	0.380
Ca, %	0.950	0.950	0.950	0.900	0.900	0.900
Na, %	0.200	0.200	0.200	0.180	0.180	0.180
<b>Determined nutrient content</b>						
Crude Fiber, %	-	-	-	2.25	3.50	4.00
CP, %	-	-	-	21.29	20.78	20.15
Lysine, %	-	-	-	1.395	1.240	1.190
Thr, %	-	-	-	0.860	0.785	0.750
Met+Cys, %	-	-	-	0.950	0.840	0.810

<sup>1</sup>Supplied the following per kilogram of diet: supplied per kg of diet: vitamin A, 13,233 IU; vitamin D<sub>3</sub>, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B<sub>12</sub>, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydriodide, 2.10 mg; selenium from sodium selenite, 0.25 mg.

<sup>2</sup>International Fiber Corporation, North Tonawanda, NY.

## PCR Amplification and Sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the primer FwOvAd\_341f and ReOvAd\_785r as previously described (Khindworth et al., 2013). Each PCR reaction contained DNA template (10 ng), 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 12.5 µL 2× Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), and water to a final volume of 25 µL. The DNA was subjected to initial denaturation at 95°C for 3 min. Amplification was then achieved by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Final extension was at 72°C for 5 min. PCR products were cleaned using AMPure XP magnetic beads (Labplan, Dublin, Ireland) and submitted to another PCR to incorporate indexes (Illumina Nextera XT indexing primers, Illumina, Sweden) to the samples. Each PCR reaction contained 5 µL of each index primer, 25 µL 2× Kapa HiFi Hot Start Ready mix (Anachem, Dublin, Ireland), and 10 µL water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were cleaned and pooled and paired-ends were sequenced at a read length of 300 nucleotides on a MiSeq platform (Illumina, Inc., San Diego, CA).

## Bioinformatics

All sequence processing was performed using MOTHUR software (Ann Arbor, MI) version 1.37.1 (Schloss et al., 2009). Sequences were paired-end and quality trimmed. Sequences containing more than 8 homopolymers nucleotides, and mismatched or ambiguous bases were removed. High-quality sequences were aligned against the SILVA database (Ribocon GmbH, Bremen, Germany) release 119 (Pruesse et al., 2007). UCHIME software (Tiburon, CA) was used to identify and remove chimeric sequences (Edgar et al., 2011). Number of sequences per sample was normalized based on the sample with the lowest number of reads for statistical comparison (Gihring et al., 2012). Operational taxonomic units (**OTUs**) were assigned at a 97% identity using the average neighbor algorithm, and taxonomic assignments were made using the Ribosomal Database Project taxonomy (**RDP**; East Lansing, MI) as described by Cole et al. (2009). Diversity indexes were calculated using MOTHUR. Representative sequences of each OTU were classified using BLASTN (Bethesda, MD) as described (Altschul et al., 1990). Predictive functions of the cecal communities were performed using Picrust software (Boston, MA) online galaxy version (Langille et al., 2013; Afgan et al., 2016). A reference OTU table was generated using Greengenes

**Table 2.** Primers used for qPCR.

Target gene	Primer sequence (5'-3')	Reference
<i>IL-6</i>	F: ATAAATCCCGATGAAGTGG R: CTCACGGTCTTCTCCATAAA	(Gao et al., 2012)
<i>IL-10</i>	F: CAATCCAGGGACGATGAAC R: GCAGGTGAAGAAGCGGTGA	(Gao et al., 2012)
<i>INF- γ</i>	F: TGAGCCAGATTGTTCGA R: ACGCCATCAGGAAGGTTG	(Gao et al., 2012)
<i>IL-1β</i>	F: AGAAGAACGCCTCGCCTGGAT R: CGCAGCAGTTGGTCAT	(Gao et al., 2012)
<i>NF-κB p65</i>	F: GTGTGAAGAACGGGAACCTG R: GGCACGGTTGTCATAGATGG	(Li et al., 2015)
<i>A20</i>	F: GACATCGTCTAACAGCTTGAA R: AGAAAAGAGGGTATCAGGCACAAC	(Li et al., 2015)
<i>GAPDH</i>	F: ACTGTCAAGGCTGAGAACCG R: CATTGATGTTGCTGGGTC	(Gao et al., 2012)
<i>Uni</i>	F: GTGSTGCAYGGYYGTCGTC R: ACGTCRTCCMCNCCTTCCTC	(Belenguer et al., 2006)
<i>BcoATscr</i>	F: GCIGAICATTCACTGGAAWSITGGCAYATG R: CCTGCCCTTGCAATRTCIAACRAANGC	(Louis and Flint, 2007)

(Berkeley, CA) core set database (DeSantis et al., 2006). A closed-reference OTU table was normalized by the 16S rDNA copy number, the metagenome was predicted and categorized by function based on the Kyoto Encyclopedia of Genes and Genomes (**KEGG**; Uji, Kyoto, Japan) pathway (Kanehisa and Goto, 2000). The obtained biome file was processed by STAMP (Halifax, Nova Scotia, Canada) version 2.1.3 (Parks et al., 2014).

### Determination of Butyryl-CoA:Acetate CoA-transferase gene by Quantitative PCR

Bacterial genomic DNA isolated from the cecal microbiota of the broilers was amplified with 16S rDNA universal and butyryl-CoA:acetate CoA-transferase primers (Table 2). Standard template DNA was prepared from *Roseburia* sp. strain A2–183 as described previously (Louis et al., 2004) and standard curves were prepared with five standard concentrations of  $10^7$  to  $10^3$  gene copies/ $\mu$ L. A pooled sample was made from 8 replicates from the same treatment and qPCR was performed in triplicate in a 20  $\mu$ L total reaction using 10  $\mu$ L SYBR Green PCR Master Mix (Bio-Rad, Foster City, CA), 10 nM final primer concentration, and 8  $\mu$ L of DNA (5 nM/ $\mu$ L). The reaction program consisted of 1 cycle at 95°C for 3 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C for the 16 s rDNA gene, and 53°C for butyryl-CoA:acetate CoA-transferase gene, and 30 s at 72°C. Data are expressed as the relative frequency of butyryl-CoA: acetate CoA-transferase genes detected per 16S rRNA gene (Louis and Flint, 2007).

### Determination of the Gene Expression in Cecal Tonsils

The preparation of the samples for the qPCR analyses was performed as described by Horn et al. (2014). Briefly, total RNA was isolated from 50 mg of the cecal tonsils tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruc-

tion. The precipitated RNA was suspended in 20  $\mu$ L of RNase free water and stored at –80°C. RNA quantity was assessed by UV spectrophotometer and then treated with DNase (Invitrogen, China). The first-strand cDNA was synthesized from 5  $\mu$ L of total RNA using oligodT primers and Superscript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Shanghai, China). Synthesized cDNA was diluted (5×) with sterile water and stored at –20°C. The real-time PCR amplification was performed in a 25  $\mu$ L reaction mixture containing 5  $\mu$ L of diluted cDNA, 12.5  $\mu$ L of 2× SYBR Green PCR Master Mix (Bio-Rad, Foster City, CA), 2.5  $\mu$ L of each primer (Table 2), and 3  $\mu$ L of PCR-grade water. The PCR procedure for A20 (ubiquitin-editing enzyme A20), interleukin 1 $\beta$  (IL-1 $\beta$ ), interleukin 6 (IL-6), interleukin 10 (IL-10),  $\gamma$  interferon ( $\gamma$  INF), and nuclear factor kappa B (NF- $\kappa$ B) consisted of heating the reaction mixture to 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 57°C, 57°C, 50°C, 55°C, 53°C, 54°C for 20 s for each primer, respectively, and 72°C for 15 s. The relative standard curve method was used to quantify the mRNA concentrations of each gene in relation to the reference gene (GAPDH). The mRNA relative abundance was calculated (Livak and Schmittgen, 2001). All samples were analyzed in duplicate.

### Statistical Analysis

The growth performance and gene expression data were analyzed as a 2-way ANOVA using the GLM procedure of the SAS system (SAS Institute, 2011). The model included the main effect of diet, SB, and their interaction. The pen was considered as the experimental unit. The means showing significant ( $P \leq 0.05$ ) treatment differences in the ANOVA were then compared using the least square mean procedure of SAS. All data were tested for normality and homogeneity of variances, using the UNIVARIATE procedure and Bartlett test of SAS system (SAS Institute, 2011), respectively.

**Table 3.** Performance of broiler chickens from 1 to 28 days of age, fed diets with different levels of reduction in energy and AA, and supplemented or not with sodium butyrate (SB).

		1 to 14 d			14 to 28 d			1 to 28 d		
		WG, g	FI, g	FCR	WG, g	FI, g	FCR	WG, g	FI, g	FCR
Without SB	Control	319	409	1.284	1,020 <sup>a</sup>	1,516 <sup>a</sup>	1.482	1,340 <sup>a</sup>	1,931 <sup>a</sup>	1.441
	-2.3% aa/-60 Kcal	307	396	1.290	971 <sup>b,c,d</sup>	1,457 <sup>a,b</sup>	1.496	1,278 <sup>b,c</sup>	1,859 <sup>a,b</sup>	1.447
	-4.6% aa/-120 Kcal	298	406	1.366	934 <sup>d</sup>	1,440 <sup>b</sup>	1.543	1,231 <sup>d</sup>	1,847 <sup>b</sup>	1.479
With SB	Control	324	406	1.257	992 <sup>a,b,c</sup>	1,466 <sup>a,b</sup>	1.479	1,316 <sup>a,b</sup>	1,872 <sup>a,b</sup>	1.424
	-2.3% aa/-60 Kcal	318	413	1.297	1,002 <sup>a,b</sup>	1,490 <sup>a,b</sup>	1.487	1,320 <sup>a,b</sup>	1,903 <sup>a,b</sup>	1.441
	-4.6% aa/-120 Kcal	308	412	1.340	961 <sup>c,d</sup>	1,490 <sup>a,b</sup>	1.551	1,268 <sup>c,d</sup>	1,902 <sup>a,b</sup>	1.499
SB										
Absence		308 <sup>b</sup>	403	1.313	975	1471	1.507	1,283 <sup>b</sup>	1,879	1.456
Presence		317 <sup>a</sup>	410	1.298	985	1,482	1.506	1,301 <sup>a</sup>	1,892	1.454
Diet										
Control		322 <sup>a</sup>	408	1.270 <sup>b</sup>	1,006 <sup>a</sup>	1,491	1.481 <sup>b</sup>	1,328 <sup>a</sup>	1,902	1.433 <sup>b</sup>
-2.3% aa/-60 Kcal		313 <sup>b</sup>	405	1.293 <sup>a,b</sup>	987 <sup>a</sup>	1,474	1.492 <sup>b</sup>	1,299 <sup>b</sup>	1,881	1.444 <sup>b</sup>
-4.6% aa/-120 Kcal		303 <sup>c</sup>	409	1.353 <sup>a</sup>	948 <sup>b</sup>	1,465	1.547 <sup>a</sup>	1,250 <sup>c</sup>	1,875	1.489 <sup>a</sup>
SEM		2.9	6.7	0.03	8.1	16.3	0.01	9.7	18.0	0.01
Source of variation					P value					
Diet		<0.001	NS	0.01	<0.001	NS	<0.001	<0.001	NS	<0.001
SB		<0.001	NS	NS	NS	NS	0.04	NS	NS	NS
Diet x SB		NS	NS	NS	0.003	0.01	NS	0.004	0.008	NS

<sup>a-d</sup>Means with different superscripts in a column differ significantly ( $P < 0.05$ ). Values are means of 8 pens (46 birds/pen).

Procedure GLM was utilized to analyze differences in microbial diversity indexes and abundance generally. Metastats was used to study if there were OTUs differentially represented between the samples ( $P < 0.05$ ). Unweighted and weighted Unifrac software (Boulder, CO) were adopted to determine differences in presence and absence of OTUs, and abundance, respectively (Lozupone et al., 2011). Welch's *t*-test was applied to compare the KEGG pathways. Significant OTUs were determined using nonparametric Wilcoxon sum-rank test ( $P < 0.05$ ) followed by linear discriminant analysis ( $\log < 2$ ) that was used to compare differences in the microbiota.

## RESULTS AND DISCUSSION

### Productive Performance

There was no interaction between nutrient density of the diet and SB supplementation in the starter phase (1 to 14 d). However, broilers fed nutritionally reduced diets had lower BW gain ( $P < 0.001$ ), and SB supplementation increased BW gain by 2.8% ( $P < 0.001$ ; Table 3). In the following phase (14 to 28 d), there was an interaction between nutrient density and SB supplementation for BW gain ( $P = 0.003$ ) and FI ( $P = 0.01$ ); wherein BW gain of birds fed SB was impaired less by the energy and amino acid reduction than unsupplemented birds. A similar interaction was observed for the overall experimental period (1 to 28 d), in which the supplementation of 0.1% of SB partially recovered the reduction in BW gain caused by the dietary energy and amino acid reduction ( $P = 0.004$ ). Supplementation of SB did not affect FCR in any of the phases evaluated. Based on these results, the molecular analysis was fo-

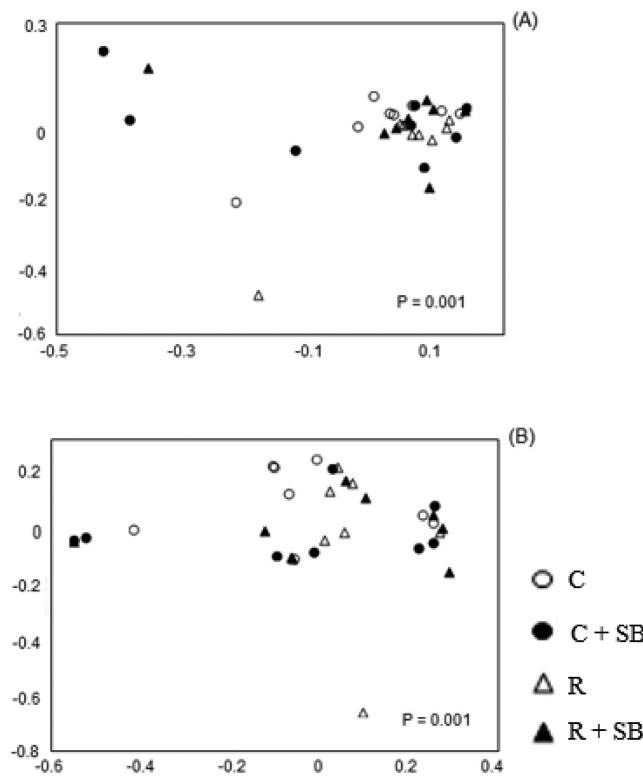
cused on the control treatment and the treatment fed a diet formulated with a reduction of 2.3% of amino acid and 60 kcal, with or without SB supplementation, totaling four treatments ( $2 \times 2$  factorial).

It has been described that butyrate can affect several host functions. For example, butyrate is a common SCFA metabolite of clostridial metabolism (Hold et al., 2003; Duncan et al., 2004; Louis et al., 2004) which can be used as an energy source for epithelial cells (Dalmasso et al., 2008). Further, butyrate has been shown to increase the expression and activity of SGLT1 and GLUT2 transporters in the brush border (Tappenden et al., 1997). Enterocyte surface receptors, such as G protein-coupled receptor (GPR) 43 and GPR41 may function as a sensor of intestinal SCFAs enhancing expression of transporters in cells as they migrate along the villus (Karaki et al., 2008) thereby enhancing feed efficiency (Adil et al., 2010). In addition to the recognized effects on intestinal metabolism, butyrate shows indirect effects that contribute to the general metabolism of animals (Guilloteau et al., 2010), including microbiota composition and function, which was also the focus of the present study.

### Diversity of the Cecal Microbiota

A total of 253,216 good quality sequences were obtained, 7,913 from each one of the 32 cecal microbiotas analyzed. The calculated sampling coverage of these samples was between 98 and 99%.

The number of OTUs, Chao index (number of OTUs comprising the microbiota), Shannon index (biodiversity based on sequences uniformity amongst OTUs), and Simpson index (richness and evenness) were unaffected by SB and/or the nutritionally reduced diet



**Figure 1.** Unweighted (A) and weighted (B) UniFrac cecal microbiota visualized via principle coordinate analysis (PCA) from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

( $P > 0.05$ ; mean OTU, Chao index, Shannon index, and Simpson index were 261, 412, 3.53, and 0.09, respectively). In order to compare the cecal microbiota of chickens, distance matrices were calculated by weighted and unweighted UniFrac and visualized via principle coordinate analysis (**PCA**). Unweighted and weighted PCA plots are presented in Figures 1A and 1B, respectively. UniFrac distances of cecal microbiotas indicated both membership (unweighted), and community structure (weighted) variations. Both analyses revealed differences associated with the presence of SB supplementation and the nutritional level of diets ( $P < 0.001$ ).

Although similar microbial richness was observed among treatments, the community structure was significantly different. Both community membership and structure contributed to differences in cecal microbial communities among diets. Analyzing the presence/absence of OTU (unweighted UniFrac) it is possible to magnify the effects of OTU present in low abundance. When OTU abundance is taken into account (weighted UniFrac), the clusterization indicates that the most abundant members of a cecal community from chickens were unaffected by dietary treatment.

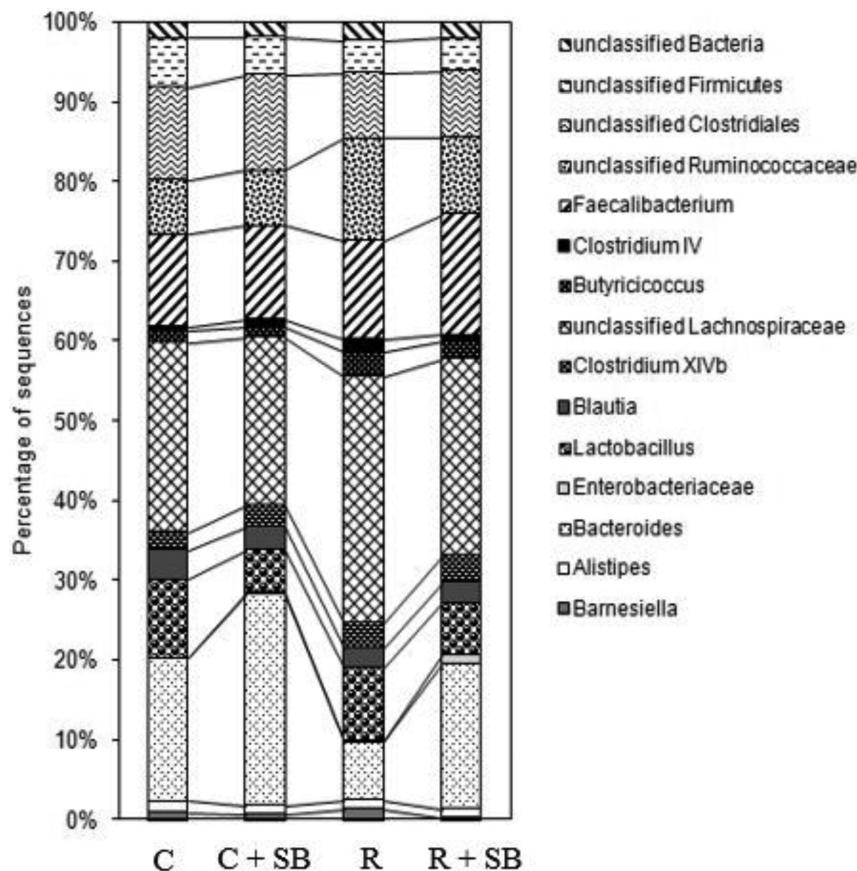
### Composition of the Cecal Microbiota

Microbial compositions showed high inter-individual variability. Overall, the microbiotas were dominated

by the phylum *Firmicutes* ( $78.1 \pm 14.7\%$  in the control group,  $70.6 \pm 27.2\%$  in the control + SB group,  $87.6 \pm 7.27\%$  in the reduced group, and  $77.5 \pm 20.8\%$  in the reduced + SB group; mean  $\pm$  SD). *Bacteroidetes* was the second most abundant ( $18.9 \pm 15.1\%$  in the control group,  $26.7 \pm 28.0\%$  in the control + SB group,  $9.0 \pm 7.21\%$  in reduced group, and  $18.5 \pm 22.5\%$  in the reduced + SB group), followed by the *Proteobacteria* phyla ( $0.08 \pm 0.08\%$  in the control group,  $0.14 \pm 0.18\%$  in the control + SB group,  $0.23 \pm 0.34\%$  in reduced group, and  $1.09 \pm 2.5\%$  in the reduced + BS group).

The phylum *Firmicutes*, the largest phylum, consisted of *Clostridiales*, *Ruminococcaceae*, *Faecalibacterium*, *Clostridium VI*, *Butyrococcus*, *Lachnospiraceae*, *Clostridium XIVb*, and *Blautia*; *Bacteroidetes* mainly consisted of *Barnesiella*, *Alistipes* and *Bacteroides*; and *Proteobacteria* consist mainly of *Enterobacteriaceae* (Figure 2). The combination of the nutritional density of the diet and the supplementation of SB affected the distribution of *Ruminococcaceae* in the cecal microbiota (interaction of nutrient density by SB;  $P = 0.04$ ). Chickens fed a nutritionally reduced diet presented a higher percentage of sequences related to *Ruminococcaceae* (11.9%) in the cecal microbiota than chicken fed a control diet supplemented or not with SB (6.5% and 6.4%, respectively). The amount of *Ruminococcaceae* observed in the cecal microbiota of birds fed a nutritionally reduced diet supplemented with SB did not differ statistically from the other groups (9.1%). When only considering nutritional density of the diet, chickens fed a nutritionally reduced diet tended to present a higher percentage of sequences in the cecal microbiota related to *Butyrococcus* ( $P < 0.06$ ) and *Ruminococcaceae* ( $P < 0.07$ ). On the other hand, chickens fed a control diet had a cecal microbiota enriched for sequences related to *Firmicutes* ( $P < 0.05$ ) and *Clostridiales* ( $P < 0.01$ ).

The phylum *Firmicutes* has been related to the ability to harvest energy from the diet, presenting higher proportion in animals with better feed efficiency (Ley et al., 2005). In the present work, lower nutritional density of the diet reduced the total number of microorganisms related to the phylum *Firmicutes*, which helps explain the impairment in BW gain in birds fed this diet; in addition, members of this phylum had their representation modified according to the different dietary treatments. We detected an over-representation of several genera, such groups related to *Ruminococcaceae* and *Butyrococcus* in the microbiota of chickens fed a nutritionally reduced diet. These groups of bacteria are well known for degradation of complex plant materials, as cellulose and hemicelluloses, being able to secrete xylanase, cellulase and beta-galactosidase and are among the most abundant groups in the cecal content (Ze et al., 2012; Biddle et al., 2013; Wei et al., 2013). A large amount of bacteria specialized in fiber degradation was expected in chickens fed nutritionally reduced diets due to the high amount of cellulose used to reduce the nutrient density of the diet. The cecal microbiota was likely modified in order to

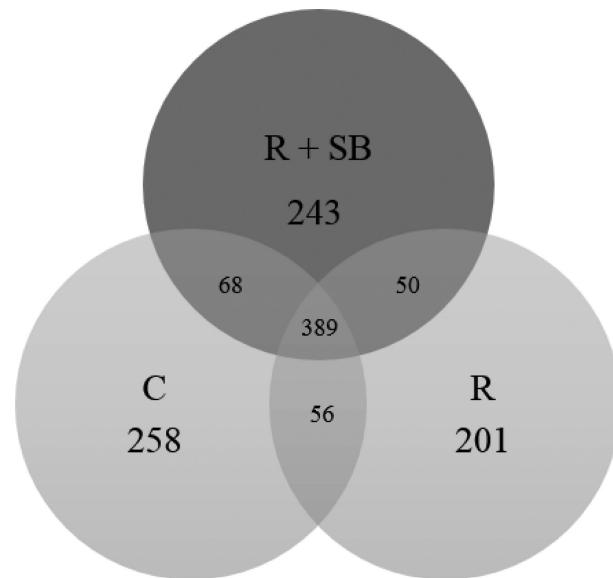


**Figure 2.** Stacked bar charts of the distribution of bacterial species detected in a 16S rDNA sequencing library created by use of cecal contents collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

degrade resistant fiber and harvest energy from the lower quality diet.

Due to the similarity in the BW of chickens fed a control diet and chickens fed nutritionally reduced diet supplemented with SB, a Venn diagram was constructed to identify the shared phylotypes between these groups, after eliminating the phylotypes shared with the group just fed nutritionally reduced diet (Figure 3). Sixty-eight OTU were identified to be unique, representing 2,380 sequences, 8.8 and 9.0% of the total species observed in the microbiota of birds fed a control or a nutritionally reduced diet supplemented with SB, respectively. Within these 68 OTU, it was observed the presence of sequences related to *Bacteria* (9 OTU), *Firmicutes* (12 OTU), *Clostridia* (5 OTU), *Clostridiales* (11 OTU), *Lachnospiraceae* (4 OTU), *Ruminococcaceae* (13 OTU), *Erysipelotrichaceae* (1 OTU), *Turibacter* (1 OTU), *Oscilibacter* (2 OTU), *Clostridium XIVb* (1 OTU), *Clostridia IV* (3 OTU), *Flavonifractor* (1 OTU), *Ruminococcus* (1 OTU), and *Bacteroides* (4 OTU) were observed.

Next, the composition of the cecal microbiota was examined using Metastats to identify specific phylotypes associated with the supplementation of SB and the nutritional density of the diet. Just two phylotypes had different abundance in the cecal microbiota of chickens receiving a control diet with or without SB supplementation. The cecal microbiota of chickens re-



**Figure 3.** Venn diagrams of the shared and unique OTUs (bacterial species) detected in the cecal contents collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

ceiving a control diet had significantly fewer species related to *Gracilibacter thermotolerans* (OTU92, 88% of similarity to RDP,  $P < 0.01$ ) and more phylotypes related to *Clostridium sulfatiphilum* (OTU124, 87%) than

the group receiving a control diet supplemented with SB ( $P < 0.004$ ).

A greater difference in the number of phylotypes was observed comparing the cecal microbiota of birds fed a control diet with that of the birds fed nutritionally reduced diet. Chickens fed a nutritionally reduced diet showed a lower percentage of sequences related to *Vallitalea pronyensis* (OTU19, 87%,  $P < 0.04$ ), *G. thermotolerans* (OTU166, 88%,  $P < 0.02$ ), *Oscilibacter valericigenes* (OTU152, 91%,  $P < 0.007$ ), *P. capillosus* (OTU22, 97%,  $P < 0.02$ ), *Bacteroides thetaiotaomicron* (OTU70, 99%,  $P < 0.0009$ ), and more sequences related to *Clostridium leptum* (OTU31, 92%,  $P < 0.03$ ), *Ruminococcus bromii* (OTU68, 95%,  $P < 0.03$ ), *F. prausnitzii* (OTU105, 93%,  $P < 0.02$ ), *G. thermotolerans* (OTU16, 88%,  $P < 0.02$ ), *Dielma fastidiosa* (OTU117, 91%,  $P < 0.01$ ), *Saccharofermentaris acetigenes* (OTU143, 91%,  $P < 0.01$ ), *Clostridium methylpentosum* (OTU113, 92%,  $P < 0.04$ ), and *Ruminococcus faecis* (OTU5, 96%,  $P < 0.007$ ) than the group fed a control diet.

In addition, comparing the cecal microbiota of birds fed a nutritionally reduced diet with that of birds fed the same diet supplemented with SB, we observed that the frequency of some phylotypes were changed. Chickens fed a nutritionally reduced diet presented a lower percentage of sequences related to *Faecalibacterium prausnitzii* (OTU69, 92%,  $P < 0.04$ ), *Clostridium spiroforme* (OTU95, 96%,  $P < 0.03$ ), *Pseudoflavonifractor capillosus* (OTU104, 89%,  $P < 0.03$ ), and *Clostridium leptum* (OTU79, 92%,  $P < 0.02$ ); and more sequences related to *Saccharofermentaris acetigenes* (OTU91, 88%,  $P < 0.03$ ), *G. thermotolerans* (OTU133, 87%,  $P < 0.02$ ), and *Odoribacter splanchnicus* (OTU185, 99%,  $P < 0.0009$ ) than chickens fed the same diet supplemented with SB.

*Faecalibacterium prausnitzii* is a butyrate-producing bacterium of species belonging to *Lachnospiraceae* have been related to good feed efficiency (Kameyama and Itoh, 2014), which may contribute to the improved performance observed in birds fed a nutritionally reduced diet supplemented with SB. *F. prausnitzii* is a component of the normal chicken microbiota (Lu et al., 2003; Lu et al., 2008) and a decreased abundance of this microorganism has been associated with inflammatory disease (Sokol et al., 2008; Fujimoto et al., 2013). Similarly, *Subdoligranulum variabile*, a phylogenetic closely related to *F. prausnitzii*, has demonstrated the ability to degrade complex carbohydrates and is closely related to several host metabolic pathways (Duncan et al., 2002; Li et al., 2008).

*Odoribacter splanchnicus*, observed in higher percentage in the microbiota of chickens fed a nutritionally reduced diet (vs. the same diet supplemented with SB), can ferment carbohydrates and produce short chain fatty acids. Acetic acid, succinic acid, and butyric acid are important for both microbial and host epithelial cell growth and has been associated with improved performance in chickens (Goker et al., 2011; Asano et al.,

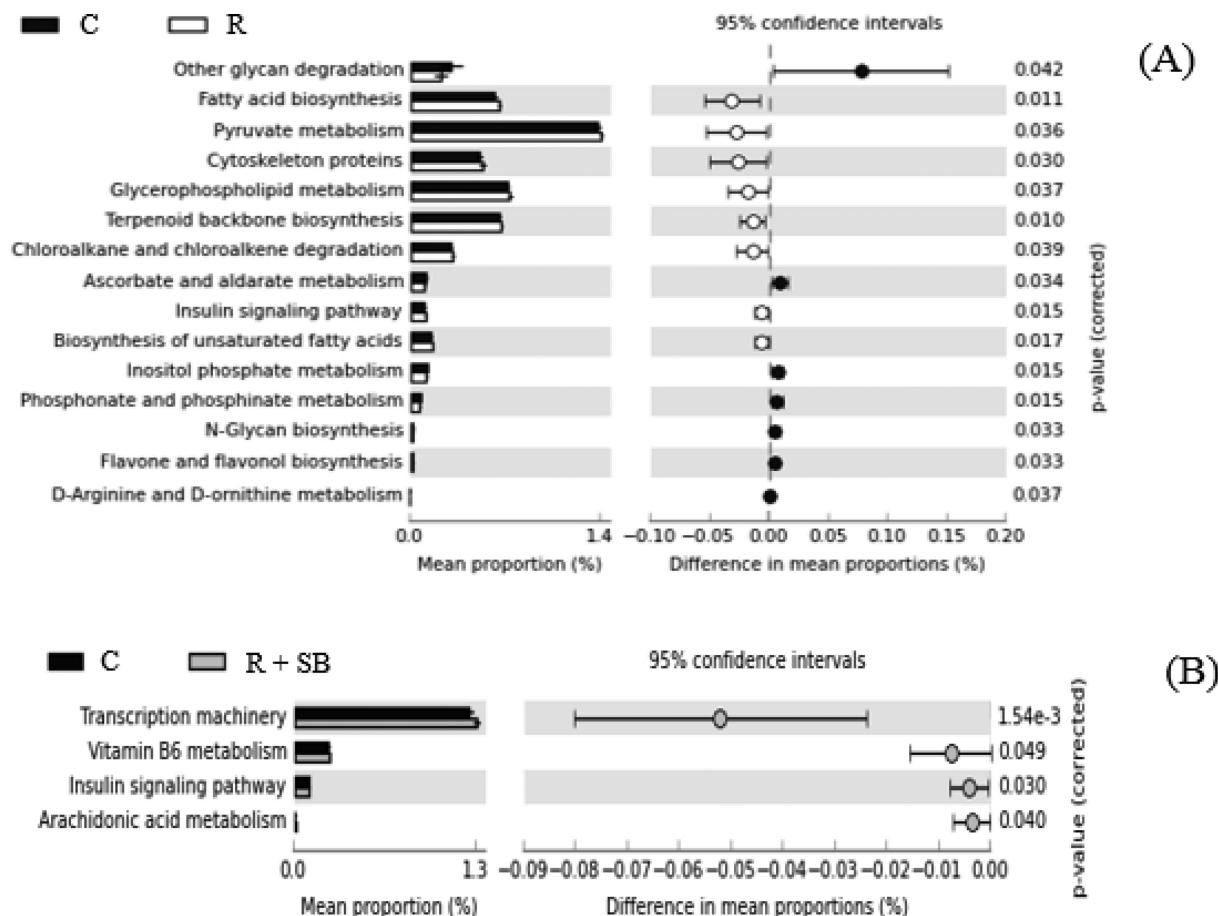
2013; Meehan and Beiko, 2014; Li et al., 2016). Non-pathogenic *Clostridia* species, such as *C. leptum*, that can degrade complex carbohydrates, were observed in higher percentage in the microbiota of chickens fed a nutritionally reduced diet supplemented with SB (vs. the unsupplemented diet). *C. leptum* has a great impact on the host metabolism and it is sensitive to dietary manipulation (Klein et al., 2016). It is also able to degrade cellulose, produce butyrate and plays an important role in the energy metabolism and development of intestinal epithelial cells (Pryde et al., 2002; Eckburg et al., 2005). *Bacteroides thetaiotaomicron* is known for its contribution to the symbiosis of the microbiota (Xu et al., 2003) and *Blautia* can use carbohydrate as a fermentable substrate and produce acetate and lactate as the major end products of glucose fermentation (Park et al., 2012; Bai et al., 2016).

Finally, the cecal microbiota of birds fed control diet and that of birds fed nutritionally reduced diet supplemented with SB was compared. Chickens fed a diet containing a nutritionally reduced level of nutrients supplemented with SB presented more sequences related to *Clostridium lactifermantans* (OTU120, 95%,  $P < 0.03$ ), *Subdoligranulum variabile* (OTU56, 99%,  $P < 0.03$ ), *R. bromii* (OTU68, 95%,  $P < 0.01$ ), *O. splanchnicus* (OTU185, 99%,  $P < 0.0009$ ), *Sporobacter termidis* (OTU177, 89%,  $P < 0.0009$ ) and less species related to *Blautia hansenii* (OTU167, 96%,  $P < 0.04$ ), *O. valericigenes* (OTU152, 91%,  $P < 0.04$ ), *G. thermotolerans* (OTU166, 88%,  $P < 0.02$ ), and *Clostridium succinogenes* (OTU128, 87%,  $P < 0.02$ ).

## Predicted Function of the Cecal Microbiota

A hypothesis has been proposed that the host and its microbiotas have evolved together and that the host genome does not encode for all of the information needed to carry all of the functions (Zaneveld et al., 2008). In the absence of transcriptome data, and since the samples were not stored at  $-80^{\circ}\text{C}$  for RNA analysis, PICRUSt was applied to predict the metagenome from 16S data and a reference genome database (Langille et al., 2013). PICRUSt can predict and compare probable functions of a wide range of samples. Few limitations of this approach must be considered: the software does not differentiate among strain level; it cannot analyze gene families if those genes are not included in the imputed database, or if the pathways are not well characterized (Langille et al., 2013); and it also assumes 100% of gene function (if the bacterium is present).

Initially, the predicted function of the cecal microbiota of chickens fed a control diet was compared to that of the group fed a nutritionally reduced diet (Figure 4a). The nutritionally reduced diet was predicted to affect ( $P < 0.05$ ) a greater number of pathways in the microbiota, especially related to carbohydrate (pyruvate metabolism) and lipid metabolism (fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, and



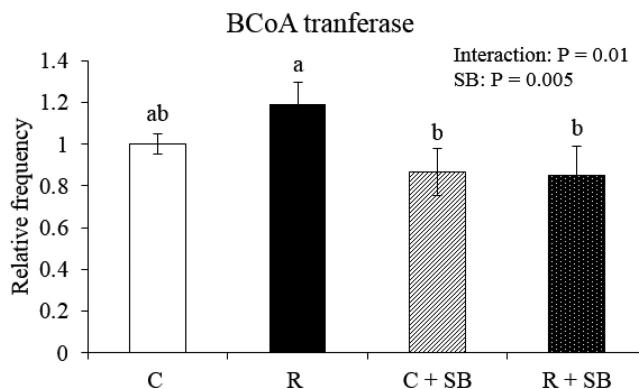
**Figure 4.** Predicted function of the cecal microbiota collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with sodium butyrate (SB) supplementation.

glycerophospholipid metabolism). However, when the predicted function of the microbiota of chickens fed a control diet and the predicted function of the microbiota of chickens fed a nutritionally reduced diet supplemented with SB were compared, fewer differences in the KEGG pathway were observed (Figure 4b). There were 15 different predicted metabolic pathways observed between the first two groups of birds, which showed that the microbiota from birds fed a nutritionally reduced diet was utilizing the cecal content in a dissimilar way since the digesta profile reaching the ceca was distinct between both groups. On the other hand, comparing the predicted function of the cecal microbiota from broilers fed control diets and the microbiota of chickens fed a nutritionally reduced diet supplemented with SB, only four metabolic pathways were statistically different.

Pyruvate metabolism is well known in the energy process (Turnbaugh et al., 2008) and is described in KEGG database as pathways containing of genes involved in SCFA production (Rampelli et al., 2013). Fiber plays a role in the SCFA production in the gut (Topping and Clifton, 2001; Brouns et al., 2007). Pathways like terpenoid backbone biosynthesis are involved in the metabolism of cofactors and vitamins (Vazquez-Castellanos et al., 2015) and cytoskeleton protein path-

ways are related to cell motility. The microbiota of chickens fed a control diet presented an increase imputed representation in the glycan biosynthesis and metabolism (N-glycan biosynthesis and other glycan degradation) probably due to the diet composition (Lang et al., 2014). Microbial fermentation transforms N-glycans from diets in SCFA affecting the composition and function of the microbiota (Koropatkin et al., 2012). The identification of the dietary carbohydrate profile could give us insights of the microbial function and validate the predicted metabolic function of the cecal microbiota. In addition, an increased frequency of pathways representing the metabolism of amino acids (phosphonate and phosphinate, and D-arginine and D-ornithine metabolism) was observed, probably due to differences in the amino acid concentration between diets.

Furthermore, we hypothesized that despite the structural changes in the cecal microbiota, butyrate allowed the normalization of the microbial cecal function. Our hypothesis was supported by the observed constancy in the imputed KEGG carbohydrate and lipid pathways in the cecal microbiota of chickens fed a control diet and a nutritionally reduced diet supplemented with SB. Few pathways had a significant distribution, as arachidonic acid metabolism, that has a role in preventing



**Figure 5.** Relative frequency of butyryl-CoA: acetate CoA transferase gene in the cecal microbiota of 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation. Values are means  $\pm$  SEM (1 bird/pen; 8 birds/treatment). <sup>a-b</sup>Means with different superscripts in a column differ significantly ( $P < 0.05$ ).

inflammation (Hyde and Missailidis, 2009), vitamin B<sub>6</sub> metabolism, also involved in the fatty acid metabolism (Horrobin, 1993; Nakamura and Nara, 2004) and bacterial transcription machinery. Unfortunately, a number of OTUs did not match public databases, thus their functions were not imputed.

### Frequency of Butyryl-CoA: Acetate CoA Gene in the Bacterial Community

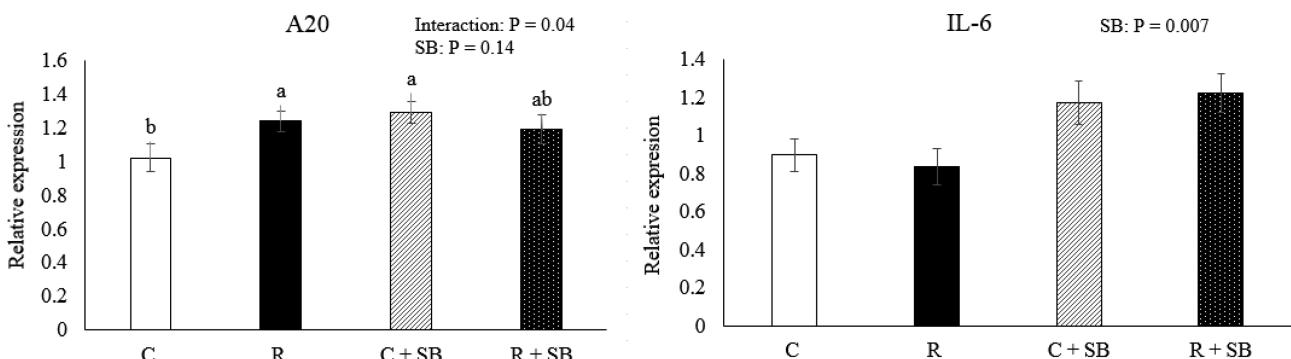
Butyryl-CoA: acetate CoA-transferase catalyzes the final step of butyrate formation, and is used by several bacterial species in the healthy gut microbiota (Duncan et al., 2004; Onrust et al., 2015). There was a significant interaction between the nutritional density of diets and SB supplementation; wherein the intestinal microbiota of chickens fed a nutritionally reduced diet without supplementation of SB showed a higher frequency of butyryl-CoA: acetate CoA gene compared to the treatments supplemented with SB ( $P = 0.01$ ; Figure 5), and was not affected by nutrient density when diets were supplemented with SB. The higher frequency of butyryl-CoA: acetate CoA-transferase in

the cecal microbiota of chickens fed a nutritionally reduced diet without SB, also had a higher frequency of butyrate-producing bacteria, such as *F. prausnitzii* and non-pathogenic *Clostridial* species. Nutritionally reduced diets were produced by adding cellulose to its composition, and likely the cecal microbiota of chickens fed this diet had a higher frequency of bacteria containing the butyryl-CoA:acetate CoA-transferase gene as a response to the composition of the cecal content. In addition, through a cross-feeding mechanism, lactic acid produced in the small intestine by lactobacilli may be consumed by butyrate-producing bacteria in the cecum (De Maesschalck et al., 2015), which may also explain the higher frequency of the butyryl-CoA:acetate CoA-transferase gene, and consequently butyrate producing bacteria, observed in the microbiota of chickens. Interestingly, SB decreased the abundance of the butyryl-CoA:acetate CoA-transferase gene in the intestinal microbiota, probably as a result of the dietary butyrate available to the host. It has been shown that the chemical composition of the intestinal ecosystem regulates not only the composition of the microbiota, but the production of butyrate (Dostal et al., 2015).

### Expression of Immune-related Genes

An interaction was observed between nutritional density and SB supplementation for the expression of A20 ( $P = 0.04$ ; Figure 6); wherein A20 was upregulated in chickens fed a nutritionally-reduced diet without SB, but was unaffected by nutritional reduction when supplemented with SB. In addition, SB supplementation upregulated IL-6 ( $P = 0.007$ ). The nutritional density of the diet nor the supplementation of SB affected the expression of IL-10,  $\gamma$  INF, NFK- $\beta$  p65, and IL-1  $\beta$  ( $P > 0.05$ ; data not shown).

Evidence suggests that a beneficial partnership has evolved between symbiotic bacteria and the immune system. Studies have shown the role of some individual bacteria in suppressing the inflammatory response during an inflammatory disease (Round and Mazmanian, 2009). However, many studies conducted to evaluate the



**Figure 6.** Relative gene expression of A20, and interleukin 6 (IL-6) in the cecal tonsils of 28 d old broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation. Values are means  $\pm$  SEM (1 bird/pen; 8 birds/treatment). <sup>a-b</sup>Means with different superscripts in a column differ significantly ( $P < 0.05$ ).

pathogenesis of inflammatory diseases showed an altered immune response against commensal gut microorganisms (Sundin et al., 2015). Few investigations have been conducted to evaluate the effects of immune modulators, such as butyrate, on the normal microbiota of chickens (Zhang et al., 2011). The expression of ubiquitin-editing enzyme A20, a cytoplasmic anti-inflammatory protein able to regulate the inflammatory response and intestinal apoptosis (Vereecke et al., 2009; Catrysse et al., 2014), was upregulated in the cecal tonsils of chickens fed a control diet supplemented with butyrate and in birds fed a nutritionally reduced diet. Ubiquitin-editing enzyme A20 is related to intestinal tolerance to lipopolysaccharide (Wang et al., 2009). Immuno-regulatory bacterial strains and butyrate producers can lead to an upregulation of A20, and beneficially modulate the Toll-like receptors 4 (TLR4) activation by reducing the activation of mitogen-activated protein kinase and nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathways and the production of proinflammatory cytokines (Song et al., 2012; Tomosada et al., 2013). An upregulation of IL-6 was observed in the cecal tonsils in the presence of SB; IL-6 is a potent, pleiotropic, inflammatory cytokine that mediates a plethora of physiological functions, including cell survival and amelioration of apoptotic signals (Kamimura et al., 2003).

## CONCLUSION

Overall, it was observed that the nutritional reduction of energy and amino acids impaired the performance of broiler chickens, but the supplementation of SB could partially counteract this effect. The cecal microbiota of chickens showed a large amount of fiber degraders and SCFA producers, especially in the groups fed a nutritionally reduced diet supplemented with SB. The nutritional reduction changed the predicted function performed by the microbiota, and the SB supplementation reduced these variations, keeping the imputed microbial function more similar to that of the control diet fed broilers. The frequency of bacterial species presenting the butyryl-CoA:acetate CoA-transferase gene related to butyrate production was increased in the microbiota of chickens fed a nutritionally reduced diet and reduced with SB supplementation. Additionally, SB supplementation was able to modulate the immune response. Butyrate is a bacterial metabolite critical to intestinal health and host performance. Based on the results herein, the use of synthetic sources of butyrate may bring benefits in terms of performance and intestinal function of broiler chickens.

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