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Assessment of Feed Value of Chicory and Lucerne for Poultry, Determination of Bioaccessibility of Their Polyphenols and Their Effects on Caecal Microbiota

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Abstract: Chicory and lucerne possess high feed value for poultry being good sources of protein and fiber. In addition, they are rich in polyphenols that help the body build an integrated antioxidant system to prevent damage from free radicals and positively modulate microbial populations in the gastrointestinal tract. These health-promoting effects of polyphenols depend on their bioaccessibility and absorption in the animal body. The present paper aimed to study the bioaccessibility of polyphenols from chicory and lucerne after subjecting the samples to gastric and intestinal phases of digestion in an in vitro model of chicken gut and assessment of their feed value by measuring the presence of fermentable substrates (in terms of gas production), SCFAs produced and their effects on gut microbiota population during in vitro cecal fermentation. Results revealed that the bioaccessibility of polyphenols varied with different polyphenol compounds. The highest bioaccessibility was recorded for *p*-hydroxybenzoic acid (90.8%) from chicory following the intestinal phase of digestion. The lowest bioaccessibility was observed for quercetin-3-rhamnoside (12.6%) from chicory after the gastric phase of digestion. From lucerne, the highest bioaccessibility was recorded for kaempferol-3-glucoside (77.5%) after the intestinal phase of digestion. Total gas production was higher for lucerne (39.9 mL/g) than chicory (28.1 mL/g). Similarly, total SCFAs production was higher after 24 h of cecal fermentation with lucerne (42.2 mmol L^{-1}) as compared to chicory (38.1 mmol L^{-1}). Results also revealed that the relative abundance of *Clostridium* was reduced with chicory (0.225%) and lucerne (0.176%) as compared to the control (0.550%) after 24 h of cecal fermentation. The relative abundance of *Streptococcus* was reduced by lucerne (4.845%) but was increased with chicory (17.267%) as compared to the control (5.204%) after 24 h of fermentation. These findings indicated that chicory and lucerne differentially affected the microbial populations during in vitro cecal fermentation.

Keywords: chicory; lucerne; polyphenols; bioaccessibility; chicken; gut microbiota; modulation; cecal fermentation; in vitro fermentation

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1. Introduction

Chicory (*Cichorium intybus* L.) and lucerne (*Medicago sativa* L.) are widely used in animal production due to their widespread existence and represent valuable feed sources for poultry being rich in protein and fiber. Lucerne has been found to contain 15% to 22%

crude protein on a dry matter basis [1] and has 1.15% and 0.27% lysine and methionine content respectively. It also contains macro and trace minerals as well as water and fat-soluble vitamins [1]. Particularly, lucerne contains highly digestible fiber that could be efficiently utilized by the birds and leads to improved production performance. The highly digestible fiber of lucerne helps to improve gut health through the positive modulation of gut microbiota that aids in digestion and assimilation of nutrients, building immunity and disease resistance [2]. The high fiber content of lucerne also helps the birds to forage and scratch through the litter like they would do normally in their natural habitat collecting any stray bits of lucerne which in turn all helps to improve the health, appearance, and welfare of poultry birds.

Chicory is also gaining a reputation for providing a good quality feed for poultry. It contains 16–27% crude protein on a dry matter basis [3] and has 1.21% lysine and 0.4% methionine (DM basis). It is also a good source of fiber. The dietary fiber in chicory is high in a type of pectin (80–90 g/kg dry matter) that is highly soluble [4]. Chicory also contains high amounts of inulin that ranges from 150–200 g/kg of the whole chicory plant [3]. Inulin is prebiotic that can promote the growth of beneficial microbes and can suppress the pathogens in the gut [5]. Therefore, chicory could be utilized to cure dysbiosis that reduces productivity in poultry [6–8]. Chicory root and stem powder are also known to improve the intestinal microenvironment, reduce endogenous nitrogen loss, and improve carcass quality broilers [4].

In addition, chicory and lucerne produce numerous bioactive compounds like polyphenols imparting therapeutic importance to these plants. In a recent study, 80 different polyphenol compounds have been reported in chicory and lucerne [9] that contribute to these plants' bioactive potential and putative health benefits. These polyphenolic compounds are well known for their prebiotic and antimicrobial properties. These properties of polyphenols can be more beneficial for poultry because of their low absorption in the upper digestive tract [10]. Due to their low absorption, about 90% of these phenolic compounds pass unaltered to the lower digestive tract where they interact with the gut microbiota [11]. These compounds can increase the number of *lactobacilli* and *bifidobacteria* [2,12] and suppress the colonization of pathogens [13]. This results in positive modulation of the microbial populations in the gastrointestinal tract that improves fermentation of feed and results in improved harvesting of nutrients [14,15].

The expression of these health-promoting effects depends on the bioaccessibility and absorption of polyphenols in the animal body [15–17] that varies significantly among different polyphenol compounds [18]. The information regarding the bioaccessibility/bioavailability of phenolic compounds in broilers is very limited. Striking dissimilarities also exist between the in vitro and ex vivo data available on this aspect [15]. Nevertheless, chicory and lucerne remained underutilized in poultry feeding despite their widespread existence and high nutritive value, and the information to compare the feed value of chicory with that of lucerne for poultry is lacking. Therefore, the present research was designed to assess the feed value of chicory and lucerne for poultry, measure the release and bioaccessibility of their polyphenols during simulated gastrointestinal digestion and study their effects on cecal microbiota in an in vitro model of the chicken gut.

2. Materials and Methods

2.1. In Vitro Digestion

2.1.1. Sample Preparation

Fresh chicory and lucerne were collected from Ellinbank and Hamilton Smart Farms, Agriculture Victoria Research, respectively. Upon collection, plant samples were dried at 60 °C for 48 h in a hot air oven. The oven-dried chicory and lucerne were ground to a fine powder using a coffee blender (MultiGrinder II, Sunbeam, Sydney, Australia) to obtain a uniform particle size before in vitro digestion and subsequent cecal fermentation.

2.1.2. Gastrointestinal Digestion

The in vitro digestion method was based on a published protocol with minor modifications [19,20]. For simulation of gastrointestinal compartments, incubation temperature was set at 41 °C instead of 37 °C to closely reflect the chicken digestive tract conditions [21]. For the simulation of crop digestion, 5 ± 0.1 g of feeds and 15 mL of 0.03 mol/L hydrochloric acid were mixed in 50-mL polypropylene centrifuge tubes, the pH was adjusted to 5.2 and incubated for 30 min.

The gastric section was simulated by adding 3000 U of pepsin (Sigma-Aldrich, Castle Hill, NSW, Australia) per g of feed and 2.5 mL of 1.5 mol/L HCl to each tube, and pH was adjusted to 1.4–2.0 and incubated at 41 °C for 45 min. Enzyme solution (pepsin solution) was prepared by adding 1.2 mg of pepsin per mL of 0.1 mol L^{-1} HCl. For simulation of the intestinal phase, 6.84 mg of 8x pancreatin (MP Biomedicals, LLC Diagnostics Division, Fountain Parkway, Solon, OH, USA) in 6.5 mL of 1.0 mol/L sodium bicarbonate solution was added to each tube (pH adjusted to 6.4–6.8). Following this, tubes were again incubated for 2 h. Hence, pre-caecal gastrointestinal digestion was completed in 3 h and 15 min.

2.2. In Vitro Cecal Fermentation

2.2.1. Cecal Slurry Preparation

The cecal digesta were obtained from a group of six-week-old male Ross 308 broiler chickens grown on a commercial broiler diet from day 1 to six weeks of age at the Animal Facility of the University of Melbourne under thermo-neutral conditions for an experiment approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia (Application ID. 20277). For the collection of cecal digesta, the caeca of the birds were aseptically removed and the digesta was collected. The cecal digesta collected were immediately transferred to the laboratory for inoculum preparation. The cecal material was weighed and an amount of pre-warmed (41 °C), anaerobic, sterile phosphate buffer (pH 7.2) was added to yield 10% (wt./vol.) slurry [22]. The diluted material was homogenized and stored at −80 °C before use.

2.2.2. Basal Media Preparation

For the preparation of basal media (pH 7.0 at 25 °C), NaCl (4.5 g), KCl (4.5 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), $CaCl_2$ (0.11 g), $NaHCO_3$ (1.5 g), $MgSO_4 \cdot 7H_2O$ (1.23 g), L-cysteine HCl (0.8 g), peptone (5 g), casein (3 g), bile salts (0.4 g), guar (1.0 g), mucin (4 g), pectin (2 g), soluble starch (5 g), tryptone (5 g), Tween 80 (1 mL), and yeast extract (4.5 g) were mixed in a volumetric flask and volume was made 1L with Milli-Q water by following the method of Fu et al. [23] and autoclaved at 121 °C for 20 min before use.

2.2.3. Cecal Fermentation

The fermentation was performed by the method proposed by Menke [24] with some modifications. 0.5 ± 0.1 g of residual sediment from gastrointestinal digestive fractions of chicory and lucerne samples were mixed with 5 mL of fermentation media and 5mL of already prepared cecal slurry in 50 mL tubes. Blank test samples were prepared by mixing cecal slurry (5 mL) with sterilized fermentation medium (5 mL) without lucerne or chicory samples. All samples were placed in 50 mL polypropylene tubes (flushed with N_2). The tubes were incubated at 41 °C for 24 h using anaerobic chambers (BD Gas pak™, BD Australia, NSW, Australia) containing an anaerobic gas generator (AN 0010 W, Oxoid®) and indicator (BR55, Oxoid®). After 12 h and 24 h of incubation, samples were collected and stored at −80 °C before being analyzed for antioxidant potential, individual polyphenols, short-chain fatty acids (SCFA), and DNA extraction.

2.3. Antioxidant Potential of Digestive Fractions

All the chemicals used in antioxidant assays were of analytical grade. These include KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$, $(NH_4)_2CO_3$, NaOH, HCl, and $CaCl_2$, 2,2-Diphenyl-1-pricrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2,4,6-tripytidyl-s-triazine (TPTZ), gallic

acid, sodium carbonate, ascorbic acid, sodium acetate, and ferric chloride. The antioxidant potential of digestive fractions was determined by high throughput 96-well plate methods [9,25]. The supernatants were collected at every digestive stage after centrifugation at 10,000× *g* for 20 min. Total phenolic content (TPC), 2,2-Diphenyl-1-pricrylhydrazyl (DPPH), 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Radical Scavenging Assay and Reducing Power Assay (RPA) were measured to evaluate the antioxidant potential of the bio-accessible portions of the digested fractions.

2.4. Separation and Analysis of Polyphenols

Aliquots of 2 mL of the supernatant from each digestive compartment after centrifugation (at 10,000× *g* for 20 min) were analyzed for individual polyphenols from absorbable fractions (released by the action of enzymes and digestive juices). Polyphenols from unabsorbable fractions (that could not be released by the action of enzymes and digestive juices) were extracted from the sediments of each digestive compartment with 80% ethanol and analyzed for determination of %age bioaccessibility. The extracts were filtered through a 0.22 µm syringe filter and stored at −20 °C for polyphenols analysis.

2.4.1. Quantification of Individual Polyphenols

Separation and quantification of individual polyphenols were achieved with a Synergi Hydro-RP (250 × 4.6 mm i.d.) column having a particle size of 4 mm (Phenomenex, Lane Cove, NSW, Australia) on Waters Alliance 2690 (Chromatograph Separation Module) HPLC system coupled to a PDA detector (Model 2998, Waters) and data acquisition and analysis were achieved with Empower Software (2010) (Shimadzu Scientific Instruments, Sydney, NSW, Australia). Mobile phase A was composed of water/acetic acid solution (98:2 *v*/*v*) and mobile phase B was composed of water/acetonitrile/acetic acid (50:49.5:0.5, *v*/*v*/*v*). The gradient used was: 90% A, 0 min; 75% A, 20 min; 65% A, 30 min; 60% A, 40 min; 45% A, 52 min; 20% A, 54 min; 90% A, 56 min with a steady flow rate of 0.8 mL/min. Gallic acid, protocatechuic acid, catechin, *p*-hydroxybenzoic acid, epicatechin, epicatechin gallate, quercetin-3-galactoside, quercetin-3-rhamnoside, kaempferol-3-glucoside, and procyanidin B2 were used to create standard curves for HPLC quantification of individual polyphenols.

2.4.2. Determination of Bioaccessibility of Polyphenols

The bioaccessibility of each polyphenol compound was calculated after the feeds were subjected to different stages of gastrointestinal digestion (Figure 1). It was calculated as the percentage of solubilized polyphenol from the supernatant at different stages of digestion relative to the total amount of the compound (soluble + insoluble) at the corresponding stages of the digestion process [23]. The following equation was used;

$$\% \text{ Bioaccessibility} = \frac{\text{Concentration of polyphenol in soluble fraction } \times 100}{\text{Concentration of polyphenol in total digest at the corresponding stage}} \quad (1)$$

2.5. Short-chain Fatty Acids Analysis

Short-chain fatty acids (SCFAs) analysis was performed with gas chromatography following a previously published method [26]. Briefly, the extraction of SCFAs from fermented samples was performed with water/formic acid (99:1, *v*/*v*) and analysis was achieved with a capillary column (SGE BP21, 12 × 0.53 mm internal diameter (ID) with 0.5 µm film thickness, SGE International, Ringwood, VIC, Australia, P/N 054473) on Agilent gas chromatography system (7890B Agilent, Santa Clara, CA, USA) coupled to a flame ionization detector (FID) with an injection volume of 2 µL. The carrier gas was helium, and the makeup gas was composed of hydrogen, nitrogen, and air. The internal standard used was 4-methyl-valeric acid. Acetic acid, propionic acid, butyric acid, and isovaleric acid were quantified and results were reported as mmol/L.

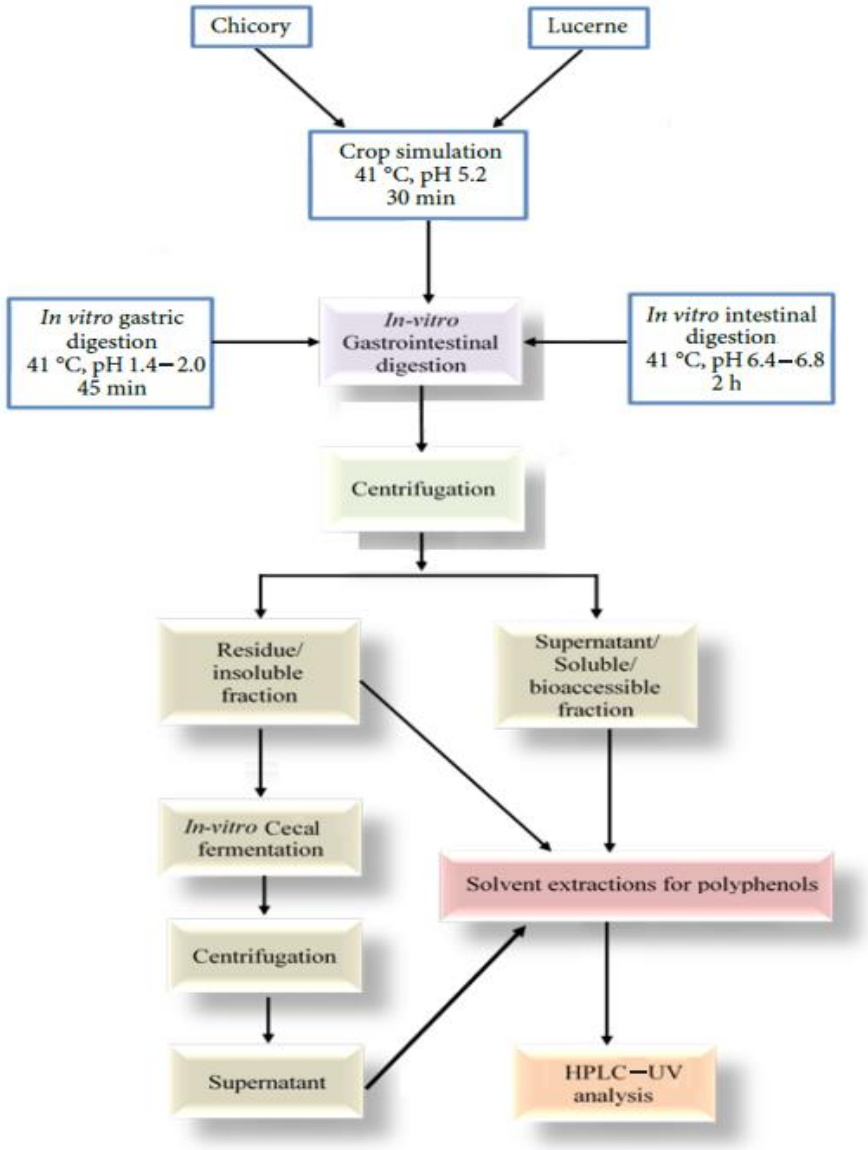

Figure 1. Schematic overview of in vitro digestion process and polyphenols analysis.

2.6. In Vitro Gas Production

In vitro gas production was determined by modifying a previously published method [27]. Briefly, ANKOM^{RF} Gas Production System was used instead of the traditional gas syringes. Approximately 30 mL of the inoculum was added to 310 mL bottles with 0.5 ± 0.1 g of the feeds. Blanks were prepared without the feeds. After removing the air from the headspace, the bottles were tightly capped with modules and incubated at 41 °C. Fermentation was carried out for 24 h and pressure was recorded after every 5 min interval. The gas pressure measured during the fermentation was converted to moles of gas produced by applying the ideal gas law. The equation used was:

$$n = p\,(V/RT) \quad (2)$$

where:

n = gas produced in moles (mol)

p = pressure in kilopascal (kPa)

V = headspace volume in the glass bottle in liters (L)

T = temperature in Kelvin (K)

R = gas constant (8.314472 L·kPa·K^{-1}·mol^{-1})

Finally, Avogadro's Law was applied to convert moles of gas to gas volume (mL) by the following formula:

$$\text{Gas volume (mL)} = n \times 22.4 \times 1000 \quad (3)$$

2.7. 16S rRNA Sequencing and Analysis

DNA extraction was performed from 0.25 g of fermented samples with a DNeasy® PowerSoil® kit (QIAGEN GmbH, Hilden, Germany) according to the instructions of the manufacturer. The V3-V4 regions of the 16S rRNA gene were amplified using target 341F primers (forward primer CCTAYGGGRBGCASCAG and reverse primer GGACTAC-NNGGGTATCTAAT). The procedure of 16S rRNA amplicons preparation followed a standard protocol. High throughput sequencing was performed on the Illumina MiSeq platform by Australian Genome Research Facilities (AGRF, Melbourne, Australia). Image analysis was performed in real-time by the MiSeq Control Software (MCS, Illumina, San Diego, CA, USA) v4.0.0.1769 and Real-Time Analysis (RTA, Tableau, Seattle, WA, USA) v1.18.54.4. Sequence data was generated on Illumina bcl2fastq 2.20.0.422 pipeline. Diversity profiling was achieved with QIIME 2 2019.7 [28]. Primer trimming and quality filtration of demultiplexed raw reads were performed with the cutadapt plugin followed by denoising with DADA2 [29] (via q2-dada2). ASVs were assigned taxonomy using the q2-feature-classifier [30] classify-sklearn naïve Bayes taxonomy classifier.

2.8. Statistical Analysis

The results are presented as mean ± standard deviation of the values of three independent analyses. Minitab Program for Windows version (Minitab Inc., State College, PA, USA) was used to conduct the statistical analysis. One-way analysis of variance (ANOVA) was applied to test the statistical significance of mean values of total phenolic contents, antioxidant activities, polyphenol contents, and short-chain fatty acids (SCFA) of the chicory and lucerne samples after in vitro digestion and cecal fermentation (p value < 0.05).

3. Results

3.1. Total Phenolic Content of Chicory and Lucerne during In Vitro Digestion and Cecal Fermentation

TPC of chicory and lucerne as well as their digestive and fermented fractions was determined to approximate the overall release of phenolics during in vitro digestion and the results are presented in Table 1. TPC of chicory and lucerne varied considerably after gastric and intestinal phases of in vitro digestion. The TPC of chicory and lucerne decreased by 9% and 31% respectively after gastric digestion as compared to the undigested samples. This compared to a 3% decrease for lucerne and a 22% increase for chicory for the intestinal phase as compared to undigested samples as TPC of both chicory and lucerne was increased after intestinal digestion as compared with the gastric phase.

Table 1. Total phenolic content (TPC) of undigested chicory and lucerne and their digestive fractions.

TPC	Chicory (mg GAE/g of Digesta)	Lucerne (mg GAE/g of Digesta)
Undigested	0.441 ± 0.04 ^{b}	0.714 ± 0.01 ^{a}
Soluble gastric fraction	0.400 ± 0.01 ^{b}	0.491 ± 0.02 ^{b}
Soluble intestinal fraction	0.540 ± 0.02 ^{a}	0.690 ± 0.01 ^{a}
Soluble fermented fraction (12 h)	0.510 ± 0.02 ^{a}	0.542 ± 0.01 ^{b}
Soluble fermented fraction (24 h)	0.500 ± 0.03 ^{a}	0.544 ± 0.02 ^{b}

Results are reported as mean ± SD of three analyses (n = 3). Different letters ^{a} and ^{b} indicate a significant difference in columns ($p \leq 0.05$).

The TPC of lucerne was significantly decreased after gastric digestion of lucerne. However, there was no significant change in the total phenolic content of chicory after the gastric phase of digestion. The TPC for both samples was higher during different time points of cecal fermentation than in the gastric phase. However, it was lower for both samples than in the intestinal phase. There was no significant difference in total phenolic contents of chicory and lucerne at different time points of cecal fermentation. Overall, the TPC of chicory and lucerne was differentially affected during in vitro gastrointestinal digestion and cecal fermentation.

3.2. Individual Polyphenols Identified and Quantified after In Vitro Digestion and Their Bioaccessibility

Identification and quantification of polyphenols from digestive fractions of chicory and lucerne were performed by comparing their retention times with that of standards. HPLC chromatograms of soluble gastrointestinal digestive fractions of chicory are shown in Figure 2 and that of lucerne are shown in Figure 3. The polyphenols quantified in the soluble and insoluble fractions (Figures S1 and S2-Supplementary materials) of in vitro gastrointestinal digestion include gallic acid, *p*-hydroxybenzoic acid, epicatechin, catechin, epicatechin gallate, protocatechuic acid, quercetin-3-galactoside, quercetin-3-rhamnoside, and kaempferol-3-glucoside and are presented along-with their bioaccessibility in Table 2.

Table 2 shows that the bioaccessibility of polyphenols varied significantly during gastric and intestinal phases of in vitro digestion of chicory and lucerne. Gallic acid was identified in digestive fractions of both chicory and lucerne. From chicory, bioaccessibility of gallic acid was 54.2% and 57.3% after gastric and intestinal phases, respectively. However, the bioaccessibility of gallic acid from lucerne was 65.6% and 47.1% after gastric and intestinal phases, respectively. Protocatechuic acid was identified only in gastrointestinal fractions of chicory. The bioaccessibility of protocatechuic acid was 55.6% and 81.8% after gastric and intestinal digestion, respectively. Bioaccessibility of catechin was 58.1% and 66.2% after gastric and intestinal phases of in vitro digestion of chicory. However, its bioaccessibility was higher from lucerne during gastrointestinal digestion and recorded at 61.3% after the gastric phase and 74.8% after the intestinal phase.

p-hydroxybenzoic acid was identified only in the digestive fractions of chicory that showed the highest bioaccessibility after intestinal digestion (90.8%). Epicatechin is an important polyphenol found in many plants. It was identified in digestive fractions of both chicory and lucerne. Epicatechin showed slightly higher bioaccessibility from chicory (58.4%) than lucerne (57.0%) after intestinal digestion. Epicatechin gallate, quercetin-3-galactoside, and kaempferol-3-glucoside were identified only in digestive fractions of lucerne. Bioaccessibility of epicatechin gallate was 49.1% and 52.9% after gastric and intestinal digestion, respectively. In comparison, the bioaccessibility of quercetin-3-galactoside was 37.5% and 59.4% after gastric and intestinal digestion of lucerne, respectively. However, the bioaccessibility of kaempferol-3-glucoside was 36.6% and 77.5% after gastric and intestinal phases of in vitro digestion. Among these, bioaccessibility of kaempferol-3-glucoside was much higher after intestinal digestion than epicatechin gallate and quercetin-3-galactoside from lucerne. Quercetin-3-rhamnoside was identified only in digestive fractions of chicory with bioaccessibility of 12.6% after the gastric phase and 56.3% after the intestinal phase. These results indicated that the bioaccessibility of polyphenols from chicory and lucerne varied significantly during different phases of gastrointestinal digestion in the simulated in vitro model of chicken gut.

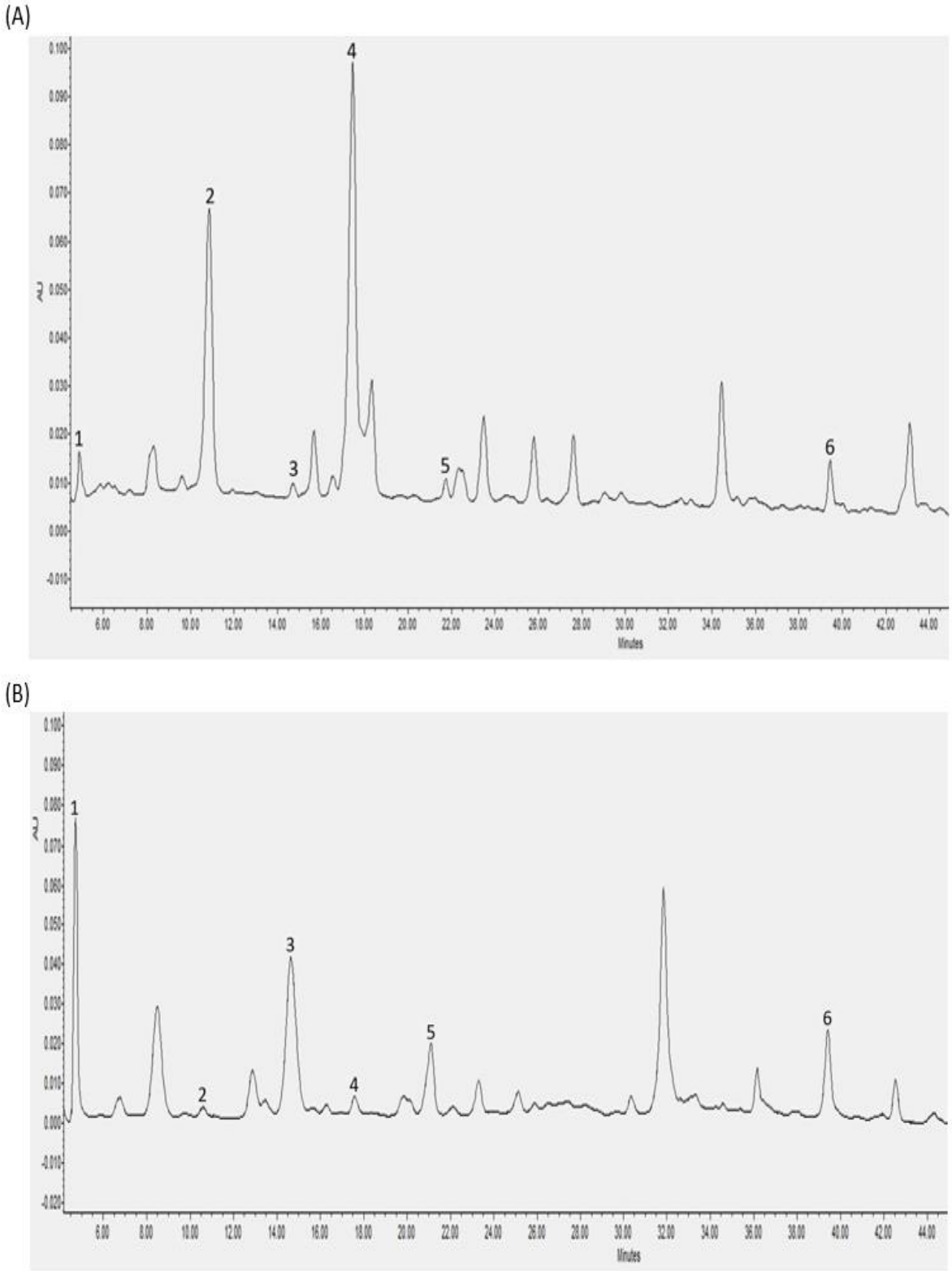

Figure 2. HPLC chromatograms of gastrointestinal fractions of chicory; (**A**) Soluble gastric fraction (extracted from supernatant), (**B**) Soluble intestinal fraction (extracted from supernatant). Polyphenols identified are (1) Gallic acid, (2) Protocatechuic acid, (3) Catechin, (4) *p*-hydroxybenzoic acid, (5) Epicatechin, and (6) Quercetin-3-rhamnoside.

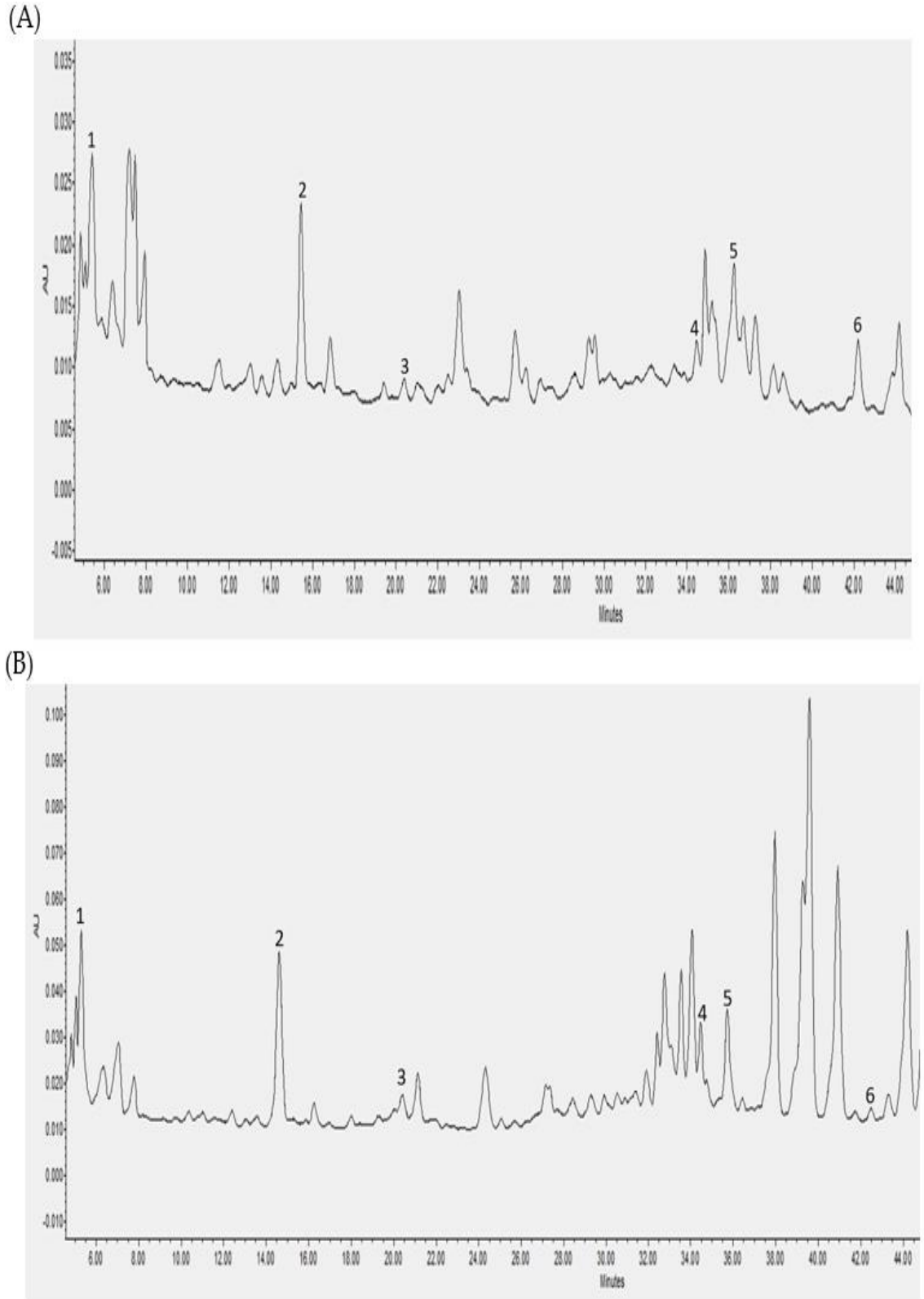

Figure 3. HPLC chromatograms of gastrointestinal fractions of lucerne; (**A**) Soluble gastric fraction (extracted from supernatant), (**B**) Soluble intestinal fraction (extracted from supernatant). Polyphenols identified are (1) Gallic acid, (2) Catechin, (3) Epicatechin, (4) Epicatechin gallate, (5) Quercetin-3-galactoside, (6) Kaempferol-3-glucoside.

Table 2. Polyphenols released during the gastrointestinal digestion and their bioaccessibility.

Compounds		Gastric		Intestinal	
		Chicory (µg/g)	**Lucerne (µg/g)**	**Chicory (µg/g)**	**Lucerne (µg/g)**
Gallic acid	FPP	20.4 ± 0.8 ^{d}	41.4 ± 1.2 ^{a}	25.9 ± 0.3 ^{c}	26.1 ± 1.3 ^{b}
	BPP	17.2 ± 0.1 ^{d}	21.7 ± 0.1 ^{b}	19.3 ± 1.2 ^{c}	29.3 ± 3 ^{a}
	TPP	37.6	63.1	45.2	55.4
	% Bioaccessibility	54.2%	65.6%	57.3%	47.1%
Protocatechuic acid	FPP	38.1 ± 0.5 ^{a}	ND	13.5 ± 0.2 ^{b}	ND
	BPP	30.4 ± 0.7 ^{a}	ND	3.2 ± 0.1 ^{b}	ND
	TPP	68.5	-	16.7	-
	% Bioaccessibility	55.6%	-	80.8%	-
Catechin	FPP	18.6 ± 0.6 ^{c}	18.7 ± 0.1 ^{c}	27.6 ± 0.4 ^{b}	44.3 ± 0.14 ^{a}
	BPP	13.4 ± 0.1 ^{c}	11.8 ± 0.2 ^{d}	14.1 ± 1.1 ^{b}	14.9 ± 0.61 ^{a}
	TPP	32	30.5	41.7	59.2
	% Bioaccessibility	58.1%	61.3%	66.2%	74.8%
p-hydroxybenzoic acid	FPP	25.4 ± 0.2 ^{a}	ND	14.8 ± 0.1 ^{b}	ND
	BPP	21.6 ± 0.1 ^{a}	ND	1.5 ± 0.1 ^{b}	ND
	TPP	47	-	16.3	-
	% Bioaccessibility	54.0%	-	90.8%	-
Epicatechin	FPP	11.6 ± 0.2 ^{a}	5.2 ± 0.1 ^{d}	7.3 ± 0.1 ^{b}	6.5 ± 0.2 ^{c}
	BPP	8.3 ± 0.1 ^{a}	6.9 ± 0.2 ^{b}	5.2 ± 0.1 ^{c}	4.9 ± 0.1 ^{d}
	TPP	19.9	12.1	12.5	11.4
	% Bioaccessibility	58.3%	42.9%	58.4%	57.0%
Epicatechin gallate	FPP	ND	13.4 ± 0.1 ^{b}	ND	14.2 ± 0.1 ^{a}
	BPP	ND	13.9 ± 0.1 ^{a}	ND	12.6 ± 0.1 ^{b}
	TPP	-	27.3	-	26.8
	% Bioaccessibility	-	49.1%	-	52.9%
Quercetin-3-galactoside	FPP	ND	13.3 ± 0.3 ^{b}	ND	15.1 ± 0.1 ^{a}
	BPP	ND	22.2 ± 0.1 ^{a}	ND	10.3 ± 0.1 ^{b}
	TPP	-	35.5	-	25.4
	% Bioaccessibility	-	37.5%	-	59.4%
Quercetin-3-rhamnoside	FPP	5.5 ± 0.1 ^{a}	ND	4.9 ± 0.1 ^{b}	ND
	BPP	38.2 ± 0.3 ^{a}	ND	3.8 ± 0.1 ^{b}	ND
	TPP	43.7	-	8.7	-
	% Bioaccessibility	12.6%	-	56.3%	-
Kaempferol-3-glucoside	FPP	ND	12.2 ± 0.1 ^{b}	ND	48.9 ± 0.1 ^{a}
	BPP	ND	21.1 ± 0.2 ^{a}	ND	14.2 ± 0.1 ^{b}
	TPP	-	33.3	-	63.1
	% Bioaccessibility	-	36.6%	-	77.5%

Results are reported as mean ± SD of three analyses (n = 3). FPP–Free polyphenols (released polyphenols) in the supernatant of SGID; BPP–Bound polyphenols (insoluble polyphenols) in the precipitate of SGID; TPP (total polyphenols); ND–Not detected. Different letters ^{a}, ^{b}, ^{c}, and ^{d} indicate significant differences in rows ($p \leq 0.05$).

3.3. Individual Polyphenols Identified and Quantified after Cecal Fermentation

As the absorption of polyphenols is low in the intestinal tract of chicken and these compounds pass to the ceca where they are exposed to degradation by the gut microbiota, analysis of polyphenols was performed after cecal fermentation of chicory and lucerne. HPLC chromatograms of soluble fermented fractions of chicory are shown in Figure 4 and that of lucerne are shown in Figure 5. Three polyphenols were quantified after cecal fermentation of lucerne, whereas two polyphenols were identified after cecal fermentation of chicory. The polyphenols identified in fermented samples include gallic acid, pyrogallol, and procyanidin B2, and are presented in Table 3.

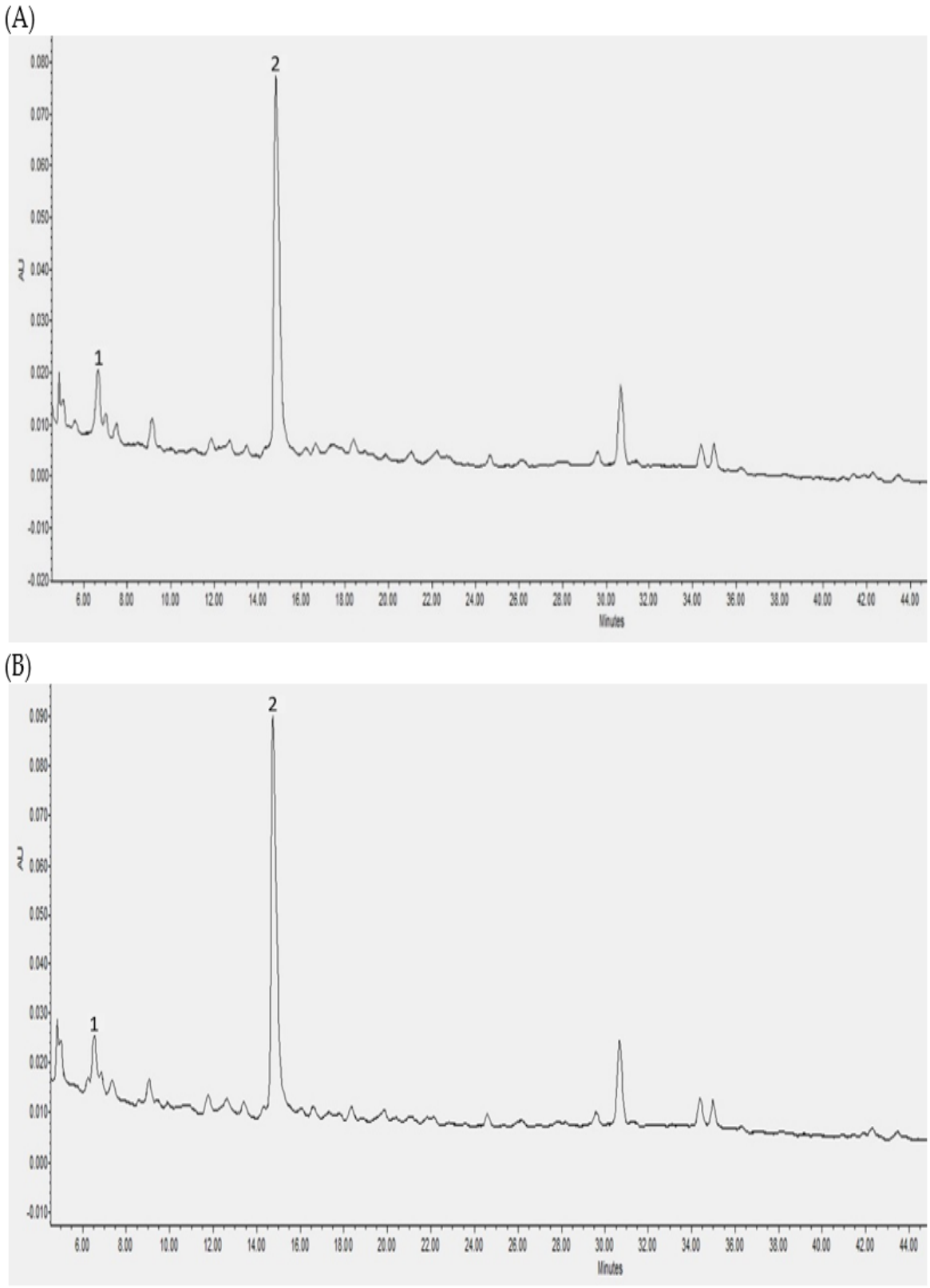

Figure 4. HPLC chromatograms of fermented fractions of chicory; (**A**) Soluble fraction of chicory after 12 h fermentation, (**B**) Soluble fraction of chicory after 24h fermentation. Polyphenols identified are (1) Pyrogallol, and (2) Procyanidin B2.

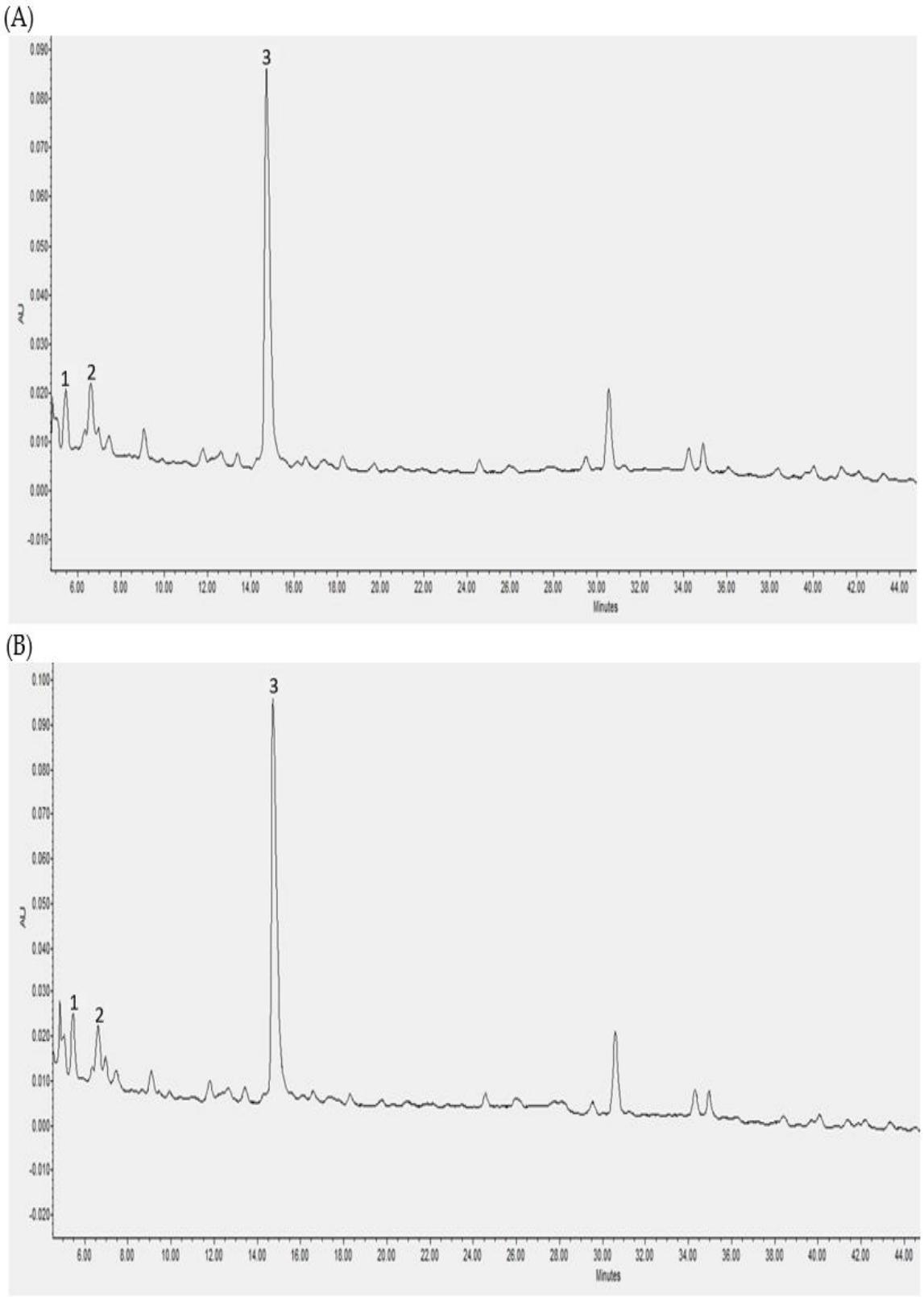

Figure 5. HPLC chromatograms of fermented fractions of lucerne; (**A**) Soluble fraction of lucerne after 12h fermentation, (**B**) Soluble fraction of lucerne after 24 h fermentation. Polyphenols identified are (1) Gallic acid, (2) Pyrogallol, and (3) Procyanidin B2.

Table 3. Contents of individual polyphenols after 12 h or 24 h cecal fermentation of chicory and lucerne.

Compounds	Fermentation 12 h		Fermentation 24 h	
	Chicory (μg/g)	Lucerne (μg/g)	Chicory (μg/g)	Lucerne (μg/g)
Gallic acid	-	21.6 ± 0.2 [b]	-	29.9 ± 2.8 [a]
Pyrogallol	191.6 ± 16.5 [c]	186.2 ± 2.8 [d]	300.6 ± 22.7 [b]	317.8 ± 8.1 [a]
Procyanidin B2	158.6 ± 1.8 [d]	176.0 ± 0.2 [b]	173.1 ± 0.7 [c]	200.4 ± 5.1 [a]

Results are reported as mean ± SD of three analyses (n = 3) on a dry weight basis. Different letters [a], [b], [c], and [d] indicate significant differences in rows ($p \leq 0.05$).

Table 3 shows that majority of the polyphenols identified in the pre-cecal gastrointestinal fractions of chicory and lucerne were not detected in the samples after cecal fermentation. This could be because either these polyphenols were degraded by microbial action or transformed into their microbial metabolites. After cecal fermentation, gallic acid was only identified and quantified in the soluble fraction of lucerne. The concentration of gallic acid was 21.6 ± 0.2 μg/g and 29.9 ± 2.8 μg/g after 12 h and 24 h of cecal fermentation, respectively. The concentration of procyanidin B2 was 158.6 ± 1.8 μg/g and 173.1 ± 0.7 μg/g in the absorbable fraction of chicory as compared to 176.0 ± 0.2 μg/g and 200.4 ± 5.1 μg/g in an absorbable fraction of lucerne after 12 and 24 h of cecal fermentation, respectively.

Pyrogallol and procyanidin B2 were detected in both chicory and lucerne. However, the concentration of both these compounds was higher for lucerne than chicory after 24 h of cecal fermentation. The concentration of pyrogallol was 191.6 ± 16.5 μg/g and 300.6 ± 22.7 μg/g in an absorbable fraction of chicory and 186.2 ± 2.8 μg/g and 317.8 ± 8.1 μg/g in the absorbable fraction of lucerne after 12 h and 24 h of cecal fermentation, respectively.

3.4. Antioxidant Activities of Chicory and Lucerne during In Vitro Digestion and Cecal Fermentation

Antioxidant activities of gastrointestinal digestive and fermented fractions of chicory and lucerne were determined by ABTS, DPPH, and reducing power ability (RPA) (Table 4). Undigested lucerne showed higher antioxidant activities than chicory determined in terms of ABTS, DPPH, and RPA. However, chicory showed higher antioxidant activities than lucerne after in vitro gastrointestinal digestion and cecal fermentation. The undigested lucerne showed the strongest antioxidant activity determined in terms of ABTS. However, the ABTS value of lucerne reduced significantly after different stages of in vitro gastrointestinal digestion and cecal fermentation. The ABTS value for chicory also showed a moderate decrease after the gastric phase of digestion, then a slight increase after the intestinal phase of digestion, and finally, showed a slight decrease after cecal fermentation. Overall, the ABTS value was higher for lucerne than chicory after the intestinal digestive phase and cecal fermentation but lower than the chicory after the gastric phase of digestion. ABTS values for chicory and lucerne were 0.224 ± 0.003 mg AAE/g and 0.163 ± 0.002 mg AAE/g after gastric phase, 0.303 ± 0.017 mg AAE/g and 0.362 ± 0.022 mg AAE/g after intestinal phase, 0.192 ± 0.008 mg AAE/g and 0.258 ± 0.009 mg AAE/g after 12 h of cecal fermentation, and 0.237 ± 0.043 mg AAE/g and 0.371 ± 0.027 mg AAE/g after 24 h of cecal fermentation, respectively.

Table 4. Antioxidant activities of undigested chicory and lucerne and their digestive fractions.

Antioxidant Activity	ABTS (mg AAE/g)		DPPH (mg AAE/g)		RPA (mg AAE/g)	
	Chicory	Lucerne	Chicory	Lucerne	Chicory	Lucerne
Undigested	0.300 ± 0.010^{a}	1.260 ± 0.020^{a}	0.123 ± 0.010^{b}	0.132 ± 0.010^{ab}	0.347 ± 0.010^{b}	0.573 ± 0.020^{a}
Soluble gastric fraction	0.224 ± 0.003^{b}	0.163 ± 0.002^{d}	0.118 ± 0.008^{b}	0.079 ± 0.003^{bc}	0.300 ± 0.012^{bc}	0.160 ± 0.005^{cd}
Soluble intestinal fraction	0.303 ± 0.017^{a}	0.362 ± 0.022^{b}	0.038 ± 0.001^{c}	0.026 ± 0.001^{cd}	0.639 ± 0.078^{a}	0.300 ± 0.007^{b}
Soluble fermented fraction (12 h)	0.192 ± 0.008^{bc}	0.258 ± 0.009^{c}	0.195 ± 0.014^{a}	0.164 ± 0.010^{a}	0.236 ± 0.008^{de}	0.135 ± 0.007^{cd}
Soluble fermented fraction (24 h)	0.237 ± 0.043^{b}	0.371 ± 0.027^{b}	0.194 ± 0.036^{a}	0.167 ± 0.002^{a}	0.221 ± 0.008^{de}	0.202 ± 0.002^{c}

Results are reported as mean ± SD of three analyses (n = 3). Different letters ^{a,b,c,d} and ^{e} indicate significant difference in columns ($p \leq 0.05$).

Undigested lucerne showed a higher value for DPPH as compared to chicory. However, after gastrointestinal digestion and cecal fermentation, the DPPH value remained higher for chicory than lucerne. The DPPH value for lucerne was reduced significantly after gastric and intestinal digestion but increased significantly after cecal fermentation. Considering 12 h and 24 h timepoints during cecal fermentation, no significant difference was observed in DDPH values of lucerne. Similarly, there was no difference in DPPH values for chicory at 12 h and 24 h timepoints of cecal fermentation. However, DPPH values were significantly higher after cecal fermentation of chicory than the DPPH values of undigested chicory and gastrointestinal digestive phases. The DPPH values for chicory and lucerne were 0.118 ± 0.008 mg AAE/g and 0.079 ± 0.003 mg AAE/g after gastric phase of digestion, 0.038 ± 0.001 mg AAE/g and 0.026 ± 0.001 mg AAE/g after intestinal phase of digestion, 0.195 ± 0.014 mg AAE/g and 0.164 ± 0.010 mg AAE/g after 12 h of cecal fermentation, and 0.194 ± 0.036 mg AAE/g and 0.167 ± 0.002 mg AAE/g after 24 h of cecal fermentation, respectively. The results indicated a similar trend for DPPH values of chicory and lucerne during cecal fermentation of the samples.

The reducing power of undigested lucerne was higher than undigested chicory. However, the reducing power of gastrointestinal digestive fractions of chicory was higher than the digestive fractions of lucerne. Similarly, the reducing power was higher for chicory than lucerne after 12 h and 24 h of cecal fermentation. The reducing power of chicory after the intestinal phase of digestion (0.639 ± 0.078 mg AAE/g) was the highest among all the gastrointestinal digestive fractions of both chicory and lucerne. The reducing power of lucerne was lower (0.300 ± 0.007 mg AAE/g) than chicory after the intestinal phase of digestion. The reducing power of lucerne was also significantly lower (0.160 ± 0.005 mg AAE/g) in comparison to that of chicory (0.300 ± 0.012 mg AAE/g) after the gastric phase of digestion. Similarly, the reducing power of lucerne was lower after 12 h (0.135 ± 0.007 mg AAE/g) and 24 h (0.202 ± 0.002 mg AAE/g) of cecal fermentation in comparison to that of chicory which showed 0.236 ± 0.008 mg AAE/g and 0.221 ± 0.008 mg AAE/g after 12 h and 24 h of cecal fermentation.

3.5. Gas Production with Chicory and Lucerne during In Vitro Cecal Fermentation

In vitro gas production provides an effective method for the assessment of feed quality and there has been an increasing interest in in vitro gas production techniques recently. Therefore, we measured total gas production over 24 h during cecal fermentation of chicory and lucerne and the results are presented in Figure 6.

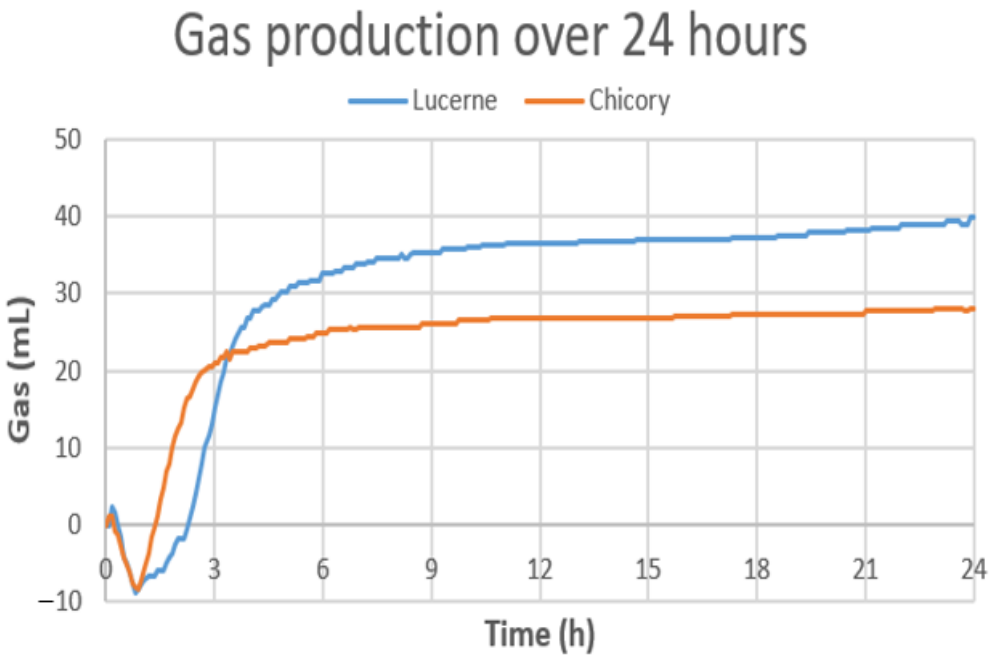

Figure 6. Gas production during cecal fermentation of chicory and lucerne.

Figure 6 shows that gas production started slightly earlier with chicory as compared to lucerne. This could be indicative of more rapid digestion of chicory. The gas production rate was considerably higher for both samples during the first three hours of cecal fermentation as indicated by the sharp rise in the curve. The gas production was 20.9 mL for chicory during the first three hours as compared to 15.3 mL for lucerne. After 3 h of fermentation, the gas production rate decreased significantly for chicory. However, gas production continued to increase, but the gas production rate was higher for lucerne as the volume of gas produced was much higher for lucerne (32.6 mL) compared to the volume of gas produced for chicory (24.9 mL) after 6 h of fermentation. The trend continued for 12 h of fermentation as gas production reached 36.6 mL and 26.7 mL for lucerne and chicory, respectively. After 12 h of fermentation, gas production plateaued indicating a decrease in gas production rate for both the samples. Overall, the gas production was significantly higher for lucerne (39.9 mL) than chicory (28.1 mL). This indicated the presence of higher fermentable substrates in lucerne as compared to chicory.

3.6. Short-chain Fatty Acids Production during In Vitro Cecal Fermentation of Chicory and Lucerne

Production of SCFAs has gained enormous importance in recent years. Therefore, the production of major SCFAs (acetic acid, propionic acid, and butyric acid) during cecal fermentation of chicory and lucerne were studied and the results of the analysis are presented in Table 5. Results showed that chicory and lucerne increased the production of SCFAs during in vitro cecal fermentation. The production of acetic acid was significantly higher with chicory and lucerne than in the control. The difference in the production of acetic acid during cecal fermentation of chicory (18.12 ± 0.05 mmol L^{-1}) and lucerne (18.25 ± 0.02 mmol L^{-1}) was not significant after 12 h of fermentation. However, this difference was significant after 24 of fermentation (Table 5). The total production of acetic acid (acetate) during 24 h of cecal fermentation was higher for lucerne (24.85 ± 0.01 mmol L^{-1}) as compared to chicory (22.56 ± 0.01 mmol L^{-1}).

Table 5. Production of short-chain fatty acids during cecal fermentation of chicory and lucerne.

SCFAs	Ctrl	Chicory		Lucerne	
		12 h	24 h	12 h	24 h
Acetic acid (mmol L^{-1})	7.35 ± 0.09 ^{a}	18.12 ± 0.05 ^{bc}	22.56 ± 0.01 ^{d}	18.25 ± 0.02 ^{bc}	24.85 ± 0.01 ^{e}
Propionic acid (mmol L^{-1})	1.40 ± 0.01 ^{a}	1.95 ± 0.01 ^{bc}	2.75 ± 0.01 ^{d}	1.96 ± 0.01 ^{bc}	2.97 ± 0.01 ^{e}
Butyric acid (mmol L^{-1})	4.79 ± 0.02 ^{a}	8.74 ± 0.01 ^{b}	12.83 ± 0.01 ^{d}	9.19 ± 0.01 ^{c}	14.42 ± 0.01 ^{e}
Total SCFA (mmol L^{-1})	13.54 ^{a}	28.81 ^{bc}	38.14 ^{d}	29.40 ^{bc}	42.24 ^{e}

Results are reported as mean ± SD of three analyses (n = 3). Different letters ^{a}, ^{b}, ^{c}, ^{d} and ^{e} indicate significant differences in rows ($p \leq 0.05$). SCFAs (short-chain fatty acids); Ctrl (control).

The production of propionic acid was slightly higher during cecal fermentation of chicory and lucerne than in the control. However, no difference was observed in the concentration of propionic acid after 12 h of cecal fermentation with chicory (1.95 ± 0.01 mmol L^{-1}) and lucerne (1.96 ± 0.01 mmol L^{-1}). A slight increase in the concentration of propionic acid was recorded with lucerne (2.97 ± 0.01 mmol L^{-1}) in comparison to chicory (2.75 ± 0.01 mmol L^{-1}) after 24 h of cecal fermentation. The production of butyric acid was higher during cecal fermentation of lucerne (14.42 ± 0.01 mmol L^{-1}) when compared with chicory (12.83 ± 0.01 mmol L^{-1}) after 24 h of fermentation

3.7. Modulatory Effects of Chicory and Lucerne on Gut Microbiota

Gut microbiota has pivotal involvement in the digestion of feed and absorption of nutrients and is markedly influenced by the type of feed. Therefore, feed is considered the single most crucial factor affecting the gut microbiota because it provides substrates for the growth of microbial communities inhabiting the gut. We studied the effects of chicory and lucerne in the modulation of gut microbiota and the results are shown in Figure 7.

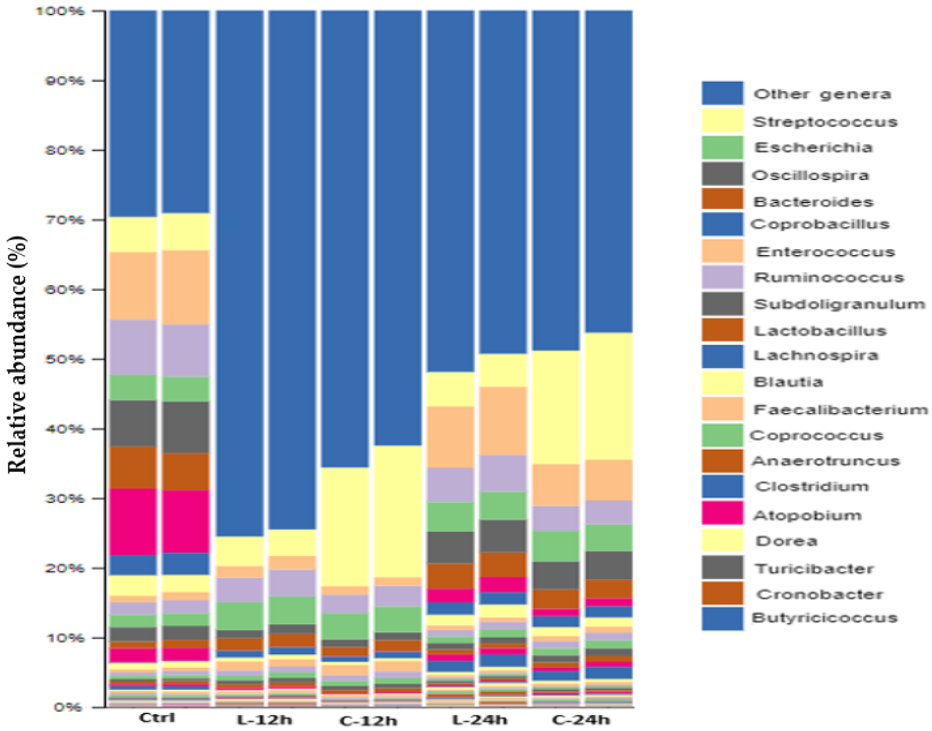

Figure 7. Effect of chicory and lucerne on the relative abundance of microbial genera. Ctrl: control; L-12 h: 12-h fermentation with lucerne; L-24 h: 24-h fermentation with lucerne; C-12 h: 12-h fermentation with chicory; C-24 h: 24-h fermentation with chicory.

Chicory and lucerne differentially modulated the cecal microbiota of broilers. The relative abundance of the genus *Enterococcus* decreased after 24 h fermentation with chicory

(0.838%) and lucerne (0.687%) as compared to the control (1.067%). The relative abundance of the genus *Streptococcus* increased after 12 h (17.981%) and 24 h (17.267%) fermentation with chicory as compared to the control (5.204%). However, lucerne decreased the relative abundance of the genus *Streptococcus* to 4.029% after 12 h fermentation and 4.845% after 24 h fermentation as compared to the control (5.204%). The relative abundance of genus *Clostridium* was decreased after 24h fermentation with chicory (0.225%) and lucerne (0.176%) as compared to the control (0.550%). When compared, lucerne was more effective in reducing the relative abundance of genus *Clostridium* than chicory. The relative abundance of genus *Escherichia* was slightly increased after fermentation with chicory (4.123%) and lucerne (4.128%) after 24 h fermentation as compared to the control (3.562%). However, no significant difference in the relative abundance of genus *Escherichia* was observed when the effects of chicory and lucerne fermentation were compared.

Lucerne fermentation had very little effect on the relative abundance of the genus *Butyricicoccus* (0.169%) after 24 h as compared to control (0.162%). However, chicory slightly reduced the relative abundance of the genus *Butyricicoccus* (0.116%). Relative abundance of genus *Oscillospira* was also reduced by chicory (4.044%) and lucerne (4.634%) after 24 h fermentation as compared to control (7.059%). However, the suppression of genus *Oscillospira* was more pronounced after 12 h fermentation with both chicory (1.088%) and lucerne (1.262%). The other genus affected by chicory and lucerne fermentation was *Ruminococcus*. The relative abundance of this genus was 1.076% with chicory and 0.997% with lucerne after 24 h fermentation.

4. Discussion

The evaluation of the bioaccessibility of polyphenols has gained increasing attention in recent years because of their paramount importance in exerting their potent health benefits. Bioaccessibility reflects the amount of a compound released after gastrointestinal digestion [31]. It provides a measure of the amount of a particular compound that can enter the circulation system to exert its bioactive functions [31]. The role of the gastrointestinal tract is very important in modulating bioaccessibility and subsequent absorption. However, variations in chemical and enzymatic conditions in the gastrointestinal tract affect the bioaccessibility of polyphenols [31]. Differences in diversity and concentration of these phenolic compounds and their distribution in plant matrices also influence their release from plant matrices and affect their bioaccessibility [32]. Therefore, significant variations in the bioaccessibility of individual polyphenols from chicory and lucerne were observed during their gastrointestinal digestion in an in vitro model of chicken gut. The bioaccessibility of polyphenols varied from 12.6% to 90.8%. *p*-hydroxybenzoic acid showed the highest bioaccessibility (90.8%) from lucerne. This compound exhibited antimicrobial activity against both *Escherichia coli* and *Staphylococcus aureus* [33]. This could explain the beneficial effects of lucerne on the modulation of broiler microbiota.

Catechin reduces lipid peroxidation and improves reproductive performance and meat quality in poultry [34]. The bioaccessibility of catechin was higher from lucerne (74.8%) than chicory (66.2%) implying the superiority of lucerne as a poultry feed supplement over chicory. Epicatechin is an important phenolic compound that delays skeletal muscle degeneration and improves survival [35]. This compound was bioaccessible from both chicory and lucerne which implies that chicory and lucerne can improve muscle degeneration and survival rate. Epicatechin gallate improves growth performance and helps to ameliorate oxidative damage in heat-stressed broilers [36]. The release of this compound from lucerne during gastrointestinal digestion in broilers could explain the effectiveness of lucerne in amelioration of oxidative stress in broilers. These polyphenols also strengthen the antioxidant defense by scavenging free radicals and reactive oxygen species (ROS). However, their health effects and physiological functions depend on their bioaccessibility [37]. As evident from the results, wide variation was observed in the bioaccessibility of polyphenols after in vitro gastrointestinal digestion of chicory and lucerne which contributed to the significant difference in antioxidant activities of digestive fractions of chicory and lucerne.

Owing to this variation in the bioaccessibility of polyphenols and the resulting bioactivities, chicory and lucerne exhibited differential effects on the modulation of cecal microbiota. The study showed that the relative abundance of various genera was affected by chicory and lucerne. Mainly, chicory and lucerne suppressed the growth of pathogens while supporting the growth of beneficial microbes. This is in agreement with the available literature that reports similar effects on the gut microbiota of chicken upon supplementation with chicory [5] and lucerne [38]. These genera include *Streptococcus, Clostridium, Enterococcus,* and *Butyricicoccus*. Chicory increased the relative abundance of *Streptococcus*. Conversely, lucerne decreased the relative abundance of *Streptococcus*. This genus is associated with reduced productive performance in poultry [39,40]. *Streptococcus* can deconjugate bile acids and the deconjugation of bile acids can decrease the digestion of lipids in broilers [41]. This implies that chicory can decrease lipid digestion in broilers. *Clostridium* is an important genus in the gut of poultry. Members of this genus especially *Clostridium perfringens are* responsible for necrotic enteritis in broilers that leads to sudden death [42]. Both chicory and lucerne reduced the relative abundance of the *Clostridium genus*, implying that supplementation of broiler feed with chicory and lucerne could help in reducing necrotic enteritis in poultry. The decrease in the *Clostridium genus* with lucerne supplementation agrees with a previous report that presented similar findings [38]. Another important genus affected by chicory and lucerne was *Butyricicoccus*. It improves the production performance of poultry and has beneficial effects on the microbiome due to the production of butyrate [43]. The relative abundance of *Butyricicoccus* was increased with lucerne and decreased with chicory after 24 h fermentation. These results indicated that lucerne could have better potential to positively modulate the gut microbiota of broilers than chicory.

Microbial populations inhabiting the cecum are also affected by the presence of fermentable substrates in the feeds and in vitro gas production technique is a reliable technique for the estimation of fermentable substrates in feeds. This is also advantageous over the in vivo studies because it is relatively inexpensive, easy to perform, and gives reliable results [44]. The presence of the fermentable substrates was studied by measuring the gas production over 24 h cecal fermentation. Gas production was higher with lucerne as compared to chicory. The higher gas production with lucerne could be attributed to the presence of slow-releasing carbohydrates [1] that served as fermentation substrates for the microbes for a longer duration. This resulted in higher production of short-chain fatty acids (SCFAs) during cecal fermentation of lucerne (42.24 mmol L^{-1}) as compared to chicory (38.14 mmol L^{-1}) over 24 h period.

SCFAs are linked with various positive physiological effects on gut health. SCFAs also exhibit probiotic and antibiotic activities and contribute to the nutrition and immune health of the host [45]. Production of three main SCFAs namely acetic acid, propionic acid, and butyric acid was studied (Table 5). Acetic acid is produced by *lactobacillus* species and is useful in the control of gastroenteritis by reducing the population of members of *Campylobacter* genera [46]. It acts as a substrate for peripheral adipogenesis by entering the liver [47]. Acetic acid also helps to reduce the pH in the gastrointestinal tract [48]. Propionic acid is produced by *Phascolarctobacteria* that are capable of fermenting monosaccharides into propionate [47]. Propionate can enter the liver and act as a substrate for gluconeogenesis [47]. Butyric acid is the most important short-chain fatty acid, and its production was significantly higher with the addition of both chicory and lucerne as compared to the control. Butyric acid is also well known for its nutritional properties for the epithelial cell and pathogen inhibitory effects in the gastrointestinal tract [49]. It acts as the major energy source for enterocytes and is essential to the health of intestinal mucosa [50]. It provides energy to the epithelial cells of the colon. Butyric acid is considered essential for intestinal health as it plays a significant role in the development of intestinal epithelium [51,52]. Besides, butyric acid exhibits bacteriostatic and bactericidal properties against pathogenic bacteria that helps to maintain the normal immunomodulatory response in poultry [46,53]. Owing to its antimicrobial properties, butyrate is linked with reduced colonization of pathogenic *Salmonella* in the cecum of chicken [54,55]. The higher production of butyric

acid with lucerne compared to chicory also augments lucerne's superiority over chicory for poultry feed supplementation.

5. Conclusions

Chicory and lucerne exhibited inhibitory effects against pathogenic microorganisms and positively modulated the cecal microbiota. Both the plants also enhanced the production of short-chain fatty acids contributing to improved nutrition. Therefore, chicory and lucerne supplementation in feed could be beneficial for poultry. By comparison, lucerne proved more effective in positive manipulation of the broiler microbiota. Lucerne also exhibited the presence of more fermentable substrates in comparison to chicory. Hence, it could be concluded that lucerne possesses a higher potential to be exploited as a feed supplement for Poultry. However, further investigations are recommended as a next step to confirm these effects in in vivo trials.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation8050237/s1. Figure S1: HPLC chromatograms of insoluble fractions of chicory; (A) Insoluble gastric fraction (extracted from the precipitate), (B) Insoluble intestinal fraction (extracted from the precipitate). Figure S2: HPLC chromatograms of insoluble fractions of lucerne; (A) Insoluble gastric fraction (extracted from the precipitate), (B) Insoluble intestinal fraction (extracted from the precipitate).

Author Contributions: Conceptualization, methodology, and validation, Y.I., F.R.D., E.N.P., and J.J.C.; investigation, Y.I., H.H.L. and O.A.; resources, F.R.D., E.N.P. and J.J.C.; writing—original draft preparation, Y.I.; writing—review and editing, F.R.D., J.J.C., E.N.P., S.K.M., and J.L.J.; supervision, F.R.D. and J.J.C.; and funding acquisition, F.R.D. and J.J.C. project administration, F.R.D. and J.J.C. All authors have read and agreed to the published version of the manuscript.

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

References

1. Mielmann, A. The utilisation of lucerne (*Medicago sativa*): A review. *Br. Food J.* **2013**, *115*, 590–600. [CrossRef]
2. Iqbal, Y.; Cottrell, J.J.; Suleria, H.A.R.; Dunshea, F.R. Gut microbiota-polyphenol interactions in chicken: A review. *Animals* **2020**, *10*, 1391. [CrossRef] [PubMed]
3. Nwafor, I.; Shale, K.; Achilonu, M. Chemical composition and nutritive benefits of chicory (*Cichorium intybus*) as an ideal complementary and/or alternative livestock feed supplement. *Sci. World J.* **2017**, *2017*, 1–11. [CrossRef] [PubMed]
4. Faramarzzadeh, M.; Behroozlak, M.; Samadian, F.; Vahedi, V. Effects of chicory powder and butyric acid combination on performance, carcass traits and some blood parameters in broiler chickens. *Iran. J. Appl. Anim. Sci.* **2017**, *7*, 139–145.
5. Khoobani, M.; Hasheminezhad, S.H.; Javandel, F.; Nosrati, M.; Seidavi, A.; Kadim, I.T.; Laudadio, V.; Tufarelli, V. Effects of dietary chicory (*Chicorium intybus* L.) and probiotic blend as natural feed additives on performance traits, blood biochemistry, and gut microbiota of broiler chickens. *Antibiotics* **2019**, *9*, 5. [CrossRef]
6. Gaskins, H.R.; Collier, C.T.; Anderson, D.B. Antibiotics as growth promotants: Mode of action. *Anim. Biotechnol.* **2002**, *13*, 29–42. [CrossRef]
7. Jeurissen, S.H.; Lewis, F.; van der Klis, J.D.; Mroz, Z.; Rebel, J.M.; ter Huurne, A.A. Parameters and techniques to determine intestinal health of poultry as constituted by immunity, integrity, and functionality. *Curr. Issues Intest. Microbiol.* **2002**, *3*, 1–14.
8. Kohl, K.D. Diversity and function of the avian gut microbiota. *J. Comp. Physiol. B* **2012**, *182*, 591–602. [CrossRef]

9. Iqbal, Y.; Ponnampalam, E.N.; Suleria, H.A.; Cottrell, J.J.; Dunshea, F.R. LC-ESI/QTOF-MS profiling of chicory and lucerne polyphenols and their antioxidant activities. *Antioxidants* **2021**, *10*, 932. [CrossRef]
10. Brenes, A.; Viveros, A.; Goni, I.; Centeno, C.; Sayago-Ayerdy, S.G.; Arija, I.; Saura-Calixto, F. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poult. Sci.* **2008**, *87*, 307–316. [CrossRef]
11. Tuohy, K.M.; Conterno, L.; Gasperotti, M.; Viola, R. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. *J. Agric. Food Chem.* **2012**, *60*, 8776–8782. [CrossRef] [PubMed]
12. Espín, J.C.; González-Sarrías, A.; Tomás-Barberán, F.A. The gut microbiota: A key factor in the therapeutic effects of (poly) phenols. *Biochem. Pharmacol.* **2017**, *139*, 82–93. [CrossRef] [PubMed]
13. Abd El-Hack, M.E.; Mahgoub, S.A.; Hussein, M.M.; Saadeldin, I.M. Improving growth performance and health status of meat-type quail by supplementing the diet with black cumin cold-pressed oil as a natural alternative for antibiotics. *Environ. Sci. Pollut. Res.* **2018**, *25*, 1157–1167. [CrossRef] [PubMed]
14. Valenzuela-Grijalva, N.V.; Pinelli-Saavedra, A.; Muhlia-Almazan, A.; Domínguez-Díaz, D.; González-Ríos, H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J. Anim. Sci. Technol.* **2017**, *59*, 8. [CrossRef]
15. Surai, P. Polyphenol compounds in the chicken/animal diet: From the past to the future. *J. Anim. Physiol. Anim. Nutr.* **2014**, *98*, 19–31. [CrossRef] [PubMed]
16. Abbas, M.; Saeed, F.; Anjum, F.M.; Afzaal, M.; Tufail, T.; Bashir, M.S.; Ishtiaq, A.; Hussain, S.; Suleria, H.A.R. Natural polyphenols: An overview. *Int. J. Food Prop.* **2017**, *20*, 1689–1699. [CrossRef]
17. Sang, V.T.; Dai Hung, N.; Se-Kwon, K. Pharmaceutical properties of marine polyphenols: An overview. *Acta Pharm. Sci.* **2019**, *57*, 217–242. [CrossRef]
18. Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S–2085S. [CrossRef]
19. Hernandez-Patlan, D.; Solis-Cruz, B.; Méndez-Albores, A.; Latorre, J.D.; Hernandez-Velasco, X.; Tellez, G.; López-Arellano, R. Comparison of PrestoBlue® and plating method to evaluate antimicrobial activity of ascorbic acid, boric acid and curcumin in an in vitro gastrointestinal model. *J. Appl. Microbiol.* **2018**, *124*, 423–430. [CrossRef]
20. Latorre, J.D.; Hernandez-Velasco, X.; Kuttappan, V.A.; Wolfenden, R.E.; Vicente, J.L.; Wolfenden, A.D.; Bielke, L.R.; Prado-Rebolledo, O.F.; Morales, E.; Hargis, B.M.; et al. Selection of *Bacillus* spp. for cellulase and xylanase production as direct-fed microbials to reduce digesta viscosity and *Clostridium perfringens* proliferation using an in vitro digestive model in different poultry diets. *Front. Vet. Sci.* **2015**, *2*, 25. [CrossRef]
21. Karunaratne, N.D.; Abbott, D.A.; Chibbar, R.N.; Hucl, P.J.; Pozniak, C.J.; Classen, H.L. In vitro assessment of the starch digestibility of western Canadian wheat market classes and cultivars. *Can. J. Anim. Sci.* **2018**, *98*, 463–476. [CrossRef]
22. Lei, F.; Yin, Y.; Wang, Y.; Deng, B.; Yu, H.D.; Li, L.; Xiang, C.; Wang, S.; Zhu, B.; Wang, X. Higher-level production of volatile fatty acids in vitro by chicken gut microbiotas than by human gut microbiotas as determined by functional analyses. *Appl. Environ. Microbiol.* **2012**, *78*, 5763–5772. [CrossRef] [PubMed]
23. Fu, S.; Augustin, M.A.; Shen, Z.; Ng, K.; Sanguansri, L.; Ajlouni, S. Bioaccessibility of curcuminoids in buttermilk in simulated gastrointestinal digestion models. *Food Chem.* **2015**, *179*, 52–59. [CrossRef] [PubMed]
24. Menke, K.H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Develop.* **1988**, *28*, 7–55.
25. Iqbal, Y.; Ponnampalam, E.N.; Cottrell, J.J.; Suleria, H.A.; Dunshea, F.R. Extraction and characterization of polyphenols from non-conventional edible plants and their antioxidant activities. *Food Res. Int.* **2022**, *157*, 111205. [CrossRef]
26. Gu, C.; Suleria, H.A.R.; Dunshea, F.R.; Howell, K. Dietary lipids influence bioaccessibility of polyphenols from black carrots and affect microbial diversity under simulated gastrointestinal digestion. *Antioxidants* **2020**, *9*, 762. [CrossRef]
27. Huang, C.-B.; Xiao, L.; Xing, S.-C.; Chen, J.-Y.; Yang, Y.-W.; Zhou, Y.; Chen, W.; Liang, J.-B.; Mi, J.-D.; Wang, Y. The microbiota structure in the cecum of laying hens contributes to dissimilar H_2S production. *BMC Genom.* **2019**, *20*, 770. [CrossRef]
28. Bolyen, E.; Rideout, J.R.; Dillon, M.R.; Bokulich, N.A.; Abnet, C.C.; Al-Ghalith, G.A.; Alexander, H.; Alm, E.J.; Arumugam, M.; Asnicar, F.; et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* **2019**, *37*, 852–857. [CrossRef]
29. Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
30. Bokulich, N.A.; Dillon, M.R.; Zhang, Y.; Rideout, J.R.; Bolyen, E.; Li, H.; Albert, P.S.; Caporaso, J.G. q2-longitudinal: Longitudinal and paired-sample analyses of microbiome data. *MSystems* **2018**, *3*, e00219-00218. [CrossRef]
31. Ed Nignpense, B.; Francis, N.; Blanchard, C.; Santhakumar, A.B. Bioaccessibility and bioactivity of cereal polyphenols: A review. *Foods* **2021**, *10*, 1595. [CrossRef] [PubMed]
32. Mrduljaš, N.; Krešić, G.; Bilušić, T. Polyphenols: Food sources and health benefits. In *Functional Food-Improve Health Through Adequate Food*; Hueda, M.C., Ed.; IntechOpen: London, UK, 2017; pp. 23–41.
33. Chaudhary, J.; Jain, A.; Manuja, R.; Sachdeva, S. A comprehensive review on biological activities of p-hydroxy benzoic acid and its derivatives. *Int. J. Pharm. Sci. Rev. Res.* **2013**, *22*, 109–115.
34. Kara, K.; Guclu, B.K.; Senturk, M.; Eren, M.; Baytok, E. Effects of catechin and copper or their combination in diet on productive performance, egg quality, egg shelf-life, plasma 8-OHdG concentrations and oxidative status in laying quail (*Coturnix coturnix japonica*). *J. Appl. Anim. Res.* **2021**, *49*, 97–103. [CrossRef]

35. Si, H.; Wang, X.; Zhang, L.; Parnell, L.D.; Admed, B.; LeRoith, T.; Ansah, T.-A.; Zhang, L.; Li, J.; Ordovás, J.M.; et al. Dietary epicatechin improves survival and delays skeletal muscle degeneration in aged mice. *FASEB J.* **2019**, *33*, 965–977. [CrossRef] [PubMed]
36. Luo, J.; Song, J.; Liu, L.; Xue, B.; Tian, G.; Yang, Y. Effect of epigallocatechin gallate on growth performance and serum biochemical metabolites in heat-stressed broilers. *Poult. Sci.* **2018**, *97*, 599–606. [CrossRef]
37. Santos, D.I.; Saraiva, J.M.A.; Vicente, A.A.; Moldão-Martins, M. Methods for determining bioavailability and bioaccessibility of bioactive compounds and nutrients. In *Innovative Thermal and Non-Thermal Processing, Bioaccessibility and Bioavailability of Nutrients and Bioactive Compounds*; Barba, F.J., Saraiva, J.M.A., Cravotto, G., Lorenzo, J.M., Eds.; Woodhead Publishing: Sawston, UK, 2019; pp. 23–54.
38. Zheng, M.; Mao, P.; Tian, X.; Guo, Q.; Meng, L. Effects of dietary supplementation of alfalfa meal on growth performance, carcass characteristics, meat and egg quality, and intestinal microbiota in Beijing-you chicken. *Poult Sci* **2019**, *98*, 2250–2259. [CrossRef]
39. Han, G.G.; Kim, E.B.; Lee, J.; Lee, J.-Y.; Jin, G.; Park, J.; Huh, C.-S.; Kwon, I.-K.; Kil, D.Y.; Choi, Y.-J. Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *Springerplus* **2016**, *5*, 911. [CrossRef]
40. Johnson, T.J.; Youmans, B.P.; Noll, S.; Cardona, C.; Evans, N.P.; Karnezos, T.P.; Ngunjiri, J.M.; Abundo, M.C.; Lee, C.-W. A consistent and predictable commercial broiler chicken bacterial microbiota in antibiotic-free production displays strong correlations with performance. *Appl. Environ. Microbiol.* **2018**, *84*, e00362-00318. [CrossRef]
41. Pan, D.; Yu, Z. Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* **2014**, *5*, 108–119. [CrossRef]
42. Caly, D.L.; D'Inca, R.; Auclair, E.; Drider, D. Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: A microbiologist's perspective. *Front. Microbiol.* **2015**, *6*, 1336. [CrossRef]
43. Eeckhaut, V.; Wang, J.; Parys, A.; Haesebrouck, F.; Joossens, M.; Falony, G.; Raes, J.; Ducatelle, R.; Immerseel, F. The probiotic Butyricicoccus pullicaecorum reduces feed conversion and protects from potentially harmful intestinal microorganisms in broilers. *Front. Microbiol.* **2016**, *7*, 1416. [CrossRef] [PubMed]
44. Bovera, F.; Morra, F.; Di Meo, C.; Nizza, A. Use of the in vitro gas production technique to study feed digestibility in domesticated ostriches (*Struthio camelus* var. *domesticus*). *Ann. Anim. Sci.* **2006**, *16*, 769–777.
45. Polansky, O.; Sekelova, Z.; Faldynova, M.; Sebkova, A.; Sisak, F.; Rychlik, I. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Appl. Environ. Microbiol.* **2016**, *82*, 1569–1576. [CrossRef] [PubMed]
46. Jha, R.; Mishra, P. Dietary fiber in poultry nutrition and their effects on nutrient utilization, performance, gut health, and on the environment: A review. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 51. [CrossRef]
47. Sun, B.; Hou, L.; Yang, Y. The development of the gut microbiota and short-chain fatty acids of layer chickens in different growth periods. *Front. Vet. Sci.* **2021**, *8*, 699. [CrossRef]
48. Dunislawska, A.; Slawinska, A.; Stadnicka, K.; Bednarczyk, M.; Gulewicz, P.; Jozefiak, D.; Siwek, M. Synbiotics for broiler chickens—In vitro design and evaluation of the influence on host and selected microbiota populations following in ovo delivery. *PLoS ONE* **2017**, *12*, e0168587. [CrossRef]
49. Meimandipour, A.; Shuhaimi, M.; Soleimani, A.; Azhar, K.; Hair-Bejo, M.; Kabeir, B.; Javanmard, A.; Anas, O.M.; Yazid, A. Selected microbial groups and short-chain fatty acids profile in a simulated chicken cecum supplemented with two strains of Lactobacillus. *Poult. Sci.* **2010**, *89*, 470–476. [CrossRef]
50. Khatibjoo, A.; Mahmoodi, M.; Fattahnia, F.; Akbari-Gharaei, M.; Shokri, A.-N.; Soltani, S. Effects of dietary short-and medium-chain fatty acids on performance, carcass traits, jejunum morphology, and serum parameters of broiler chickens. *J. Appl. Anim. Res.* **2018**, *46*, 492–498. [CrossRef]
51. Panda, A.; Rao, S.; Raju, M.; Sunder, G.S. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. *Asian-Australas. J. Anim. Sci.* **2009**, *22*, 1026–1031. [CrossRef]
52. Maki, J.J.; Klima, C.L.; Sylte, M.J.; Looft, T. The microbial pecking order: Utilization of intestinal microbiota for poultry health. *Microorganisms* **2019**, *7*, 376. [CrossRef]
53. Du, K.; Bereswill, S.; Heimesaat, M.M. A literature survey on antimicrobial and immune-modulatory effects of butyrate revealing non-antibiotic approaches to tackle bacterial infections. *Eur. J. Microbiol. Immunol.* **2021**, *11*, 1–9. [CrossRef] [PubMed]
54. Sunkara, L.T.; Achanta, M.; Schreiber, N.B.; Bommineni, Y.R.; Dai, G.; Jiang, W.; Lamont, S.; Lillehoj, H.S.; Beker, A.; Teeter, R.G. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. *PLoS ONE* **2011**, *6*, e27225. [CrossRef] [PubMed]
55. Sunkara, L.T.; Jiang, W.; Zhang, G. Modulation of antimicrobial host defense peptide gene expression by free fatty acids. *PLoS ONE* **2012**, *7*, e49558.