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In Vivo Study of the Effect of Sugarcane Bagasse Lignin Supplementation on Broiler Chicken Diet as a Step to Validate the Established Chicken Gastrointestinal Tract *In Vitro* Model

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Abstract: Since the global restrictions on antibiotics in poultry systems, there has been a growing demand for natural and sustainable feed additives for disease prevention and poultry nutrition. This study evaluated the effects of incorporating sugarcane bagasse (SCB) lignin into broiler chicken diets. The performance of the chickens, including body weight, feed intake, and mortality, as well as intestinal histomorphometry, and cecum content pH, microbiota, and volatile fatty acids were assessed. In addition, we also aimed to validate an *in vitro* gastrointestinal tract (GIT) model developed by Carvalho et al. (2023). One hundred and eight 1-day-old Ross 308 chicks were randomly and equally divided into two groups. The first group was fed a basal diet (BD group), while the second group was fed a basal diet supplemented with 1% (*w*/*w*) SCB lignin (BD + SCB lignin group) for 36 days. The *in vivo* conditions of the chicken GIT were replicated in an *in vitro* model. In the *in vivo* study, SCB lignin increased cecum acetate and butyrate levels while reducing *Bifidobacterium* and *Enterobacteriaceae*, without affecting productivity (body weight, feed intake, and mortality). The *in vitro* assessment reflected microbiota trends observed *in vivo*, although without statistical significance. The divergence in organic acid production between the *in vivo* and *in vitro* conditions likely resulted from issues with inoculum preparation. This study demonstrates that SCB lignin incorporation positively influences cecal microbiota composition without impacting the animals' productivity and physiology, suggesting its potential as a functional feed additive. For a more reliable *in vitro* model, adjustments in inoculum preparation are necessary.

Keywords: chicken; cecal microbiota; fermentation; *in vivo*; *in vitro* model; organic acids

1. Introduction

The poultry sector plays a crucial role in contributing to global sustainable development and engaging with the United Nations (UN), particularly in achieving the Sustainable Development Goals (SDGs). The poultry industry can directly address at least five SDGs: SDG 2—zero hunger, SDG 3—good health and well-being, SDG 9—industry, innovation and infrastructure, SDG 12—responsible consumption and production, and SDG 13—climate

action. It is critical to emphasize the nutritional value of meat and eggs as sources of protein, essential amino acids, minerals, and vitamins, which are required for human health and general well-being [1].

In response to global public health, the poultry industry in certain countries has limited or even prohibited the use of antimicrobial growth promoters (AGP) or in-feed antibiotics (IFAs) due to their hazards, mainly related to the increase in antibiotic-resistant microorganisms. These additives were used for disease treatment and prevention and to boost parameters such as body weight, average daily gain (ADG), and feed conversion ratio (FCR) [2–4]. As a result of these restrictions, the poultry industry is pursuing new, cost-effective, and sustainable feed additives that promote animal health, growth, and feed efficiency while also controlling infectious diseases [2,4].

One promising strategy is the use of undervalued agri-food wastes as animal feed additives. These by-products not only provide a valuable source of underused nutrients but also employ a more eco-friendly, sustainable, and cost-effective supply of nutritional additives [5]. The use of agri-food byproducts in poultry feeding is becoming more widespread and a popular practice [5]. Another consumer trend that is gaining popularity is the demand for more natural, antibiotic-free, organic, and free-range production systems [6]. However, given the susceptibility of chickens to a potentially vast array of infections, there is an urgent need for alternative strategies to replace IFAs [6,7].

Feed accounts for 60–75% of overall production costs and is a key component of poultry production, affecting animal performance and development [8–10]. During the development of new functional feed additives, the evaluation of their bioactivities is a step that helps screen the potential of each additive to be used and tested *in vivo*, especially the capacity to modulate the animals' intestinal microbiota. This is important to avoid inadequate feeding and to prevent economic losses on the producer's side. *In vitro* gastrointestinal tract (GIT) simulation models can be used to perform such assessments before proceeding with *in vivo* trials.

Chicken *in vitro* GIT models can be tools that allow these assessments to be performed quickly, cheaply, with less work associated, and most importantly, with few or no ethical concerns, allowing for a reduction in the number of *in vivo* studies [11,12]. Most of the chicken *in vitro* GIT models use batch or chemostatic models similar to the one performed by de Carvalho et al. (2023) [13], which is based on four stages: the oral phase, the gastric phase, the intestinal phase, and the cecum fermentation phase. Through *in vitro* simulation methodology, Fangueiro et al. (2023) [7] discovered that the addition of 1% (*w*/*w*) sugarcane bagasse (SCB) lignin in mash feed had a greater promotion of *Bifidobacterium* in the chicken cecum than mash feed without supplementation or pellet feed supplemented with 1% SCB lignin. This finding is significant, as the gut microbiota is directly connected to animal health and growth performance, which ultimately affects productivity. Nowadays, there is an increased interest in beneficial microbiota modulation through the use of natural and organic feed additives to meet sustainability and consumer demands [11,14,15]. However, there is a critical gap that should be addressed when assessing microbiota modulation: the absence of data in the current state-of-the-art literature on comparing established chicken *in vitro* GIT models with *in vivo* animal assays.

This study aims to address this gap by evaluating the effects of 1% (*w*/*w*) SCB lignin supplementation on broiler chicken physiology, performance, and cecum microbiota modulation *in vivo*. Simultaneously, an *in vitro* modulation assay based on the method developed by Carvalho et al. (2023) [13] was conducted to compare the cecum bacterial growth and metabolic activity between the two approaches, as a validation of this method.

2. Materials and Methods

2.1. Sugarcane Bagasse Lignin

The lignin used in this study was extracted according to Fangueiro et al. (2023) [7] from SCB, kindly provided by Raízen (São Paulo, Brazil).

2.2. Animal In Vivo Assay

2.2.1. Experiment Location and Ethics Statement

The animal trials were performed at the experimental poultry unit of the Animal Science Department of Universidade de Trás-os-Montes e Alto Douro (UTAD), Portugal. All procedures of the trial complied with the assumptions regulated by the organism responsible for animal welfare (ORBEA (Animal Welfare and Ethics Body); Inf Gesdoc nº 1899-e-DZ-2021) of the university, following the provisions of Decree-Law no. 113/2013 of 7 August, which transposes into Portuguese law the aforementioned Directive no. 2010/63/EU on the protection of animals used for scientific purposes.

2.2.2. Basal Diet Composition

The basal diet (BD) composition is shown in Table 1. This basal diet is formulated to meet or exceed the bird's nutritional needs for each stage of its development: 1st phase—starter; 2nd phase—grower; and 3rd phase—finisher [16]. The feed presentation chosen for all diets used in this study was mash, due to the positive outcomes of the SCB lignin supplementation on this matrix, previously reported by Fangueiro et al. (2023) [7].

2.2.3. Experimental Design and Sample Collection Protocol

A total of 108 1-day-old male Ross 308 broiler chicks were used in this study. The chicks were housed in groups of 3 birds, in parks with wood shavings flooring, with automatic nipple drinkers and feeders for access to water and feed. Randomly, 18 parks (54 chicks) were assigned to treatments: (1) BD and (2) BD supplemented with 1% (*w*/*w*) of SCB lignin.

At 21 days of age, 8 parks/treatments were chosen randomly, and 20 birds were slaughtered to collect the intestinal contents, and the remaining birds were excluded from the performance trial. Thus, this trial had 18 parks/treatments from 1 to 21 days of age and 10 parks/treatments from 21 to 36 days of age, as described in Table 2.

Table 1. Composition and estimated nutritional value of basal diet (BD).

		1st Phase Starter	2nd Phase Grower	3rd Phase Finisher
Time of Consumption (days)		**1 to 12**	**12 to 21**	**21 to 36**
Feed Presentation			**Mash**	
Ingredients (g/kg)	Corn Plus	365.30	407.10	448.90
	Soybean meal 44	329.10	306.30	283.40
	Wheat Plus	150.00	150.00	150.00
	Extruded whole soybean	50.00	40.20	30.30
	Soy oil	31.60	35.80	40.00
	Soy protein concentrate Hp300	25.00	12.50	---
	Calcium carbonate powder	13.10	12.40	11.70
	Arbocel Rc Fine	5.50	7.20	8.90
	Monocalcium phosphate	8.80	8.00	7.30
	Avi Plus Broiler Nn 0.5 Phase 1	5.00	5.00	5.00
	Novipel Sp	3.00	3.00	3.00
	Dl-methionine	3.10	2.70	2.30
	L-Lysine	2.40	2.10	1.80
	Salt	2.30	2.40	2.40
	Molistar L	1.50	1.50	1.50
	Sodium bicarbonate	1.30	1.20	1.00
	Mycofix Plus 5.E	1.00	1.00	1.00
	Sodium butyrate	1.00	1.00	1.00
	L-threonine	0.70	0.50	0.30
	L-tryptophan	0.10	0.10	0.10
	L-valine	0.30	0.20	---

Table 1. *Cont.*

		1st Phase Starter	2nd Phase Grower	3rd Phase Finisher
Time of Consumption (days)		**1 to 12**	**12 to 21**	**21 to 36**
Feed Presentation			**Mash**	
Estimated nutritional value	Dry matter (%)	88.00	87.90	87.80
	Organic matter (%)	82.10	82.40	82.60
	Apparent metabolizable energy (Kcal/kg)	3000.00	3050.00	3100.00
	Crude protein (%)	22.20	20.40	18.60
	Methionine (%)	0.60	0.60	0.50
	Methionine + Cystine (%)	1.00	0.90	0.80
	Lysine (%)	1.40	1.20	1.10
	Threonine (%)	0.90	0.80	0.70
	Tryptophan (%)	0.30	0.30	0.20
	Arginine (%)	1.50	1.40	1.20
	Crude fat (%)	6.20	6.50	6.80
	Starches (%)	33.60	36.20	38.80
	Sugars (%)	4.80	4.40	4.00
	Total phosphorus	0.60	0.60	0.50
	Calcium (%)	0.90	0.90	0.80
	Phytic calcium (%)	1.00	1.00	0.90
	Crude fiber (%)	4.10	4.00	4.00
	Neutral detergent fiber (%)	11.50	11.60	11.70

Table 2. Number of broilers and parks per treatment for the *in vivo* assay testing the basal diet (BD) with and without sugarcane bagasse (SCB) lignin supplementation.

	Period (Days)		
Treatment	**1 to 12**	**12 to 21**	**21 to 36**
BD	54	54	30
BD + 1% SCB lignin	54	54	30
Total number of animals	108	108	60
Animal per park	3	3	3
Number of parks per treatment	18	18	10

The animals were received on their 1st day of life and reared up to 36 days of age inside a light program with 18 h/day and minimum intensity of 20 lux, following European standards of animal welfare (e.g., the area per animal and the environment—temperature, light intensity, photoperiod).

For the performance trial, the body weight of birds was measured at 1, 12, 21, and 36 days of age. Feed intake, ADG, the FCR (i.e., feed ingested/ADG), and mortality were determined for periods 1 to 12, 12 to 21, 21 to 36 days, and 1 to 36 days of age.

At day 21, 20 birds per treatment, i.e., a total of 40 birds, were slaughtered by cervical dislocation, and the cecum was removed to collect the cecal content. Before their slaughter, the animals underwent a fasting period of 1–2 h. The remaining animals in these parks were excluded.

At 36 days of age, 20 birds of each treatment underwent a fasting period of 1–2 h, were weighed and slaughtered. After slaughter, they were bled, eviscerated, plucked, and had their heads and feet removed to obtain carcasses. The carcass yield was calculated based on the carcass weight and live weight. The development of the digestive tract was evaluated (10 birds/treatment) by measuring the length of the duodenum–jejunum, ileum, cecum, and rectum (these measurements were expressed both in absolute terms and relative to the live weight of the animal at the time of slaughter). Samples from these birds' initial section of the ileum were collected to measure intestinal villi and absorption area.

The workflow of the animal *in vivo* assay carried out is demonstrated in Figure 1.

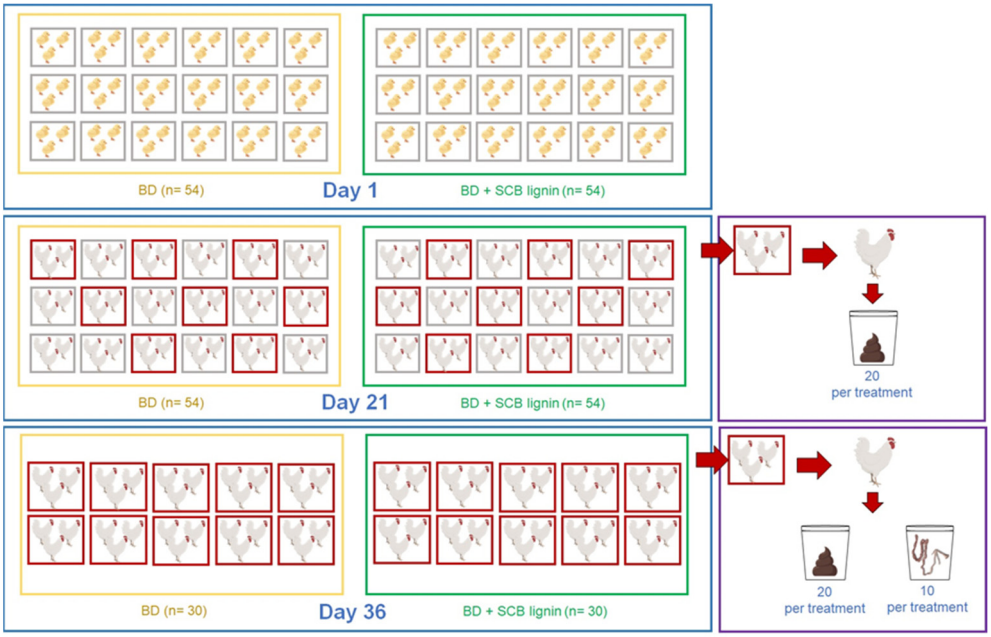

Figure 1. Animal *in vivo* assay work flowchart for testing the basal diet (BD) without and with sugarcane bagasse (SCB) lignin supplementation.

2.2.4. Ileum Sample Collection

The morphometry of the ileum was measured in all birds slaughtered at 36 days (10 birds per treatment). Ileum and jejunum tissue samples were prepared for light microscopy as follows: segments with 5 cm of jejunum were cut and immediately fixed in 10% neutral formalin, processed in an automated tissue processor (Shandon Hipercenter XP, Thermo Fisher Scientific, Waltham, MA, USA), and embedded in paraffin wax (Histoplast Shandon, Thermo Fisher Scientific, Waltham, MA, USA). Three-micrometer paraffin sections were cut on a Biocut 2035 microtome (Leica Microsystems, Buffalo Grove, IL, USA) and stained with hematoxylin and eosin. Slides were evaluated on a Nikon DXM1200 digital still camera (Nikon, Tokyo, Japan). Measurements were conducted according to a modified method of Iji, Saki, and Tivey (2001) [17]. For each segment of the small intestine, fifteen villi were evaluated: villi height, villi width at the crypt, and the tip and crypt depth were measured (Figure 2). Based on these villi measurements, the absorption area was calculated.

2.2.5. Cecum Content Collection

The cecum was removed, clipped on both sides with a string, properly identified, and stored in a clean, tamper-proof 1 L specimen container (Sigma, St. Louis, MO, USA). The containers were placed in 27.5 L hermetic seal containers (Araven S.L., Zaragoza, Spain) with anaerobic generator atmosphere sachets (Thermo Fischer Scientific, Waltham, MA, USA) closed, and only open at the time of dividing the cecum contents for the different analyses (after a collection period of 3–5 h). The cecal content from each cecum was squeezed into an empty pre-weighed 180 mL flask (VWR, Radnor, PA, USA), weighed, and then divided for the following purposes: 1 g for bacterial DNA extraction and quantification by quantitative polymerase chain reaction (qPCR); 1 g for the quantification of short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) by gas chromatography (GC); 1 g for quantification of bacterial viability and pH measurement by culture plating and using a SevenCompact pH meter (Mettler Toledo, Urdorf, Switzerland), respectively; and 3 g for cecal pool inoculum.

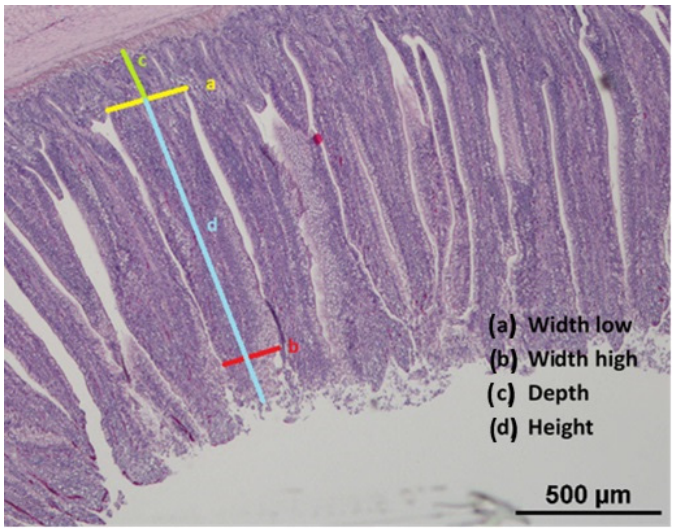

Figure 2. Image of ileum sample and measurements.

2.3. In Vitro Model Protocol

2.3.1. Preparation of Cecal Inoculum

The cecal content was obtained from 20 of 36-day Ros 308 broiler chickens with an average weight of 1.90 kg, fed with the BD treatment during the animal trial. The cecal inoculum used was prepared with an equal amount of individual cecum content destined to prepare pooled cecal inoculum (3 g of cecal material from each bird), following the method described by de Carvalho et al. (2023) [13], and stored at −80 °C.

2.3.2. Chicken Gastrointestinal Tract Simulation Model

An *in vitro* GIT simulation model described in de Carvalho et al. (2023) [13] was carried out. A gastro-intestinal digestion and absorption protocol was performed on two different feeds: (1) BD and (2) BD supplemented with 1% (*w*/*w*) SCB lignin. Batch culture fermentations inoculated with cecal inoculum were used to simulate cecum fermentation. The vessel conditions were as follows: vessels 1–2 contained 1% (*w*/*v*) of digested BD; vessels 3–4 contained 1% (*w*/*v*) of digested BD supplemented with 1% SCB lignin; and vessels 5–6 served as the inoculum control (IC) with no substrate added (negative control). This protocol was performed in duplicate, i.e., each condition had four fermentation vessels.

From each vessel, at time points 0, 24, and 48 h, samples of 10 mL were aseptically taken and processed for SCFAs and lactate analysis, total ammonia nitrogen quantification, and bacterial enumeration, according to de Carvalho et al. (2023) [13].

2.4. Bacterial Enumeration

2.4.1. Bacterial Enumeration by Culture-Dependent Methods

Individual and pooled cecum contents were diluted in 0.1% (*w*/*v*) peptone water (Sigma, St. Louis, MO, USA) in decimal dilutions and platted in different culture media, as described in Carvalho et al. (2023) [13].

2.4.2. Bacterial Enumeration by Culture-Independent Methods

The DNA samples were extracted from the cecum contents of 21- and 36-day-old broiler chickens, as well as from cecal fermentation samples collected at 0, 24, and 48 h. The extraction, quantification, final DNA concentration for each sample, the qPCR reactions, cycling conditions, specific bacterial DNA standard curves, the analysis of melting curves, and qPCR product purity were according to de Carvalho et al. (2023) [13]. The targeted groups, primer sequences, amplicon sizes, and literature references are described in Carvalho et al. (2023) [13]. Triplicates were performed for each sample and control.

2.5. Determination of Volatile Fatty Acids in the Cecum Contents

From each broiler chicken cecum squeezed, 1 g of the individual cecum contents was stored at −80 °C until further analysis. The extraction of SCFAs and BCFAs from the cecum content samples (in duplicates) was carried out according to Scortichini et al. (2020) [18] for fecal samples and the organic phase was analyzed by Agilent 8860 GC LL autosampler equipped with a split/splitless injector and a flame ionization detector (FID) (Agilent, Santa Clara, CA, USA). The capillary chromatographic column used was a CP-Wax 58 (FFAP CB), with a length of 50 m, a diameter of 0.25 mm, and a film thickness of 0.20 µm (Agilent, Santa Clara, CA, USA). The GC injector was set and maintained at 280 °C. The oven temperature was initially set to 40 °C for 3 min, programmed at a rate of 20 °C/min up to 160 °C, and then at 40 °C/min up to 245 °C, resulting in a total run time of 17 min. The carrier gas was hydrogen at a flow rate of 2 mL/min. The FID temperature was maintained at 250 °C. The composition and concentration of SCFAs were compared to the standard solutions previously prepared in diethyl ether. Acetate, propionate, and butyrate (Sigma-Aldrich, St. Louis, MO, USA) were prepared in concentrations ranging from 1 to 40 mM, while isobutyrate, isovalerate, and valerate (Sigma-Aldrich, St. Louis, MO, USA) were prepared in concentrations ranging from 0.1 to 3 mM.

2.6. Determination of Organic Acids Produced during In Vitro Fermentation

The supernatants collected after centrifugation of the fermentation time point samples were filtered (0.22 µm) and directly analyzed by Agilent 1260 II series HPLC (Agilent, Santa Clara, CA, USA) in duplicates, as described in de Carvalho et al. (2023) [13].

2.7. Measurement of Total Ammonia Nitrogen Concentration

Total ammonia nitrogen concentration was measured according to de Carvalho et al. (2023) [13].

2.8. Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics 27 software (IBM, Chicago, IL, USA). The data's normality was evaluated using the Shapiro–Wilk test. As the samples followed a normal distribution (except for mortality), means were compared considering a 95% confidence interval, using an independent samples *t*-test for the data related to animal growth measurement, pH, bacterial enumeration by culture-dependent and independent methods, SCFAs, and BCFAs quantification on animal cecum contents. A one-way ANOVA, followed by Tukey's post hoc test, was used to analyze the data related to bacterial and metabolic profiles in the simulation of cecum fermentations. Mortality was analyzed by χ^2 test.

3. Results

3.1. Animal In Vivo Assay

3.1.1. Animal Performances

The live weight of the birds at different ages, submitted for each treatment, is shown in Table 3.

Table 3. Effect of feeding a basal diet (BD), with and without sugarcane (SCB) lignin, on the average weight of birds per park (g, mean ± SD) at different ages during the *in vivo* assay.

Treatment	Age (Days)			
	1	**12**	**21**	**36**
	Average Body Weight (g)			
BD	43.52 ± 1.69	225.96 ± 21.58	651.03 ± 51.69	1904.60 ± 87.55
BD + 1% SCB lignin	43.46 ± 1.64	217.15 ± 23.11	638.26 ± 43.23	1814.37 ± 143.70
Number of replicates per treatment (parks)	18	18	18	10

The ADG per bird, the feed intake, and the FCR are shown in Table 4. Concerning the impact of each treatment on the ADG of the chickens, no differences were found at all ages ($p > 0.05$). There were also no significant effects of treatments on the feed intake daily by the birds ($p > 0.05$). The FCR was similar for both feeding conditions for all the controlled periods.

Table 4. Effect of feeding a basal diet (BD), with or without sugarcane bagasse (SCB) lignin, on the average daily gain (ADG) of birds per park (g/day, mean ± SD), bird feed intake per park (g/day, mean ± SD), and feed conversion ratio (FCR) (mean ± SD) in different periods of the *in vivo* assay.

Treatment	Period (Days)			
	1 to 12	**12 to 21**	**21 to 36**	**1 to 36**
	ADG per park (g/day)			
BD	15.20 ± 1.79	47.23 ± 4.20	82.72 ± 4.59	51.67 ± 2.43
BD + 1% SCB lignin	14.47 ± 1.95	46.79 ± 3.23	78.72 ± 7.44	49.18 ± 4.00
	Feed intake per park (g/day)			
BD	25.38 ± 3.72	62.83 ± 4.97	127.43 ± 5.61	77.91 ± 2.80
BD + 1% SCB lignin	25.82 ± 4.13	62.63 ± 4.09	124.65 ± 10.11	75.90 ± 5.14
	FCR			
BD	1.77 ± 0.20	1.33 ± 0.05	1.54 ± 0.04	1.51 ± 0.03
BD + 1% SCB lignin	1.99 ± 0.40	1.34 ± 0.06	1.59 ± 0.07	1.55 ± 0.05
Number of replicates per treatment (parks)	18	18	10	10

Concerning bird mortality, no significant effects of the treatment conditions tested were detected during the trial period. Animal death occurred between the beginning of the trial and the 12th day of this study ($p > 0.05$). In the BD condition, two birds died (3.70% mortality), while in the supplemented condition, three birds died (5.56% mortality). After this period, no more deaths were recorded.

3.1.2. Carcass Yield and Digestive Tube Development

The results for carcass yield and digestive tract development are shown in Table 5. There were no significant effects of treatments on the carcass yield of broiler chickens at 36 days of age nor on the development of the digestive tube and intestinal villi and absorption area ($p > 0.05$), except for the small intestine (duodenum-jejunum and the ileum), which presented greater length in the BD treatment ($p < 0.05$). In addition, no differences were observed in the intestinal villi dimensions and absorption area between the two diets tested ($p > 0.05$) (Table 6).

Table 5. Effect of feeding a basal diet (BD), with or without sugarcane bagasse (SCB) lignin, on carcass yield (%, mean ± SD) and intestine sections at 36 days of age in absolute terms (cm, mean ± SD).

Treatment	Carcass Yield	Duodenum-Jejunum	Ileum	Cecum	Rectum
	(%)	**cm**			
BD	72.98 ± 0.03 [a]	96.97 ± 5.21 [b]	72.25 ± 7.16 [b]	16.59 ± 1.67 [a]	8.84 ± 0.82 [a]
BD + 1% SCB lignin	73.85 ± 0.02 [a]	92.44 ± 6.01 [a]	65.88 ± 5.52 [a]	15.94 ± 1.69 [a]	8.44 ± 0.70 [a]
		cm/kg			
BD		51.91 ± 4.03 [a]	38.57 ± 3.30 [a]	8.90 ± 1.14 [a]	4.75 ± 0.67 [a]
BD + 1% SCB lignin		53.58 ± 7.11 [a]	38.20 ± 5.39 [a]	9.22 ± 1.33 [a]	4.87 ± 0.55 [a]

[a,b] Means within the same column, marked with the same letter, do not differ from each other ($p > 0.05$).

Table 6. Effect of feeding a basal diet (BD), with or without sugarcane bagasse (SCB) lignin, on live weight at slaughter (cm/kg, mean ± SD) and the development and absorptive area of intestinal villi.

Treatment	Height	Width	Depth	Absorption Area	Height/Width	Depth/Width
	(μm)			(mm^2)		
BD	973.55 ± 182.07	111.39 ± 9.49	154.15 ± 21.64	0.34 ± 0.07	8.77 ± 1.62	1.39 ± 0.18
BD + 1% SCB lignin	1119.20 ± 171.02	117.49 ± 9.20	160.90 ± 18.65	0.41 ± 0.08	9.57 ± 1.51	1.37 ± 0.13

3.1.3. Bacterial Cell Numbers

The viable bacterial cell counts of cecal contents by culture-dependent methods from this animal trial in three different culture media are depicted in Figure 3a and Table S1. The pH of the cecal contents is shown in Figure 3b and Table S1.

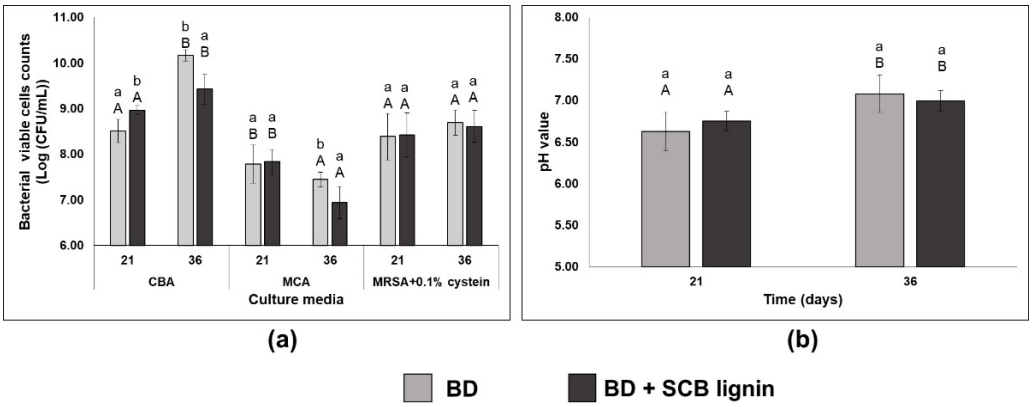

Figure 3. (**a**) Bacterial viable cell counts (log (CFU/mL), mean ± SD) of the cecal contents in different culture media and (**b**) pH values of the cecal contents diluted at 10% (*w*/*v*) in phosphate-buffered saline (PBS) obtained from the chickens fed with a basal diet (BD), without and with sugarcane bagasse (SCB) lignin supplementation. ^{a,b} Means in the culture media and pH value between sampling times, marked with the same letter, do not differ from each other ($p > 0.05$). ^{A,B} Means in culture media and pH values between sampling times for each treatment, marked with the same letter, do not differ from each other ($p > 0.05$). CBA—Columbia blood agar; MCA—MacConkey agar; MRSA—de Man, Rogosa, and Sharpe agar.

In general, the impact of treatment was only observed at 21 days in the total anaerobic bacteria, with the addition of SCB lignin leading to a higher count of this bacteria type ($p < 0.05$). Nevertheless, this effect was inverse at 36 days, when feeding with BD without SCB lignin led to a greater amount of total anaerobic bacteria and Gram-negative-enteric bacteria ($p < 0.05$).

The treatments did not impact the cecal LAB/*Bifidobacterium* ($p > 0.05$). The treatment did not affect the pH of the broilers' cecal contents at both collection days ($p > 0.05$) (Figure 3b). However, the age of the animals did affect it, and the pH of the cecal content was higher at 36 days in both treatments compared to 21 days ($p < 0.05$).

The quantification of bacterial DNA using qPCR for the various groups studied is shown in Figure 4a–f and Table S2. Figure 4b,d show no values at 21 days for Bacteroidetes and *Bacteroides*. At 21 days, the values obtained for these groups in both treatments were low and, in most cases, not even detected; therefore, they were classified as not detected (Nd). This occurrence is not rare and has been reported in other studies similar to this one [15].

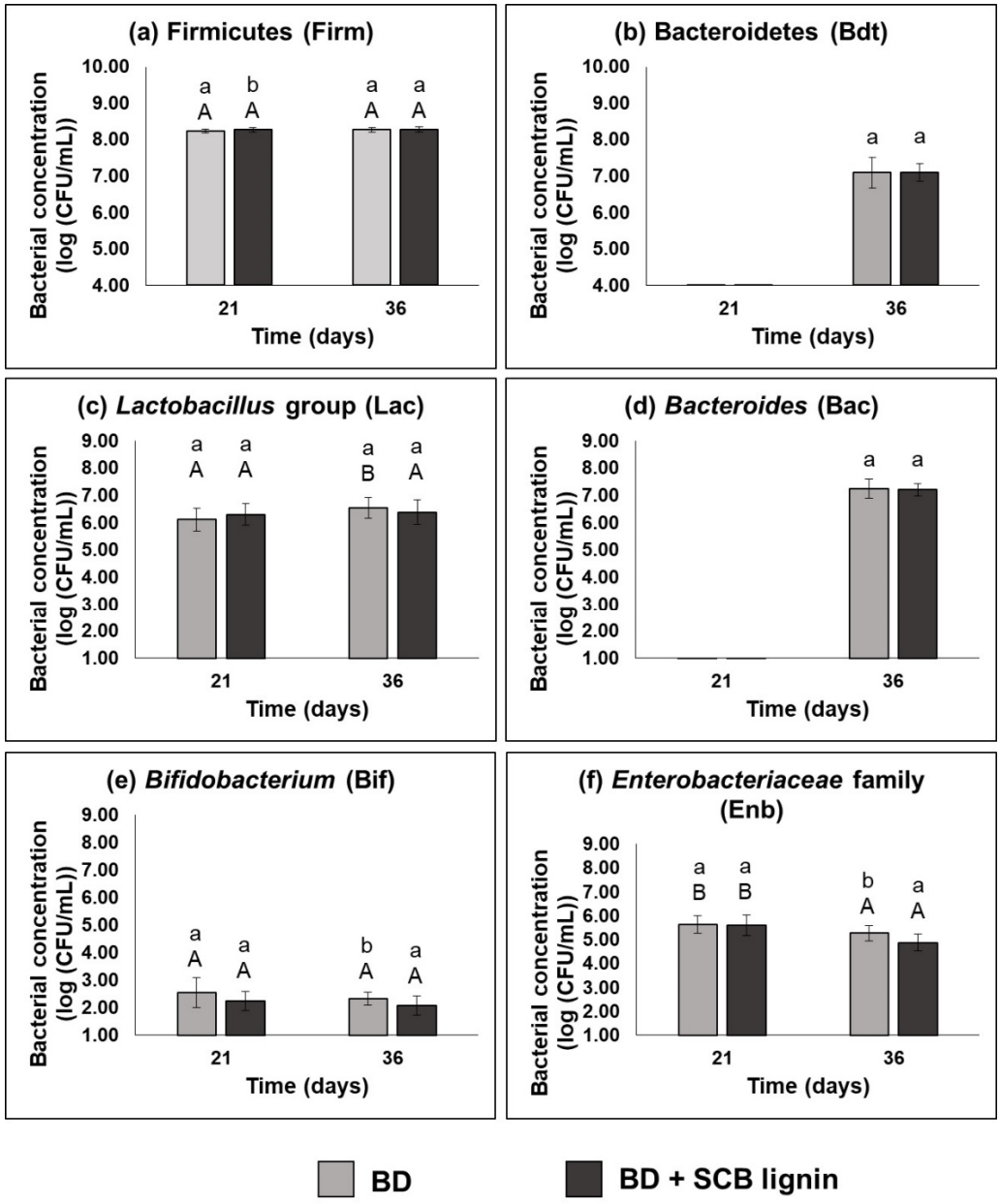

Figure 4. Bacterial quantification (log (CFU/mL), means ± SD) of the different bacterial populations in cecal contents obtained from the broiler chickens fed with a basal diet (BD), with or without sugarcane bagasse (SCB) lignin supplementation: (**a**) Firmicutes, (**b**) Bacteroidetes, (**c**) *Lactobacillus* group, (**d**) *Bacteroides*, (**e**) *Bifidobacterium*, and (**f**) *Enterobacteriaceae* family. [a,b] Means within the bacterial group across sampling times, marked with the same letter, do not differ from each other ($p > 0.05$). [A,B] Means within the bacterial group across sampling times for each treatment, marked with the same letter, do not differ from the other ($p > 0.05$).

The impact of the diet was shown at 21 days for Firmicutes, in which the feeding supplemented with SCB lignin led to an increase in this group's bacterial concentration. At 21 days, this group showed higher amounts for both treatments (equivalent to 8.25 log (CFU/mL)), followed by *Lactobacillus* (equivalent to 6.20 log (CFU/mL)), and *Enterobacteriaceae* (equivalent to 5.62 log (CFU/mL)). The *Bifidobacterium* was the bacterial group present in the lowest quantity (equivalent to 2.39 log (CFU/mL)). At 36 days, the condition SCB lignin had a lower number of *Bifidobacterium* and *Enterobacteriaceae* regarding the BD treatment. For both treatments, Firmicutes (equivalent to 8.27 log (CFU/mL)) were the most abundant, followed by *Bacteroides* and Bacteroidetes (equivalent to 7.22 and 7.10 log

(CFU/mL), respectively), *Lactobacillus* (equivalent to 6.46 log (CFU/mL)), *Enterobacteriaceae* (equivalent to 5.07 log (CFU/mL)). Once again, *Bifidobacterium* was the bacterial group with the lowest quantity (equivalent to 2.20 log (CFU/mL)).

Regarding the bacterial group composition present in the broiler chicken cecum during the *in vivo* study, there was an increase in *Lactobacillus* and a decrease in *Enterobacteriaceae* between days 21 and 36 ($p < 0.05$) with BD treatment, while the rest of the bacterial groups remained constant. For the SCB lignin treatment, only the reduction in *Enterobacteriaceae* was observed during the assay.

3.1.4. Short-Chain Fatty Acid and Branched-Chain Fatty Acid Concentrations

The concentrations of the different SCFAs (acetate (Figure 5a), propionate (Figure 5b), butyrate (Figure 5c), and valerate(Figure 5e)) and BCFAs (isobutyrate (Figure 5d) and isovalerate (Figure 5f)) present in the cecum contents of 21- and 36-day-old chicken broilers are presented in Figure 5 and Table S3.

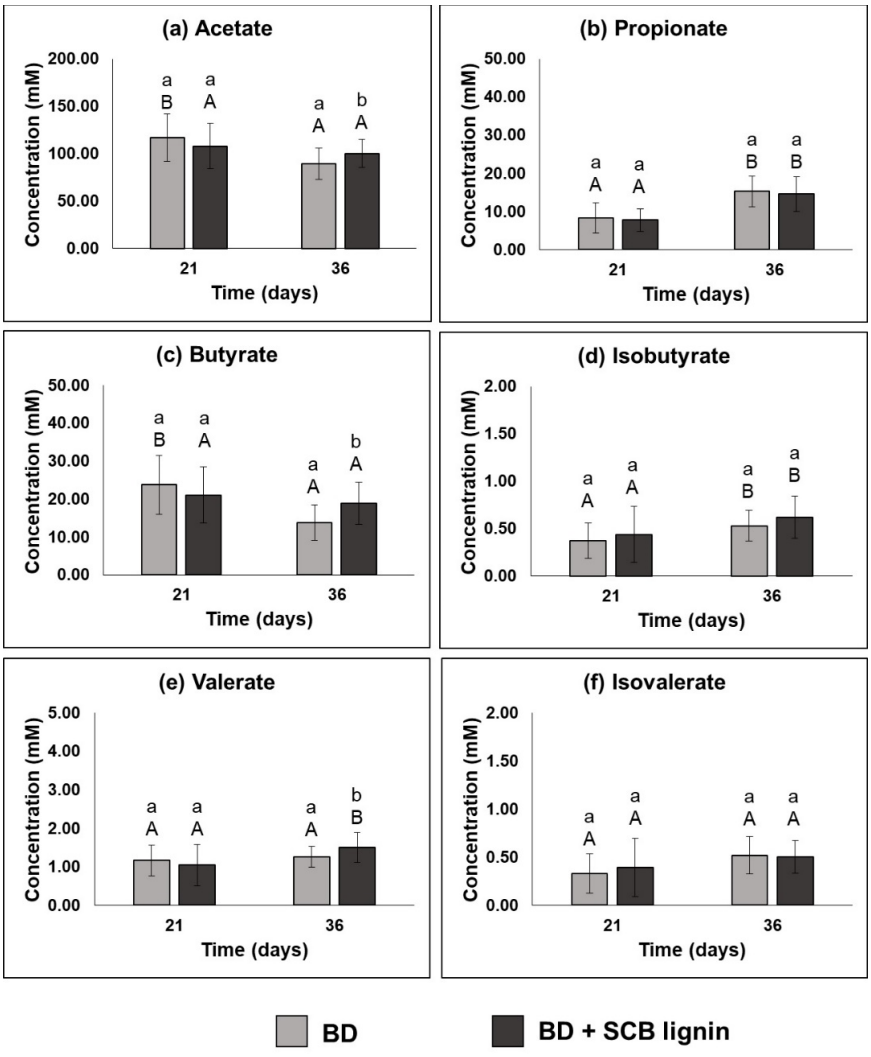

Figure 5. Concentration (mM, means ± SD) of the SCFAs and BCFAs present in the cecum contents obtained from the broiler chickens fed with a basal diet (BD), with or without sugarcane bagasse (SCB) lignin: (**a**) acetate, (**b**) propionate, (**c**) butyrate, (**d**) isobutyrate, (**e**) valerate, and (**f**) isovalerate. [a,b] Means within the organic acid across each sampling times marked with the same letter do not differ from each other ($p > 0.05$). [A,B] Means within the organic acid across sampling times for each treatment, marked with the same letter, do not differ from each other ($p > 0.05$).

At 21 days, no differences were found between the feed treatments used; however, at 36 days, differences in the concentrations of acetate, butyrate, and valerate were observed. The animals fed the SCB lignin treatment had higher concentrations of these acids compared to the animals fed the control treatment ($p < 0.05$), while the concentrations of remaining acids (i.e., propionate, isobutyrate, and isovalerate) were not significantly different for the same conditions ($p > 0.05$).

During the *in vivo* study, a reduction in the concentration of acetate and butyrate and an increase in propionate and isobutyrate were observed with the BD treatment, while in the treatment supplemented with SCB lignin, an increase in propionate, isobutyrate, and valerate was observed.

3.2. In Vitro Model Protocol

3.2.1. Bacterial Profile in Fermentations

Figure 6 and Table S4 present the bacterial modulation that occurred during cecal fermentations under the different conditions assessed. Recall that the conditions used were IC (negative control and corresponding to a fasting state), BD (baseline control and a standard response to a specific feed matrix), and BD + SCB lignin (tested condition).

At 0 h, the primers that quantified the largest DNA amount were those of the Firmicutes (equivalent to 8.28 log (CFU/mL)), followed by Bacteroidetes, *Bacteroides*, and *Lactobacillus* (equivalent to 6.16–6.27 log (CFU/mL)), which had the same average bacterial concentrations. *Enterobacteriaceae* followed with the equivalent of 5.19 log (CFU/mL), while the lowest amount of bacterial DNA was quantified for *Bifidobacterium* (equivalent to 1.54 log (CFU/mL)). At this initial fermentation point, the three conditions showed differences in Firmicutes, Bacteroidetes, and *Bacteroides* ($p < 0.05$), in contrast with *Lactobacillus*, *Bifidobacterium*, and *Enterobacteriaceae* ($p > 0.05$). The IC condition had higher values of Bacteroidetes and *Bacteroides* in comparison with the other two conditions ($p < 0.05$). The BD condition presented the highest values of Firmicutes at the beginning of the fermentations ($p < 0.05$).

At 24 h, the only differences observed among the three conditions were for *Lactobacillus* and *Bifidobacterium*, in which the IC condition showed the lowest concentration for both bacterial groups (equivalent to 6.09 and 1.73 log (CFU/mL), respectively). In contrast, both bacteria were at the highest concentrations (equivalent to 6.79 and 2.96 log (CFU/mL), respectively) in the BD condition and were only significantly different from the other conditions for the *Lactobacillus* quantification ($p < 0.05$).

At 48 h, the IC presented the highest bacterial amount of Firmicutes, Bacteroidetes, *Bacteroides*, and *Lactobacillus* and the lowest count of *Bifidobacterium* ($p < 0.05$). At 48 h, the IC condition showed the highest amount of *Enterobacteriaceae* (equivalent to 7.48 log (CFU/mL)), which was not significantly different from the BD condition (equivalent to 7.39 log (CFU/mL)) ($p > 0.05$). A slight, but not significant, decrease was observed with the addition of SCB lignin to BD (equivalent to 7.07 log (CFU/mL)) ($p > 0.05$). The condition with the highest value of *Bifidobacterium* was the BD condition (log 5.58 log CFU/mL); however, the addition of SCB lignin (5.21 log (CFU/mL) did not affect this value significantly ($p > 0.05$).

Throughout the fermentation, both feed conditions had statistically identical bacterial profiles, except for *Lactobacillus* at 24 h, when the BD condition showed greater growth promotion ($p < 0.05$). Both feed conditions had different fermentation profiles compared to the IC condition during the fermentation.

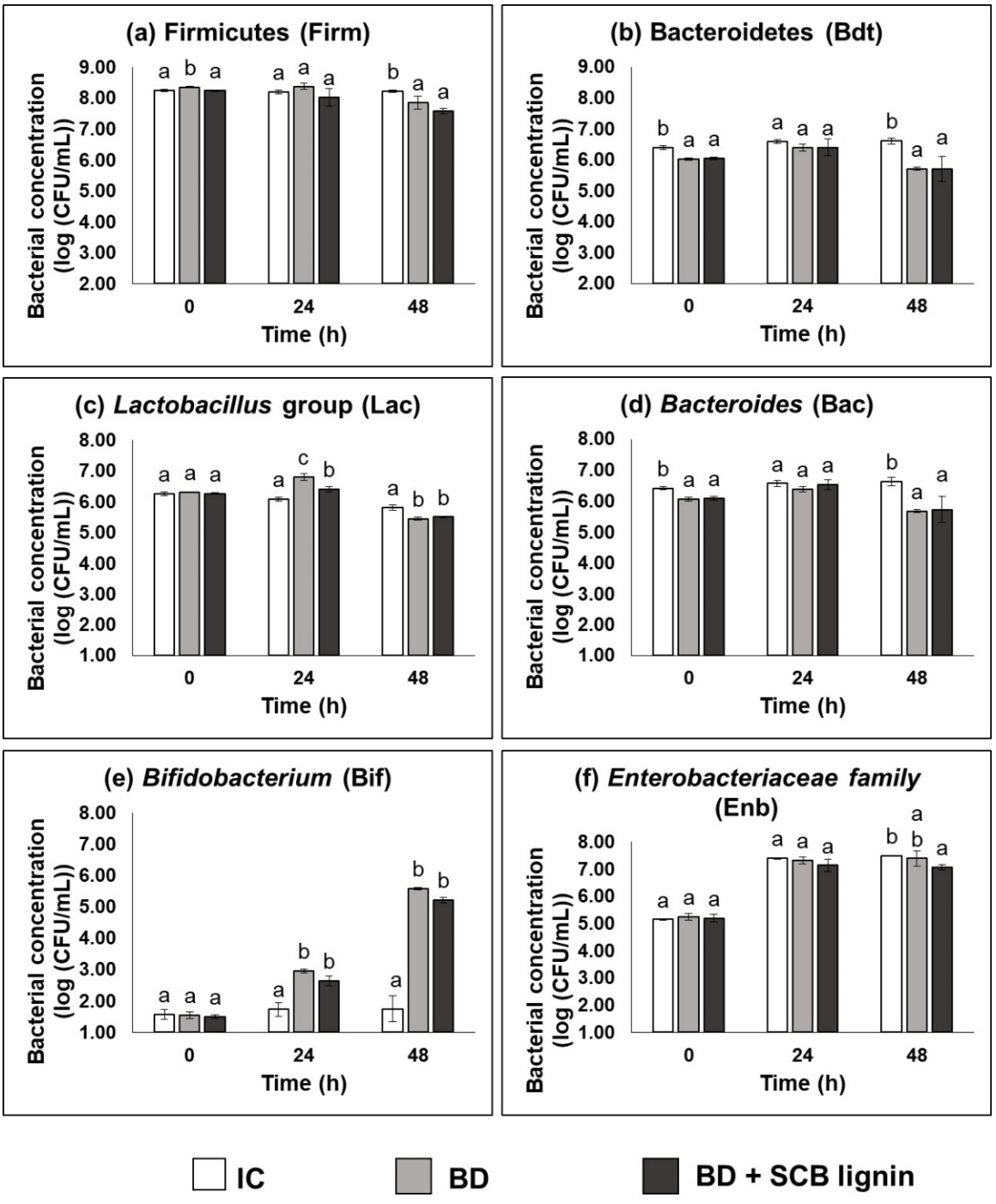

Figure 6. Bacterial quantification (log (CFU/mL), means ± SD) of the different bacterial populations in cecal fermentations for the condition inoculum control (IC), basal diet (BD), with and without sugarcane bagasse (SCB) lignin supplementation: (**a**) Firmicutes, (**b**) Bacteroidetes, (**c**) *Lactobacillus* group, (**d**) *Bacteroides*, (**e**) *Bifidobacterium*, and (**f**) *Enterobacteriaceae* family. [a,b,c] Means of each bacterial concentration at a given sampling time, marked with the same letter, do not differ from each other ($p > 0.05$).

3.2.2. Organic Acids Profile of Cecal Fermentations

The concentrations of organic acids (i.e., lactate, acetate, propionate, and butyrate) produced during the cecal fermentations are depicted in Figure 7a–d and Table S5. Isobutyrate, isovalerate, and valerate were also assessed; however, the quantified values were very

low (<1 mM) or absent (concentrations below the quantification limit of the equipment) throughout the fermentation. For this reason, these were not included.

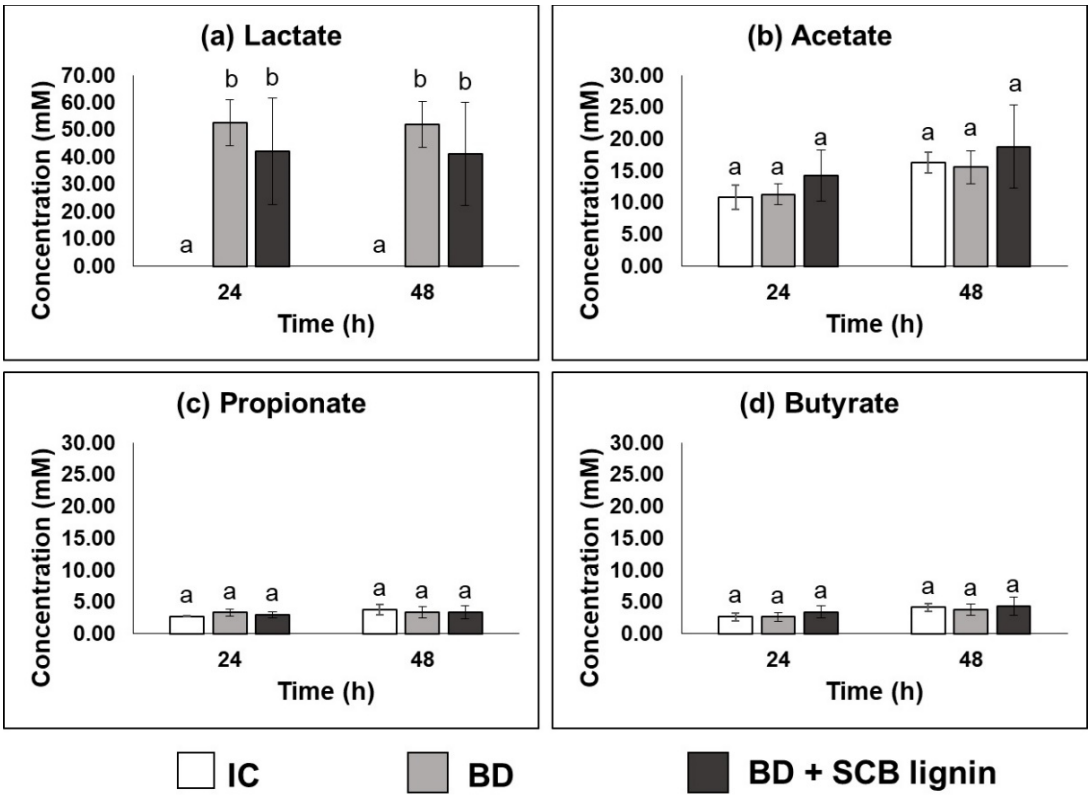

Figure 7. Concentrations (mM, mean ± SD) of the different organic acids produced during the cecal fermentation for the condition inoculum control (IC), basal diet (BD), with and without sugarcane bagasse (SCB) lignin supplementation: (**a**) lactate, (**b**) acetate, (**c**) propionate, and (**d**) butyrate. [a,b] Means of each organic acid at a given sampling time, marked with the same letter, do not differ from each other ($p > 0.05$).

Lactate was the most abundant organic acid produced throughout the fermentation, followed by acetate, propionate, and butyrate. At the end of the fermentation, the conditions with the higher SCFAs production (i.e., acetate + propionate + butyrate) were BD + SCB lignin (26.51 mM), followed by IC (24.15 mM) and BD (22.62 mM).

Lactate production was only observed in the conditions that contained substrates (i.e., BD and BD + SCB lignin). The two feed conditions assessed were not significantly different ($p > 0.05$) from each other but were different from the IC conditions at 24 and 48 h.

The production of the three quantified SCFAs increased throughout the fermentation; however, there were no significant differences ($p > 0.05$) between them. The SCFA production ratio (acetate/propionate/butyrate) was 4:1:1 throughout the fermentation for all conditions.

3.2.3. Total Ammonia Nitrogen Profile

The total ammonia nitrogen concentration produced during the cecal fermentations is depicted in Figure 8 and Table S6. At 0 h, there was a significant difference in total ammonia nitrogen between the tested conditions, with both feed conditions showing higher values than the IC condition ($p < 0.05$). However, at 24 and 48 h, the condition with the highest concentration of total ammonia nitrogen was the IC condition ($p < 0.05$), while both feed conditions showed no significant difference ($p > 0.05$) throughout the fermentations.

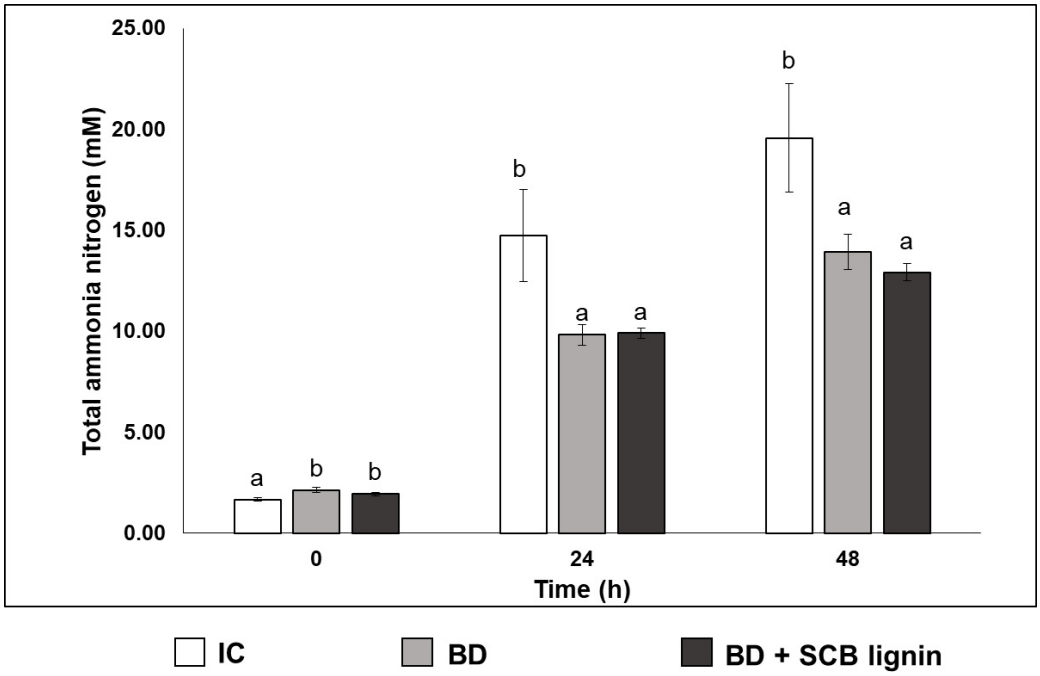

Figure 8. Concentration of total ammonia nitrogen (mM, means ± SD) produced during cecal fermentation for the condition inoculum control (IC), basal diet (BD), with and without sugarcane bagasse (SCB) lignin supplementation. [a,b] Means of total ammonia nitrogen at a given sampling time, marked with the same letter, do not differ from each other ($p > 0.05$).

4. Discussion

The animal *in vivo* assay performed in this study evaluated the impact of 1% SCB lignin on broiler chicken live weight, growth performance, intestinal morphology, cecum bacterial population, and volatile fatty acids (SCFAs and BCFAs) at several ages (1, 12, 21, and 36 days). These parameters were evaluated because the addition of prebiotics is associated with positive effects on gut microbiota modulation, which is directly related to an improvement in gut health and performance [2,19].

The results shown in Tables 3 and 4 demonstrate that the addition of SCB lignin to the chicken feeds had no impact on the animals' growth variables. The body weight, ADG, feed intake, FCR, and mortality results were also evaluated to determine the impact of SCB lignin incorporation into BD, as these parameters are indicators of poultry welfare, feed efficiency, production, and quality [20,21]. In terms of animal mortality, it was observed that it occurred during the early weeks in both feed treatments. This higher mortality in the first week of life was expected, since this is the period in which the animals' systems and organs are still immature, and several factors can negatively influence animal welfare [21,22].

The body weight of the broiler chickens at the start of this study (Table 3) ranged between 40 and 44 g, indicating good chick quality [21]. The quality of 1-day-old chicks was directly related to the survival rate in the first week of life [21]. The results obtained for ADG, FCR, and mortality (Tables 3 and 4) are consistent with those observed in a 2016 study involving 358 broiler farms across seven European Union country members [23].

Other parameters evaluated in this *in vivo* study were the GIT morphometrics (intestinal section measurements and intestinal villi dimensions and absorption area), which are crucial for assessing welfare and disease resistance, as the small intestine has an important role in proper nutrient absorption [24,25]. The size of the small intestine is an important parameter, since a longer small intestine may result in better nutrient absorption, which may lead to better animal performance and productivity parameters [26,27]. The causes behind the reduction in the length of the small intestine can be related to poor feed efficiency, inappropriate nutrient absorption, or altered gut microbiota [28,29].

In this study, the broiler chickens fed with BD supplemented with 1% SCB lignin had a shorter small intestine compared to the control group, which did not affect the animals' carcass yield or the length of other GIT regions (cecum and rectum), compared to the control group, nor did it affect the animals' performance and productivity parameters (Table 5). In addition, there were no differences in the dimensions of intestinal villi or the absorption area between the broiler chicken fed with the two diets tested (Table 6). This information is important, since the measurement of the villus height, crypt depth, and the villus/crypt ratio is the gold standard in the evaluation and the study of the impact of feed ingredients/additives on animal intestinal health [30]. An example of a negative impact on intestinal animal health is a decrease in villus length, which is common in intestinal health problems, while a positive impact would be an increase in the gut surface area that is related to an increase in body weight [24,30]. Therefore, the supplementation of 1% SCB lignin did not have a positive nor a negative effect on the intestinal villi, thus not affecting the nutrient absorption in the GIT. However, a study by Leite et al. (2024) [31] on broiler chickens reported that the inclusion of 1% lignin in their basal diet increased productive performance and nutrient digestibility, with positive effects on parameters such as body weight, ADG, feed intake, and intestinal development. These differences in outcomes may be attributed to the specific type of lignin used between these two studies and its production method.

The gut microbiota composition changes with animal aging and is directly related to animal health and productivity [19,32,33]. This study used both culture-dependent (plating) and culture-independent (qPCR) methods to quantify the targeted bacteria present in the cecal samples of 21- and 36-day-old broiler chickens, thus complementing each method's quantification limitations [33].

The main and most common bacterial groups present in the cecal microbiota include the phyla Firmicutes and Bacteroidetes, as well as the families/genera *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, and *Enterobacteriaceae* (all *Enterobacteriaceae* are Gram-negative bacteria/enteric bacteria) [19,34]. The fact that some of these bacteria are linked to either positive or negative markers of performance—for example, strains of *Lactobacillus* and *Bacteroides* are linked to improvement while strains of *Enterobacteriaceae* are linked to bad performance—was another factor for the selection of these bacteria [19,35].

The pH values of the cecal contents in both treatments and time points decreased within the predicted ranges, i.e., between 6 and 7 [36]. No impact of the treatment was detected on the pH of the cecal contents (diluted to 10%) (Figure 3b). The microbial composition at 21 and 36 days showed different amounts of bacterial groups, using the two quantification methods. Recall that at 36 days, the animals fed with chicken feed supplemented with 1% SCB lignin had lower counts of Gram-negative/enteric bacteria (plating) and *Enterobacteriaceae* (qPCR). An increase in *Bifidobacterium* in the animals fed with SCB lignin was expected, based on the results previously published by Fangueiro et al. (2023) [7]; however, this was not observed, and the group fed with SCB lignin had a lower amount of *Bifidobacterium* compared to the control diet. On the other hand, there was a decrease in *Enterobacteriaceae*, which are bacteria related to enteropathogenic bacteria [19,35]. One possible explanation for the differing outcomes between the findings of this study and those reported by Fangueiro et al. (2023) [7] could be related to variations in the experimental conditions. These include differences in the composition of the basal diet tested, the broiler chicken growth conditions, and the age at which the cecal contents were collected. It is well established that factors such as diet and age can significantly influence the intestinal microbiota, leading to different bacterial modulations under similar feeding conditions [34,37].

The GIT microbiota and their metabolites play an important role in the poultry production systems by helping the development of intestinal structure, digestion, protection, and host immune system [22,32]. Since the cecum is the primary site of fermentation in the GIT and the section with the highest abundance of SCFAs and microbial diversity,

the microbiota profile and metabolites, such as SCFAs and BCFAs, present in the animals' cecum were assessed at 21 and 36 days of life.

The values for SCFAs and BCFAs presented in Figure 5 and Table S3 agree with the scientific literature. Previous studies have analyzed microbiota metabolites from 38 to 42 days (regardless of their diet), and the concentration order found was acetate > butyrate > propionate > valerate > isobutyrate, and isovalerate [38–40]. As observed in Figure 5 and Table S3, the supplementation of SCB lignin promoted the increase in acetate, butyrate, and valerate at 36 days compared to the broiler chickens that consumed the BD treatment. Thus, at the metabolic level, these results suggest a positive impact of incorporating SCB lignin in the broilers' diet, as these acids, especially acetate and butyrate, have multiple beneficial effects (e.g., acetate is associated with the promotion of mucin; butyrate contributes to the maintenance of intestinal epithelial cell integrity) [32,34].

To ensure a more accurate comparison between the *in vivo* study and the *in vitro* results, a GIT *in vitro* simulation was carried out according to the method described by de Carvalho et al. (2023) [13]. For the *in vitro* assessment validation, the presence and production of bacteria, volatile fatty acids, and total ammonia nitrogen were evaluated during the cecum fermentation simulation to compare them with the results of the animal assay.

The simulation model carried out in this study was a static *in vitro* model with batch fermentations of 48 h, which is the recommended fermentation period to perform batch fermentations, as more than 48 h can deplete nutrients and lead to the accumulation of fermentation metabolites, which restrict fermentation and the growth of the microorganisms [12,41].

The inoculum used was prepared from a pooled cecum content of 36-day-old broilers fed with BD. In addition, the production of total ammonia nitrogen produced in the fermentation by the proteolytic activity of the cecum microbiota was assessed, due to the negative impact that ammonia has on poultry performance, poultry house workers, and the environment [7,14].

The ratio of the bacterial groups identified at 0 h of the cecal fermentations (Figure 6 and Table S4) was identical to that determined in the bacterial quantifications of individual cecal contents previously performed (Figure 4 and Table S2). This bacterial ratio present in fermentations was expected, since the cecal inoculum used in fermentations was a pooled sample of cecal contents. The use of pooled inoculum increased bacterial biodiversity and reduced inter-donor variability, allowing the sample to be more representative of the chicken cecum microbiota [42].

At 24 h of fermentation, the BD condition revealed a greater promotion of bacterial growth; however, this was statistically significant only for the growth of *Lactobacillus*. On the other hand, at 48 h, there were no statistical differences ($p > 0.05$); however, the condition BD + SCB lignin had a lower number of *Bifidobacterium* and *Enterobacteriaceae*, a result that was also observed in the in vivo study. Therefore, at 48 h of fermentation, the same modulation trends were observed for both *in vivo* and *in vitro* studies, with the difference that they were statistically significant only for the *in vivo* study ($p < 0.05$). The number of cecal fermentation reactors in the *in vitro* assessment should be increased ($n > 4$) to be statistically relevant.

Regarding the organic acids produced during cecal fermentation, the lactate cross-feed did not occur (Figure 7 and Table S5). Lactate is an important organic acid produced during the intestinal anaerobic fermentation and it is converted by the cecal microbiota into acetate, propionate, and butyrate. Normally, lactate does not accumulate in the healthy intestinal lumen [43]. This lactate cross-feeding mechanism, more precisely lactate-utilizing bacteria (e.g., *Bacteroides* and *Roseburia*) has a major role in maintaining a healthy GIT [43]. In the cecal fermentation simulations performed by Fangueiro et al. (2023) and de Carvalho et al. (2023) [7,13], the production of lactate was observed at 24 h, with values similar to the ones obtained in the present study. However, at 48 h, no lactate was detected.

The SCFAs concentrations obtained by Fangueiro et al. (2023) and de Carvalho et al. (2023) [7,13] at 48 h were higher than the ones obtained in the present study. As an

example, the IC and control diet in this study showed a concentration of SCFAs of 24.15 and 22.62 mM, respectively, while in Fangueiro et al. (2023) and de Carvalho et al. (2023) [7,13], the values were 32 and 69–71 mM, respectively, for similar conditions. The reason for the lower production of SCFAs in the present study is related to the accumulation of lactate that was not consumed, which means that the lactate-consuming bacteria present in the cecal inoculum did not exhibit the normally expected activity. This may well be related to some issues associated with the preparation of the inoculum, especially to the conditions of transport and preparation in anaerobic conditions, which promoted the inhibition of its activity.

In the cecal fermentations carried out, there were no differences between the feed treatments assessed. The only difference observed was that the IC condition did not produce lactate throughout the 48 h fermentation.

This *in vitro* study supports Fangueiro et al.'s (2023) [7] conclusion that adding 1% SCB lignin to mash feed has no significant impact on the amount of intestinal ammonia that is produced.

5. Conclusions

The addition of 1% SCB lignin in mash feed had a positive effect, which was especially observed in the 36-day-old broiler chickens' cecum microbiota and metabolites, namely the decrease in *Enterobacteriaceae*, bacteria associated with harmful impact on broiler chicken health and performance, along with an increase in acetate, butyrate, and valerate. The mentioned supplementation had no impact on the animals' productivity and physiology.

Although the same trends in microbiota modulation were observed in both *in vivo* and *in vitro* studies, there was no statistical difference between the different conditions tested in the *in vitro* assay, showing the need to increase the number of cecum fermentation simulations to a number higher than four. Additionally, the production of organic acids during the *in vitro* assessment did not follow the same variation observed in the *in vivo* assay, which we strongly believe to be associated with the inoculum preparation, which affected the lactate-using bacteria. In addition, during the *in vitro* assessment, the tested supplementation did not impact intestinal ammonia production activity.

This study demonstrates the importance of the inoculum preparation step, specifically concerning time duration, anaerobic conditions, and the number of cecum fermentation simulations (which exceeded four simulators).

The results from the chicken GIT in the *in vitro* model developed demonstrate that this model can be used as an initial screening technique for potential functional ingredients in avian nutrition (such as SCB lignin) before the tested ingredients reach the animal assay stage. This allows us to reduce the number of unnecessary animal assays and the costs associated with them. However, some adjustments regarding the preparation of the cecal inoculum used in the model are necessary to improve the technique's reproducibility and reliability.

Overall, this study shows that 1% SCB lignin in mash feed, while not improving the animals' productivity parameters, also did not negatively affect these parameters or the animals' physiology. SCB lignin can be regarded as a possible sustainable feed supplement derived from sugarcane byproducts, due to the positive effects observed. In addition, it allows us to conclude that the presented method for *in vitro* studies shows great potential for predicting the impact of target ingredients on a host's GIT.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su16208946/s1, Table S1: Viable bacterial cell counts (log (CFU/mL), mean ± SD) of the cecal contents in different culture media and pH values of the cecal contents diluted at 10% (*w*/*v*) in phosphate-buffered saline (PBS); Table S2: Bacterial quantification (log (CFU/mL), means ± SD) of the different bacterial populations in cecal contents for different treatments; Table S3: Concentration (mM, means ± SD) of the short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) present in the cecum contents of the animals used in the animal trial; Table S4: Bacterial quantification (log (CFU/mL), means ± SD) of the different bacterial populations in cecal

fermentations; Table S5: Concentration (mM, means ± SD) of the organic acids produced during cecal fermentation; Table S6: Concentration (mM, means ± SD) of total ammonia nitrogen produced during cecal fermentation.

Author Contributions: Conceptualization, N.M.d.C., A.L.A., P.S.C., C.S.S.O. and A.R.M.; methodology, N.M.d.C., C.G.d.S., C.M.C., D.O.-M., J.T., J.L.M. and V.P.; validation, A.R.M.; formal analysis, N.M.d.C.; investigation, N.M.d.C., C.G.d.S., C.M.C., C.C., J.F.F., B.H., D.O.-M., J.T., J.L.M. and V.P.; resources, D.O.-M., J.T., J.L.M., V.P. and A.R.M.; data curation, N.M.d.C., C.G.d.S., D.O.-M., J.T., J.L.M. and V.P.; writing—original draft preparation, N.M.d.C.; writing—review and editing, D.O.-M., J.T., J.L.M., V.P., D.L.O. and A.R.M.; visualization, N.M.d.C.; supervision, D.O.-M., J.T., J.L.M., V.P., A.L.A., P.S.C., C.S.S.O., M.E.P. and A.R.M.; project administration, D.O.-M., J.T., J.L.M., V.P., A.L.A., P.S.C., C.S.S.O., M.E.P. and A.R.M.; funding acquisition, M.E.P. and A.R.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Ethics Committee of Universidade de Trás-os-Montes e Alto Douro (UTAD), Portugal.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

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

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