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PHAGE Study: Effects of Supplemental Bacteriophage Intake on Inflammation and Gut Microbiota in Healthy Adults

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Abstract: The gut microbiota is increasingly recognized as an important modulator of human health. As such, there is a growing need to identify effective means of selectively modifying gut microbial communities. Bacteriophages, which were briefly utilized as clinical antimicrobials in the early 20th century, present an opportunity to selectively reduce populations of undesirable microorganisms. However, whether intentional consumption of specific bacteriophages affects overall gut ecology is not yet known. Using a commercial cocktail of *Escherichia coli*-targeting bacteriophages, we examined their effects on gut microbiota and markers of intestinal and systemic inflammation in a healthy human population. In a double-blinded, placebo-controlled crossover trial, normal to overweight adults consumed bacteriophages for 28 days. Stool and blood samples were collected and used to examine inflammatory markers, lipid metabolism, and gut microbiota. Reductions in fecal *E. coli* loads were observed with phage consumption. However, there were no significant changes to alpha and beta diversity parameters, suggesting that consumed phages did not globally disrupt the microbiota. However, specific populations were altered in response to treatment, including increases in members of the butyrate-producing genera *Eubacterium* and a decreased proportion of taxa most closely related to *Clostridium perfringens*. Short-chain fatty acid production, inflammatory markers, and lipid metabolism were largely unaltered, but there was a small but significant decrease in circulating interleukin-4 (Il-4). Together, these data demonstrate the potential of bacteriophages to selectively reduce target organisms without global disruption of the gut community.

Keywords: bacteriophage; cytokines; gut microbiota; gastrointestinal; inflammation; short-chain fatty acid

1. Introduction

Intestinal health and the gut microbiota have increasingly been linked to various chronic health outcomes. Imbalances in the gut microbiota resulting from poor diet, stress, antibiotic use, and other

lifestyle and environmental factors are associated with the development of intestinal inflammation and bowel irregularities [1,2]. Several autoimmune and metabolic conditions and even mental health may also be rooted in the gut and influenced by its microbial residents [2]. As a result, there is a growing interest in identifying dietary supplements that favorably modulate gut microbial populations. According to Transparency Market Research, the U.S. market for probiotics, prebiotics, and other digestive aids currently exceeds $68.8 billion and is expected to reach $83.5 billion by 2022 [3]. Thus, there exists a critical need to identify safe and effective methods of manipulating gut microbiota to promote overall health and well-being.

Bacteriophages (or phages) are among the candidates being explored as potential microbial modifiers in promoting intestinal health [4]. These ubiquitous, bacteria-targeting viruses exhibit a high degree of host specificity, suggesting utility for selectively reducing pathogenic or pro-inflammatory bacteria in the microbial milieu. The antibacterial activity of phages was first observed in the waters of the Ganges and Jumna rivers in India in 1896 [5]. In 1917, Felix d'Herelle demonstrated their clinical relevance by isolating and applying phages to treat numerous bacterial infections [6]. However, despite promising early results, the concept of phage therapy lost momentum with the introduction of broad spectrum antibiotics, which allowed the treatment of bacterial diseases without the need to identify a specific causal organism [7]. The host specificity of phages, which has limited their widespread application as clinical antimicrobials, may be advantageous when considering their use as microbiota-modulating dietary supplements. While antibiotics can cause or exacerbate microbiota imbalances or dysbiosis [8], phages offer the opportunity to subtly and selectively modify the gut microbiota. Several bacteriophages are "Generally Recognized As Safe" (GRAS) for human consumption by the U.S. Food and Drug Administration (FDA). Recently, we demonstrated that oral ingestion of a bacteriophage cocktail in dietary supplement form was safe and tolerable in a healthy adult population [9].

The objective of the current study was to determine how daily consumption of supplemental *Escherichia coli*-targeting phages (commercially sold as PreforPro®) influences the gut microbiota of healthy adults with self-reported gastrointestinal distress. In addition, we determined the effects on microbial production of short-chain fatty acids (SCFAs), as well as explored whether phage consumption alters lipid metabolism and parameters of local and systemic inflammation. Here, we report that 28 days of phage consumption did not substantially alter the global gut microbiota profiles of most individuals, but did reduce populations of the target bacteria, *E. coli*, as well as modify a number of individual bacterial species, including an increase in amplicon sequence variants (ASVs) mapping to *Eubacterium spp*., which is one of the most abundant genera in the healthy human gut [10]. We also saw a reduction in the circulating pro-inflammatory cytokine interleukin 4 (Il-4), which has been associated with autoimmune and allergic responses in human populations [11]. These data highlight the potential of bacteriophages for selective modification of targeted microbial species without inducing dysbiosis.

2. Materials and Methods

2.1. Participant Characteristics

Forty-three healthy adults aged 18–65 with self-reported gastrointestinal issues were enrolled in the study. Eligibility was determined by a telephone or in-person screening prior to obtaining informed consent. Exclusion criteria included (a) a previous diagnosis of gastrointestinal or metabolic conditions, cancer, liver, or kidney diseases; (b) pregnancy or breastfeeding;(c) smoking;(d) use of antibiotics in the last 2 months; and (e) current medication or dietary supplement use that may impact gut microbiota. Participants were asked to maintain their regular diet and exercise habits throughout the study, refrain from supplemental prebiotics or probiotics, and limit alcohol consumption to 1 drink per day or no more than 7 drinks per week. Additional study criteria and participant demographics are reported in Gindin et al. [9]. Of the total enrolled participants (n = 43), 36 completed at least one

arm of the study and 32 of those completed the entire study. The data shown here represent analysis from these 36 participants.

2.2. Study Design

The study was conducted as a randomized, double-blind, placebo-controlled crossover intervention trial, with two 28 day intervention periods and a washout period of at least two weeks between treatments. The treatments consisted of 4 supplemental bacteriophage strains (LH01-*Myoviridae*, LL5-*Siphoviridae*, T4D-*Myoviridae*, and LL12-*Myoviridae*) at a titer of 10^6 phages per dose included in PreforPro® commercial capsules prepared by Deerland Enzymes (Kennesaw, GA, USA). The placebo was rice maltodextrin and coconut triglycerides, which were also the carrier materials used in the treatment capsules. Participants were asked to consume one 15 mg capsule per day during the treatment and placebo periods. The study was conducted at the Human Performance Clinical Research Laboratory (HPCRL) at Colorado State University. At each clinic visit (t = 0 and t = 28 days for each intervention period, 4 visits in total),participants provided a fresh stool sample that had been collected at home and returned to the clinic within 24 h of their visit. A fasted blood sample was also collected. Collected stool and plasma samples were stored at −80 °C prior to analysis. A detailed study protocol has been previously published [9]. The study protocol was approved by the Institutional Review Board (IRB) for Human Subjects Research at Colorado State University, CSU protocol #16-6666HH, and all participants provided written informed consent prior to beginning the study. The study is also registered at clinicaltrials.gov as NCT03269617 [9].

2.3. DNA Extraction and Sequencing

Collected stool samples were thawed and subsampled with sterile cotton swabs. Fecal DNA was then extracted from the swabs using the FastDNA® KIT (MP Biomedical; Santa Ana, CA, USA) following modified manufacturer's instructions that included optimization with additional wash steps. Sequencing libraries were constructed by PCR amplification of the V4 region of the *16s* rRNA gene using primers 515F and 806R following the protocol for the Earth Microbiome Project [12]. Amplicons were purified using AxyPrep Mag PCR clean-up beads (Axygen; Corning, NY, USA), and amplicons were quantified with a Quanti-iT PicoGreen dsDNA Assay Kit (Invitrogen; Eugene, OR, USA) and pooled in equimolar ratios prior to sequencing at the Colorado State University Genomics Core facility using a 2 × 250 MiSeq flow cell (Illumina, San Diego, CA, USA).

2.4. Microbiota Analysis

Paired-end sequence reads were concatenated, and all combined 16s sequences were filtered, trimmed, and processed with the DADA2 [13] implementation included in the open source bioinformatics tool myPhyloDB version 1.2.1 [14]. Briefly, all primers were removed from each sequence using the open source Python program Cutadapt [15], and amplicon sequence variants (ASVs) were inferred using the default pipeline in DADA2. Each sequence variant identified in DADA2 was classified to the closest reference sequence contained in the GreenGenes reference database (Vers. 13_5_99) using the usearch_global option (minimum identity of 97%) contained in the open source program VSEARCH [16].Analysis of covariance (ANCOVA), DiffAbund, and principle coordinates analysis (PCoA) analyses were conducted in myPhyloDB, and MicrobiomeAnalyst [17] was used to calculate alpha diversity scores. Data were filtered to remove unassigned reads and phylotypes with ≥25% zeros. The raw sequencing data are available upon request, and links to the metadata and.biom files are included with the Supplementary Materials.

2.5. Short-Chain Fatty Acids

Short chain fatty acids (SCFA) were extracted from frozen fecal samples and analyzed as previously described [18]. Briefly, fecal aliquots were extracted in acidified water (pH 2.5) containing an internal standard of 5mM of ethylbutyric acid. Suspended samples were homogenized and

sonicated, followed by centrifugation to remove particulate matter. Supernatant was analyzed on a Gas Chromatograph with Flame Ionization Detection (GC-FID; Agilent 6890 Plus GC Series, Agilent 7683 Injector series, GC Column: TG-WAXMS A 30mx 0.25mm × 0.25µm). The run program was as follows: Initial temp = 100 °C for 1 min, max temp = 300 °C, equilibration time = 1 min with a ramp rate of 8.0 for 1 min to a final temp of 180 °C, followed by a ramp rate of 20.0 to a final temp of 200 °C for 5 min. Post temperature was 50 °C. The front inlet was split (10:1), 240 °C, 16.57psi. Peak areas were normalized to the internal standard (5 mM ethyl butyric acid, Retention Time (RT) = 9.2) and quantified using standard curves (acetic acid, RT = 5.5; propionate, RT = 6.7; butyrate, RT = 7.7) from dilutions of commercial stocks.

2.6. Fecal Triglycerides

Fecal triglycerides were assessed using the Triglycerides Assay Kit (Cayman Chemicals, Ann Arbor, MI). Briefly, 75 mg of homogenized fecal sample was suspended in 1xNP40 reagent containing protease inhibitors. Samples were centrifuged at 4 °C for 10 min at 10,000 rpm. Supernatant was diluted 1:5 with 1xNP40, and absorbance at 530–550nm was measured after incubation for 15 min at room temperature. Triglyceride quantity was determined by fitting to standard curves.

2.7. Local Inflammation and Immune Responses

Fecal secretory immunoglobulin A (sIgA) and fecal calprotectin were analyzed using the Human Secretory IgA ELISA Assay Kit and Calprotectin ELISA Assay kits (Eagle Biosciences, Amherst, NH, USA).

2.8. Systemic Inflammation

Systemic inflammation was assessed by measuring plasma levels of C-reactive protein (CRP), as well as several chemokines and cytokines. CRP levels were assessed using Human hsCRP ELISA kits (BioVender LLC., Asheville, NC, USA) according to the manufacturer's instructions. In addition, 13 different chemokines and cytokines, which included GM-CSF, IFNγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, and TNF-α, were measured using the Milliplex MAP Human High Sensitivity T Cell panel (Millipore Sigma, Burlington, MA, USA). All samples were processed according to the manufacturers' protocols and analyzed on a Luminex instrument (LX200; Millipore, Austin, TX, USA).

2.9. Plasma Lipids

Lipid panels (total cholesterol (TC), high-density lipoprotein cholesterol (HDL), triglycerides (TRIGs), nonhigh-density lipoprotein cholesterol (nHDL), total cholesterol/HDL ratio (TC/HDL), low-density lipoprotein cholesterol (LDL), and very low-density lipoprotein cholesterol (VLDL)) were assayed within one hour of blood collection using a Piccolo Xpress Chemistry Blood Analyzer (Abaxis, Union City, CA, USA).

2.10. Statistical Analysis

To evaluate the effects of the starting sequence, differences between baseline levels were assessed for each sequence (A-B, B-A). Continuous data were tested for normality prior to performing linear regression analysis. A linear mixed model approach, controlling for sequence and repeated measures, was used to compare treatment effects within each time point and between time points within a treatment group. A p-value of 0.05 was used to assess statistical significance. Prior to statistical analysis, microbiota data were normalized using Laplace smoothing followed [19] by subsampling with replacement (rarefaction (keep) command) in MyPhyloDB [14]. Data were rarefied to 31,037 sequence reads using 100 iterations. Amplicon sequence variants (ASVs) that were present in less than 25% of the total samples were excluded from analyses. An analysis of covariance (ANCOVA)

model was used to assess taxonomic differences across treatment groups, and a genewise negative binomial generalized linear model (GLM) (EdgeR [20]) was used to determine differential distribution of taxa between treatments. Measures of alpha (CHAO1 estimates, Shannon and Simpson diversity indices) and beta diversity (Bray–Curtis distances) were statistically analyzed using nonparametric Kruskal–Wallis tests.

3. Results

Study compliance and the safety and tolerability of phage consumption have previously been reported [9]. Here, we present the results of the *16s rRNA* sequencing and targeted metabolite analysis from stool samples as well as plasma lipids and markers of inflammation.

3.1. Gut Microbiota and Metabolite Analysis

Bacterial sequences were classified to seven phyla, with the majority being represented by Firmicutes, followed by Bacteroidetes and minor components including Actinobacteria, Proteobacteria, and Verrucomicrobia. There were no significant differences in bacterial taxa between treatment groups and time points (Figure 1A and Figure S1). In addition, richness estimates (CHAO1) and α-diversity, calculated as Shannon and Simpson's indices, did not vary across groups (Table S1). PCoA visualization of Bray–Curtis distances did not show any significant clustering between treatment groups or time points (Figure 1B).

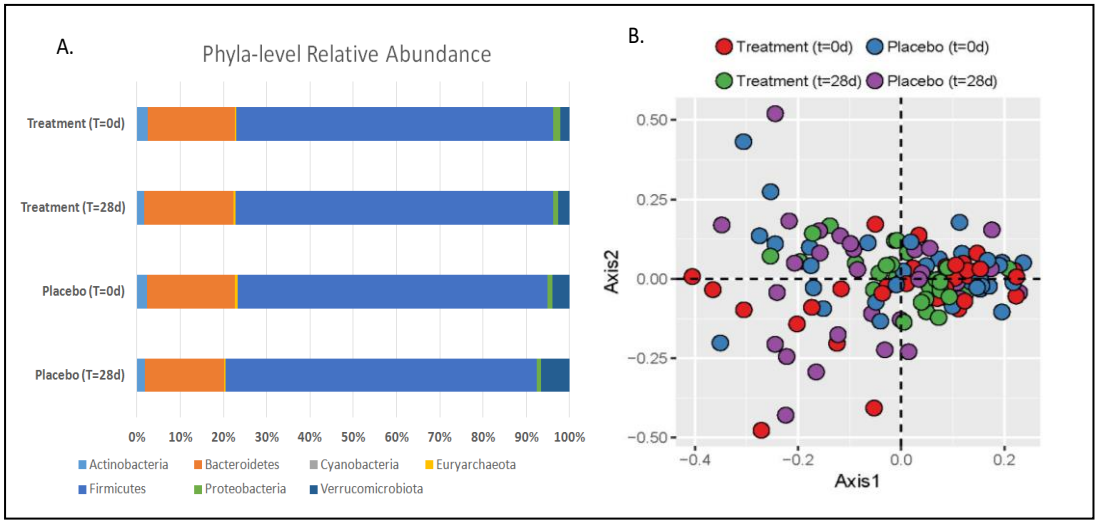

Figure 1. (**A**) Relative abundance of bacterial phyla detected in stool samples at baseline and after 28days for both phage (treatment) and placebo study periods. No significant differences were detected by analysis of covariance (ANCOVA) at $p < 0.05$. (**B**) Principle coordinates analysis (PCoA) with nonmetric dimensional scaling of species-level Bray–Curtis distances. Stress = 0.191; perMANOVA (1000 permutations) *Pr* (>*F*) = 0.996.

Because the supplemented phage cocktail specifically targeted *E. coli*, we identified ASVs in each sample that mapped to *E. coli* (gg_111717). Only 21 total participants had detectable levels of *E. coli* prior to starting the treatment period. Baseline levels varied significantly among participants, ranging from 0.01%–3.2% of total reads. The response rate was ~71%, with 15 of the 21 participants that had detectable levels of *E. coli* prior to starting the treatment period showing reduced or undetectable levels after treatment (responder subpopulation), while only 47% of the individuals with detectable levels of *E. coli* prior to the placebo period showed reduction after 28 days. Overall, *E. coli* levels were significantly reduced after phage treatment ($p = 0.03$ for the total study population and $p = 0.02$ when only including participants with baseline *E. coli*, but not after placebo ($p = 0.85$ for the total study population and $p = 0.78$ for participants with baseline *E. coli*)) (Figure 2). On average, the number of

E. coli reads were reduced by ~40% after treatment and by only 14% after the placebo, although there was a great deal of variability between participants.

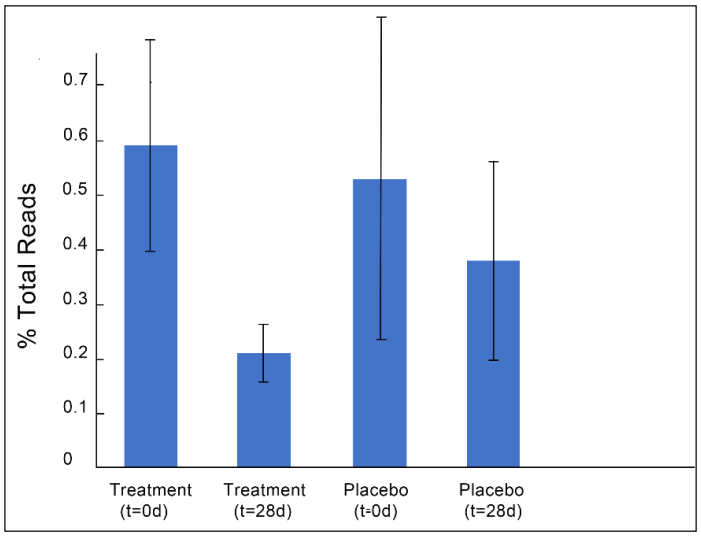

Figure 2. (**A**) Percent of total reads represented by amplicon sequence variants (ASVs) mapping to *Escherichia. coli* for each treatment and time point. (**B**) Change in *E. coli* levels from baseline values after treatment or placebo consumption. Data represents only individuals with baseline *E. coli* levels (n = 21). Error bars represent SEM.

Several microbial taxa were significantly correlated with fecal levels of *E. coli*, regardless of intervention period. *Oxalobacter formigenes* and ASVs assigned to the Lachnospiraceae family were negatively correlated with *E. coli* populations. Conversely, several ASVs identified as belonging to the Ruminococcaceae family were positively correlated with *E. coli*, as was *Desulfovibrio* (Figure 3).

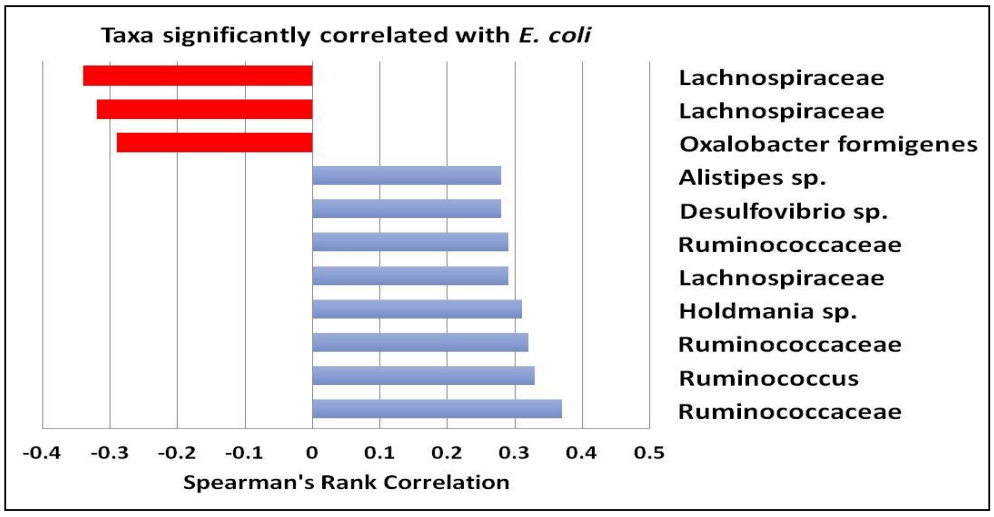

Figure 3. Using Spearman's rank, several ASVs were found to be significantly negatively correlated (red bars) or positively correlated (blue bars) with *E. coli* ASVs. Significant values were considered $q < 0.10$.

There was only one bacterial ASV that increased significantly ($q < 0.10$) after phage consumption in the responder subpopulation (those who had baseline *E. coli* and saw reductions with treatment), *Bifidobacterium bifidum*, which was ~5.5-fold higher compared to baseline measures (mean t = 0 = 0.13; mean t = 28 = 1.1; log fold change (logFC) = 2.7, Counts Per Million (CPM) = 8.1, likelihood ratio = 6.7;

False Discover Rate (FDR) = 0.06). There were also several treatment-associated taxa changes noted in the total study population, which included 36 participants that completed at least one arm of the study, regardless of whether *E. coli* was detected in baseline samples. Most notably, three ASVs representing potentially pathogenic or inflammation-associated taxa were reduced relative to the placebo, including an ASV mapping to *Clostridium perfringens*, a food-borne pathogen and minor component of the commensal flora [21], which was reduced by about 75%. In addition, two ASVs representing species of *Eubacterium* were increased by 4–5-fold after phage consumption compared to the placebo (Figure 4).

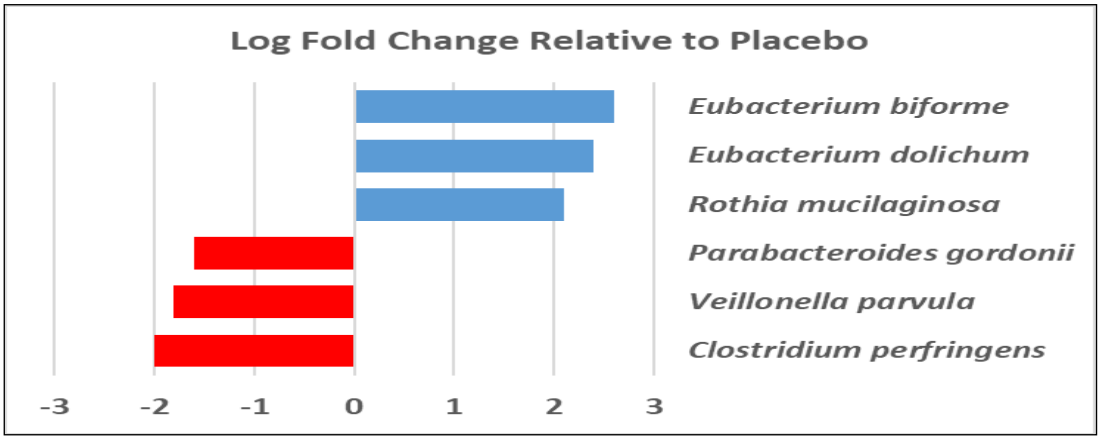

Figure 4. Using a negative binomial generalized linear model (GLM) (EdgeR), we identified several taxa that significantly ($q < 0.10$) differed from placebo levels after 28days of phage consumption. Red bars represent taxa reduced with phage treatment, and blue bars represent taxa that were increased.

To assess functional changes in the microbiota, we also assayed fecal short-chain fatty acid (SCFA) concentrations. Acetate was the most abundant SCFA, averaging 10–20 mM/gram of stool across all treatments and timepoints (Figure S2A). Propionate and butyrate were detected in levels of approximately 2–4 mM/gram of stool (Figure S2B,C). There were no significant differences in any of the SCFAs detected across timepoints or between treatment periods, although there was a trend toward decreased acetate from the baseline level within the placebo period ($p = 0.06$; *Confidence Interval* (*CI*) = −0.09 to 3.15).

3.2. Stool and Plasma Lipid Profiles

Since the microbiota also plays a role in lipid absorption [22,23], we measured total triglyceride levels in stool. Although there was significant interindividual variability, there were no significant differences in fecal triglycerides across timepoints or between treatment periods (Table 1), although there was a significant period effect noted for participants starting on the placebo and transitioning to the treatment group ($p = 0.03$; CI = −8.93 to −0.49). Likewise, there were no significant changes across time periods or between treatments for plasma lipids associated with phage consumption. However, there was a statistically significant change in total cholesterol to HDL ratio (TC/H) between the baseline (t = day 0) and day 28 of the placebo period ($p = 0.045$; CI = 0.00 to 0.18), possibly driven by a trend toward reduced HDL cholesterol during this period ($p = 0.08$; CI = −3.87 to −1.75) (Table 1). However, these statistical differences are unlikely to have any clinical or biological relevance.

3.3. Immunological and Inflammatory Markers

We examined several stool and blood markers indicative of inflammatory state and immunological activity. In stool, we measured calprotectin and secretory immunoglobin A (sIgA). Calprotectin was below the detection limits of our assay for the majority of samples tested (data not shown): sIgA was detectable, and the majority of samples fell within clinically normal ranges (510–2040 µg/mL). Therewere no significant differences in this parameter across timepoints or between treatment periods

(Figure S3), although there was large variability between individuals and even between timepoints for select individuals. In plasma, we analyzed C-reactive protein using a high-sensitivity ELISA assay (hsCRP) as well as a panel of 13 human T-cell-derived cytokines. There were no significant responses across timepoints or between treatments in these parameters, with one exception. Interleukin-4 (Il-4) was significantly reduced from baseline after 28 days of phage consumption ($p = 0.002$; CI = −15.63 to −3.67) (Table 2).

Table 1. Stool and plasma lipid profiles.

	Treatment ($t = 0$)	Treatment ($t = 28$)	Placebo ($t = 0$)	Placebo ($t = 28$)
Fecal Triglycerides (mg/dL)	6.70 (±0.70)	8.12 (±0.89)	9.25 (±1.14)	7.46 (±0.79)
Cholesterol (mg/dL)	189.70 (±4.93)	187.18 (±4.83)	192.03 (±5.76)	189.35 (±6.08)
LDL (mg/dL)	103.48 (±3.56)	100.82 (±4.23)	106.76 (±4.85)	103.06 (±4.67)
vLDL (mg/dL)	20.21 (±1.82)	20.52 (±1.87)	19.62 (±1.94)	19.85 (±1.94)
HDL (mg/dL)	65.06 (±2.51)	65.24 (±2.58)	65.74 (±2.33)	63.91 (±2.84)
nHDLc (mg/dL)	123.61 (±4.89)	122.85 (±4.98)	126.21 (±5.68)	125.62 (±5.40)
TC/H	3.03 (±0.13) [a]	3.01 (±0.14) [a]	3.02 (±0.13) [a]	3.11 (±0.14) [b]
Plasma Triglycerides (ng/dL)	99.94 (±9.11)	102.61 (±9.46)	97.74 (±9.70)	99.41 (±9.77)

Data represent means (±SD). Statistically different values are denoted with different letters ($p<0.05$). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL), nonhigh-density lipoprotein cholesterol (nHDL), low-density lipoprotein cholesterol (LDL), and very low-density lipoprotein cholesterol (vLDL).

Table 2. Plasma C-reactive protein (CRP) and cytokines.

	Treatment ($t = 0$)	Treatment ($t = 28$)	Placebo ($t = 0$)	Placebo ($t = 28$)
hsCRP (mg/mL)	1.76 (±0.51)	1.79 (±0.52)	1.56 (±0.41)	2.45 (±0.68)
GMCSF (pg/mL)	80.69 (±9.99)	80.54 (±10.35)	83.17 (±9.78)	80.59 (±10.10)
IFN-γ (pg/mL)	12.67 (±1.12)	12.29 (±1.02)	14.21 (±1.86)	13.81 (±1.89)
Il-10 (pg/mL)	24.13 (±3.30)	23.53 (±2.85)	26.03 (±3.75)	24.83 (±3.84)
Il-12 (pg/mL)	3.57 (±0.33)	3.57 (±0.32)	3.75 (±0.35)	3.55 (±0.37)
Il-13 (pg/mL)	23.05 (±5.53)	22.16 (±5.71)	25.39 (±6.06)	24.5 (±5.73)
Il-1β (pg/mL)	1.76 (±0.13)	1.71 (±0.10)	1.85 (±0.13)	1.73 (±0.12)
Il-2 (pg/mL)	2.24 (±0.14)	2.15 (±0.18)	2.46 (±0.28)	2.36 (±0.31)
Il-4 (pg/mL)	69.48 (±5.75) [a]	59.83 (±4.43) [b]	63.79 (±4.95) [a,b]	61.71 (±3.88) [a,b]
Il-5 (pg/mL)	8.16 (±3.26)	5.55 (±1.35)	7.85 (±2.71)	6.38 (±1.57)
Il-6 (pg/mL)	3.37 (±0.33)	3.43 (±0.33)	3.76 (±0.38)	3.82 (±0.41)
Il-7 (pg/mL)	13.37 (±1.07)	13.39 (±1.14)	13.91 (±1.16)	13.35 (±1.12)
Il-8 (pg/mL)	4.14 (±0.70)	4.24 (±0.77)	4.51 (±0.83)	4.45 (±0.81)
TNFα (pg/mL)	4.45 (±0.29)	4.18 (±0.25)	4.23 (±0.24)	4.09 (±0.26)

Values represent mean (± SEM). Statistically significant differences are denoted by different letters ($p < 0.01$). Il: Interleukin; GMCSF: Granulocyte Macrophage Colony Stimulating Factor; TNF: Tumor Necrosis Factor.

4. Discussion

Bacteriophages offer a novel and selective means of modifying the gut microbiota, thereby influencing the intestinal environment without causing global perturbations that can lead to microbial dysbiosis. In the current study, we confirmed that phage treatment was not associated with global perturbations in the gut microbiota, as evidenced by a lack of differences in community descriptors such as richness and α-diversity between treatments or over time. Furthermore, no clustering of groups was apparent on a PCoA plot of Bray–Curtis distances. On the contrary, disruption of microbial communities by antibiotics and other pharmaceutical treatments can predispose individuals to dysbiosis and create ecological niches where pathogens can establish a foothold, as is commonly

seen with *Clostridium difficile* infections [8,24]. Thus, phages represent a novel means of selectively modifying the microbiota without causing global disruptions to community structure.

Although no global changes to the microbiota were apparent with phage treatment, there were a few significant alterations in certain members of the microbial community. Importantly, *E. coli*, which is the target host for the consumed phage consortium, was significantly reduced at the end of the treatment period. Populations of several bacterial taxa were also positively or negatively correlated with levels of *E. coli*. While it is difficult to discern whether there were specific ecological interactions driving these associations, both *E. coli* and sulfate-reducing bacteria (SRB), such as *Desulfovibrio*, have been associated with higher clinical activity indices and sigmoidoscopy scores in rectal biopsies of patients with Inflammatory Bowel Disease (IBD) [25–27].One study also reported higher *Clostridium perfringens* and lower *Eubacterium* spp. associated with disease severity [25]. Interestingly, consumption of bacteriophages in the current study resulted in a more than 4-fold reduction of *C. perfringens* as well as 4–5-fold increases in two ASVs mapping to taxa in the genus *Eubacterium*. *Eubacterium* reductions have also been associated with several inflammatory conditions in the gastrointestinal tract [28,29]. *Eubacterium* spp. are butyrate producers, and as such may be important in stimulating enterocyte turnover and maintaining tight barrier junctions. However, we did not observe an overall increase in stool butyrate with the bacteriophage treatment. This could have been due to a high level of interindividual variation in SCFA production masking responses at the population level. It may also have been due to insufficient levels of fiber intake, as this is the substrate for SCFA production. Finally, another interesting observation was the positive correlation between the sulfate-reducing bacteria (SRB) *Desulfovibrio* and *E. coli* ASVs across the population. Although the physiological role of SRB in the gastrointestinal tract is still not well understood, it is commonly accepted that production of hydrogen sulfide could have direct inflammatory, cytotoxic, and genotoxic effects in the gut that could reduce epithelial barrier integrity [30]. These data suggest that further investigation of bacteriophages is warranted to determine the therapeutic value of treating individuals with inflammatory bowel conditions.

Another observation associated with phage consumption was a reduction of circulating Il-4. This cytokine is released during Th2 responses, which are associated with the promotion of IgE and eosinophilic responses to atopy [31]. Although the mechanisms relating Il-4 reduction to phage consumption are unclear, some studies have demonstrated that bacterial lipopolysaccharide (LPS) induces Il-4 production via a MyD88 and TRAM-dependent pathway [32]. Although we do not have direct evidence of reduced circulating LPS, we have previously reported lower aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in whole blood samples collected after treatment compared to the placebo control [9]. In a rodent model, AST and ALP increased after exposure to LPS [33]. Circulating LPS is associated with systemic inflammation and increased cytokine release [34]. Therefore, it is plausible that the phage treatment resulted in lower circulating LPS, which may drive reductions in Il-4. These data suggest that future experiments are justified to further explore mechanistic links between Il-4 and phage consumption as well as to examine the effect of the bacteriophage cocktail in a human population with atopic dermatitis and other allergic atopies.

A major strength of the current study was its crossover design, in which each individual served as his or her own control. This was advantageous given the inter-person variability of the microbiota, as well as individual responses to a stimulus. An additional strength of this study design was the double blinding, thus minimizing participant and researcher bias. Despite these strengths, there were several shortcomings that limited our ability to interpret the data. For example, the phage cocktail prescribed has been shown in animal models to reduce target *E. coli* populations and stimulate the growth of probiotic species [35]. However, the variability in detectable baseline levels of both *E. coli* and these probiotic species in our sample pool made it difficult to replicate these findings in a human population. However, this product is currently marketed as a dietary supplement, and thus it is important to determine how it impacts the global microbiota in a healthy population. While we did see a phage-associated increase in taxa mapping to *Bifidobacterium bifidum* in a subpopulation

of responders, examining the influence of the co-administration of phages with particular probiotic species may be necessary to establish whether they enhance probiotic survival and efficacy in the gastrointestinal tract. Another limitation was the lack of a diet and physical activity assessment, as these parameters may influence microbiota composition. While participants were asked to remain on their typical diet and exercise regime and were excluded from the study if they indicated that they were actively trying to lose weight, tracking these metrics would have been an additional measure to assess compliance. Thus, although some of the observed changes in specific bacterial populations may have been in response to changes in these external factors, the study was designed to minimize these effects.

In conclusion, bacteriophage consumption caused minimal disruption to the gut microbiota, but did elicit minor changes that may be viewed as beneficial overall. Specifically, the reduction of *E. coli*, decreased proportions of potentially pro-inflammatory bacteria, and increases in fermentative taxa that are capable of butyrate production suggest a shift toward a healthier gut environment. As gut dysbiosis continues to be associated with human disease and the medical community is confronted with anti-microbial "superbugs", bacteriophages offer an additional resource to combat these issues. This study merely broaches the edge of this potential, and along with our previously published data [9], confirms the safety and tolerability of phage consumption in a human population. These data support further studies on the microbiota modulatory potential of bacteriophages for use as a dietary supplement and possibly as a therapeutic agent in clinical populations of intestinal inflammation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/3/666/s1, Figure S1: Bar plots of relative abundance at varying taxonomic resolutions. Unclassified samples were removed from the analysis at each taxonomic level. Figure S2: Fecal short chain fatty acid levels across treatments and time. Figure S3: Fecal sIGA levels. Table S1: Microbial alpha diversity parameters.

Author Contributions: T.C.W. and T.L.W. conceptualized the study, acquired funding, and obtained regulatory board approvals. M.G. served as the clinical coordinator, which included obtaining consent from and scheduling participants, collecting biofluids and anthropomorphic data at clinic visits, and managing data collection and storage. N.D.M.G. and E.F. assisted with clinical sample collection, lipid panels, and blood processing. H.P.F., J.S.V., T.L.W., and S.L. processed wet lab samples and conducted laboratory assays. Microbiota data were analyzed by D.K.M. and T.L.W. Statistical analysis was conducted by S.R. Data interpretation was conducted by T.L.W., D.K.M., H.P.F., and T.C.W. Drafting the manuscript was the responsibility of H.P.F., T.C.W. and T.L.W., with all authors contributing to editing and approving the final manuscript.

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Conflicts of Interest: Funding was provided by Deerland Enzymes to the Think Healthy Group, Inc., through an unrestricted educational grant. T.C.W. is the Principal and CEO of the Think Healthy Group, Inc., a food science and nutrition consulting firm dedicated to advancing cutting-edge research and public health through engagement with industry, academia, government, media, and nongovernmental organizations. All conflicts can be found on his website at www.drtaylorwallace.com. Deerland Enzymes played no role in the study design, data collection, analysis, or interpretation and presentation of results. All other authors declare no conflicts of interest.

References

1. Gentile, C.L.; Weir, T.L. The gut microbiota at the intersection of diet and human health. *Science* **2018**, *362*, 776–780. [CrossRef] [PubMed]
2. Schippa, S.; Conte, M.P. Dysbiotic events in gut microbiota: Impact on human health. *Nutrients* **2014**, *6*, 5786–5805. [CrossRef] [PubMed]
3. Nutraceutical News. Available online: http://www.nutra-insurance.com/nutraceutical-news (accessed on 25 February 2019).

4. Gordillo Altamirano, F.L.; Barr, J.J. Phage Therapy in the Postantibiotic Era. *Clin. Microbiol. Rev.* **2019**, *32*, e00066-18. [CrossRef] [PubMed]
5. Abedon, S.T.; Thomas-Abedon, C.; Thomas, A.; Mazure, H. Bacteriophage prehistory: Is or is not Hankin, 1896, a phage reference? *Bacteriophage* **2011**, *1*, 174–178. [CrossRef] [PubMed]
6. Ackermann, H.W. On an invisible microbe antagonistic to dysentery bacilli. *C. R. Acad. Sci.* **1917**, *165*, 373–375.
7. Kutateladze, M.; Adamia, R. Bacteriophages as potential new therapeutics to replace or supplement antibiotics. *Trends Biotechnol.* **2010**, *28*, 591–595. [CrossRef] [PubMed]
8. Francino, M.P. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. *Front. Microbiol.* **2016**, *6*, 1543. [CrossRef] [PubMed]
9. Gindin, M.; Febvre, H.P.; Rao, S.; Wallace, T.C.; Weir, T.L. Bacteriophage for Gastrointestinal Health (PHAGE) Study: Evaluating the Safety and Tolerability of Supplemental Bacteriophage Consumption. *J. Am. Coll. Nutr.* **2018**, *38*, 68–75. [CrossRef]
10. Finegold, S.M.; Sutter, V.L.; Mathisen, G.E. Chapter 1—Normal Indigenous Intestinal Flora. In *Human Intestinal Microflora in Health and Disease*; Hentges, D.J., Ed.; Academic Press: Cambridge, MA, USA, 1983; pp. 3–31.
11. May, R.D.; Fung, M. Strategies targeting the IL-4/IL-13 axes in disease. *Cytokine* **2015**, *75*, 89–116. [CrossRef]
12. Gilbert, J.A.; Jansson, J.K.; Knight, R. The Earth Microbiome project: Successes and aspirations. *BMC Biol.* **2014**, *12*, 69. [CrossRef]
13. 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]
14. Manter, D.K.; Korsa, M.; Tebbe, C.; Delgado, J.A. myPhyloDB: A local web server for the storage and analysis of metagenomic data. *J. Biol. Database Curation* **2016**. [CrossRef]
15. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10–12. [CrossRef]
16. Rognes, T.; Flouri, T.; Nichols, B.; Quince, C.; Mahé, F. VSEARCH: A versatile open source tool for metagenomics. *J. Life Environ. Sci.* **2016**, *4*, e2584. [CrossRef]
17. Dhariwal, A.; Chong, J.; Habib, S.; King, I.L.; Agellon, L.B.; Xia, J. MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* **2017**, *45*, W180–W188. [CrossRef]
18. Sheflin, A.M.; Borresen, E.C.; Kirkwood, J.S.; Boot, C.M.; Whitney, A.K.; Lu, S.; Brown, R.J.; Broeckling, C.D.; Ryan, E.P.; Weir, T.L. Dietary supplementation with rice bran or navy bean alters gut bacterial metabolism in colorectal cancer survivors. *Mol. Nutr. Food Res.* **2017**, *61*, 1500905. [CrossRef]
19. Manning, C.D.; Schütze, H. *Foundations of Statistical Natural Language Processing*; The MIT Press: Cambridge, MA, USA, 1999.
20. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010**, *26*, 139–140. [CrossRef]
21. Adlerberth, I.; Wold, A.E. Establishment of the gut microbiota in Western infants. *Acta Paediatr.* **2009**, *98*, 229–238. [CrossRef]
22. Martinez-Guryn, K.; Hubert, N.; Frazier, K.; Urlass, S.; Musch, M.W.; Ojeda, P.; Pierre, J.F.; Miyoshi, J.; Sontag, T.J.; Cham, C.M. Small Intestine Microbiota Regulate Host Digestive and Absorptive Adaptive Responses to Dietary Lipids. *Cell Host Microbe* **2018**, *23*, 458–469. [CrossRef]
23. Semova, I.; Carten, J.D.; Stombaugh, J.; Mackey, L.C.; Knight, R.; Farber, S.A.; Rawls, J.F. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. *Cell Host Microbe* **2012**, *12*, 277–288. [CrossRef]
24. Brown, K.A.; Khanafer, N.; Daneman, N.; Fisman, D.N. Meta-analysis of antibiotics and the risk of community-associated Clostridium difficile infection. *Antimicrob. Agents Chemother.* **2013**, *57*, 2326–2332. [CrossRef]
25. Fite, A.; Macfarlane, S.; Furrie, E.; Bahrami, B.; Cummings, J.H.; Steinke, D.T.; Macfarlane, G.T. Longitudinal analyses of gut mucosal microbiotas in ulcerative colitis in relation to patient age and disease severity and duration. *J. Clin. Microbiol.* **2013**, *51*, 849–856. [CrossRef]

26. Kassinen, A.; Krogius-Kurikka, L.; Mäkivuokko, H.; Rinttilä, T.; Paulin, L.; Corander, J.; Malinen, E.; Apajalahti, J.; Palva, A. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology* **2007**, *133*, 24–33. [CrossRef]
27. Malinen, E.; Krogius-Kurikka, L.; Lyra, A.; Nikkilä, J.; Jääskeläinen, A.; Rinttilä, T.; Vilpponen-Salmela, T.; von Wright, A.J.; Palva, A. Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. *World J. Gastroenterol.* **2010**, *16*, 4532–4540. [CrossRef]
28. Gobert, A.P.; Sagrestani, G.; Delmas, E.; Wilson, K.T.; Verriere, T.G.; Dapoigny, M.; Del'homme, C.; Bernalier-Donadille, A. The human intestinal microbiota of constipated-predominant irritable bowel syndrome patients exhibits anti-inflammatory properties. *Sci. Rep.* **2016**, *6*, 39399. [CrossRef]
29. Matsuoka, K.; Kanai, T. The gut microbiota and inflammatory bowel disease. *Semin. Immunopathol.* **2015**, *37*, 47–55. [CrossRef]
30. Scanlan, P.D.; Shanahan, F.; Marchesi, J.R. Culture-independent analysis of desulfovibrios in the human distal colon of healthy, colorectal cancer and polypectomized individuals. *FEMS Microbiol. Ecol.* **2009**, *69*, 213–221. [CrossRef]
31. Muir, A.B.; Benitez, A.J.; Dods, K.; Spergel, J.M.; Fillon, S.A. Microbiome and its impact on gastrointestinal atopy. *Allergy* **2016**, *71*, 1256–1263. [CrossRef]
32. Sherry, C.L.; Kim, S.S.; Dilger, R.N.; Bauer, L.L.; Moon, M.L.; Tapping, R.I.; Fahey, G.C., Jr.; Tappenden, K.A.; Freund, G.G. Sickness behavior induced by endotoxin can be mitigated by the dietary soluble fiber, pectin, through up-regulation of IL-4 and Th2 polarization. *Brain Behav. Immun.* **2010**, *24*, 631–640. [CrossRef]
33. Sherer, F.; Van Simaeys, G.; Kers, J.; Yuan, Q.; Doumont, G.; Laute, M.; Peleman, C.; Egrise, D.; Lahoutte, T.; Flamand, V. Dynamic molecular imaging for hepatic function assessment in mice: Evaluation in endotoxin-induced and warm ischemia-reperfusion models of acute liver failure. *J. Liver* **2015**, *4*, 1000170.
34. Cani, P.D.; Amar, J.; Iglesias, M.A.; Poggi, M.; Knauf, C.; Bastelica, D.; Neyrinck, A.M.; Fava, F.; Tuohy, K.M.; Chabo, C. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. *Diabetes* **2007**, *56*, 1761–1772. [CrossRef]
35. Deerland Enzymes. Available online: https://www.deerlandenzymes.com/preforpro/the-science/ (accessed on 18 January 2019).

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Review

Dietary Polyphenol, Gut Microbiota, and Health Benefits

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Abstract: Polyphenols, which are probably the most important secondary metabolites produced by plants, have attracted tremendous attention due to their health-promoting effects, including their antioxidant, anti-inflammatory, antibacterial, anti-adipogenic, and neuro-protective activities, as well as health properties. However, due to their complicated structures and high molecular weights, a large proportion of dietary polyphenols remain unabsorbed along the gastrointestinal tract, while in the large intestine they are biotransformed into bioactive, low-molecular-weight phenolic metabolites through the residing gut microbiota. Dietary polyphenols can modulate the composition of intestinal microbes, and in turn, gut microbes catabolize polyphenols to release bioactive metabolites. To better investigate the health benefits of dietary polyphenols, this review provides a summary of their modulation through in vitro and in vivo evidence (animal models and humans), as well as their possible actions through intestinal barrier function and gut microbes. This review aims to provide a basis for better understanding the relationship between dietary polyphenols, gut microbiota, and host health.

Keywords: dietary polyphenols; host health; gut microbiota; biotransformation

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1. Introduction

Polyphenols, described as plants' secondary metabolites, are probably the most abundant antioxidants in our daily life. The main dietary sources of these compounds include fruits, vegetables, grains, green tea, coffee, etc. [1]. Total dietary polyphenol intake is as high as 1 g per day for each adult, which is about 10-times higher than the intake of Vitamin C, and even 100-times higher than that of Vitamin E and carotenoids [2]. During the last few decades, there has been tremendous research output related to the health-promoting effects of polyphenols, including their antioxidant, anti-inflammatory, antibacterial, anti-adipogenic, and neuro-protective activities [3,4].

It has been reported that most dietary polyphenol intake remains unabsorbed in the small intestine, while the unabsorbed parts may accumulate in the large intestine and are extensively metabolized by the gut microbiota [5]. Therefore, intestinal microbiota play an important role in the biotransformation and metabolism of the original polyphenolic structures into low-molecular-weight metabolites, which can be readily absorbed and contribute to host healthy benefits. However, little is currently known regarding the possible mechanism among dietary polyphenols, gut microbes, and host health.

Dietary polyphenols influence gut microbiota compositions in the host, which further affect the host's metabolism. In turn, intestinal microbiota can metabolize polyphenols into bioactive, low-molecular-weight phenolic metabolites to modulate the regulatory metabolism network. In this regard, this review aims to provide an assessment of dietary polyphenols' biological significances on host health, a summary of their modulation through in vitro and in vivo evidence (animal models and humans), as well as their possible action through intestinal barrier function and gut microbes.

2. Dietary Polyphenols and Their Sources

Dietary polyphenols are one of the most abundant and widely distributed natural products in plants. At present, according to the structure, dietary polyphenols are divided into four categories: phenolic acids, flavonoids (the largest subclass of polyphenols), polyphenolic amide, and other non-flavonoids (Figure 1). Phenolic acids can be further divided into two main types, benzoic acid and cinnamic acid derivatives based on C1–C6 and C3–C6 backbones [6]. Flavonoids include flavonoids, flavanones, isoflavones, chalcones, flavanols, flavonols, flavanonols, anthocyanins, and so on [7]. Polyphenolic amides have N-containing functional substituents, two such groups are capsaicinoids and avenanthramides. The non-flavonoids include mainly stilbenes and lignans. In addition to phenolic acids, flavonoids, and phenolic amides, there are several non-flavonoid polyphenols found in foods that are considered important to human health, such as resveratrol, ellagic acid and its derivatives, curcumin, etc. The remarkable feature of the chemical structure is that it has a different amount of phenolic hydroxyl groups, which can be divided into phenolic monomers and polymerized polyphenols. Phenolic monomers include flavonoids and non-flavonoids. The former generally involves a common carbon skeleton of diphenyl propane in which two benzene rings are connected by a linear three-carbon chain, while the latter is two benzene rings connected by the vinyl group [8]. Polymeric polyphenols are oligomers or polymers polymerized by monomers known as tannins.

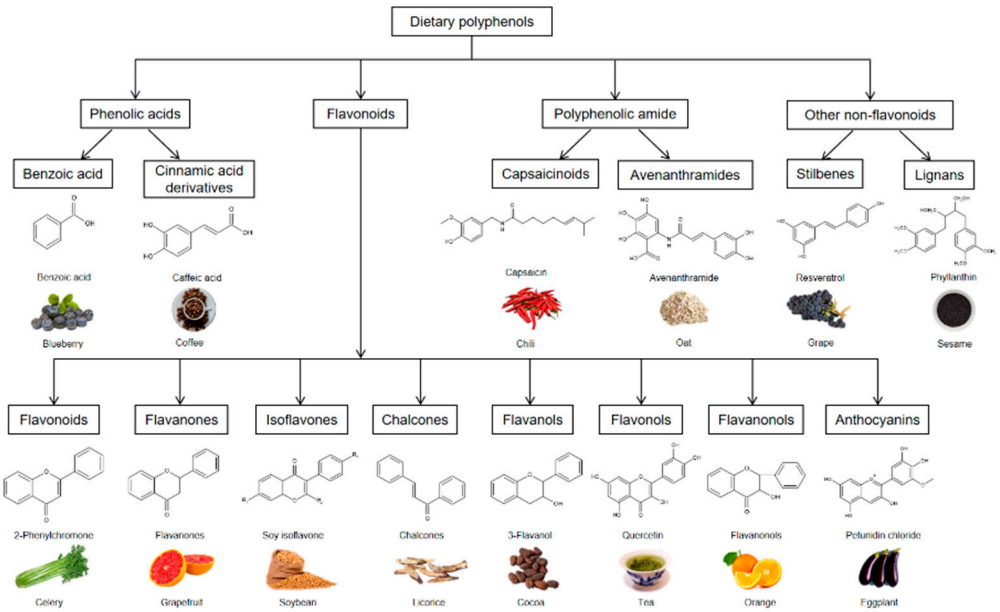

Figure 1. Classification of dietary polyphenols and their sources.

Polyphenols are widely distributed in nature, including in fruits, vegetables, cereals, beans, tea, coffee, honey, and red wine, which are the main sources of dietary polyphenols. Specifically, caffeic acid and ferulic acid are the most common phenolic acids in food. Caffeic acid is abundant in vegetables, fruits, and coffee, while ferulic acid is mainly distributed in rice bran, wheat bran, and other cereals. Among the flavonols, quercetin is the most common, which is commonly found in onions. Flavanols or flavan-3-ols are often commonly called catechins, which are abundant in red wine, chocolate, and lotus root. Isoflavones are mainly found in the leguminous family of plants. Anthocyanidins in plants mainly exist in glycosidic forms, which are commonly referred to as anthocyanins [9] and are largely distributed in strawberries, blueberries, and cherries. Some polyphenols have N-containing functional substituents, such as capsaicinoids in chili peppers and avenanthramides in oats, which belong to polyphenolic amides. The second major non-flavonoid group mainly consists of stilbenes, with resveratrol being the main representative, which is found in red

and purple grape skins and grape wine. Another important nonflavonoid group is the lignans, which exist in bound forms in flax, sesame, and many grains.

3. Dietary Polyphenols and Their Biological Significance

As the most general plant-derived bioactive components in our diet, dietary polyphenols have received tremendous attention among nutritionists, food researchers, and consumers. Phenolic compounds are generally involved in defenses against plant pathogens and atmospheric agents, including bacteria, fungi, and viruses, and many abiotic stresses like drought, salinity, and UV. Polyphenols exhibit antimicrobial and antioxidant properties that can help plants to evade pathogenic infections and, at the same time, protect the major tissues from the toxic effects of reactive oxygen species [10]. Currently, they represent a topic of great scientific attention due to interest in their biological significance for humans. Both in vitro and in vivo studies have shown their health-promoting effects, including their antioxidant, anti-inflammatory, antibacterial, anti-adipogenic, and neuro-protective activities.

3.1. Antioxidant Properties

The effectiveness of phenolic compounds in the inhibition of oxidative processes is potentially related to their reactive species scavenging activity. Due to the structure of the hydroxyl group on the benzene ring, polyphenols scavenge free radicals by H-atom transfer from the active OH group of the polyphenol to the free radical [6]. This allows polyphenols to indirectly activate antioxidant responses and generate non-toxic levels of intermediates, specifically the electrophilic forms of hydroquinone and quinone [11]. On the other hand, polyphenols inhibit the formation of or deactivation of the active species and precursors of free radicals, thus reducing the rate of oxidation and ultimately suppressing the generation of free radicals. They donate an electron to the free radical, neutralizing the radicals, and causing themselves to become stable (less reactive) radicals, thus stopping the reactions [12]. Treatment of HepG2 cells with (−)-epigallocatechin-3-gallate from green tea stimulates the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), which modulates the expression of antioxidant genes [13]. Resveratrol improves antioxidant defenses in pancreatic tissue because it enhances the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) [14].

3.2. Anti-Inflammatory Properties

Oxidative-stress-induced inflammation is mediated by the activation of cellular signaling processes of nuclear factor-kappa B (NF-kB) activation and activator protein-1 (AP-1) DNA binding [15]. It affects the expression of pro-inflammatory genes such as interleukin-1beta (IL-1β), IL-6, tumor necrotic factor alpha (TNF-a), and inducible nitric oxide synthase (iNOS) [16]. Preclinical and clinical studies suggest that polyphenols are able to express anti-inflammatory properties [17]. Although the precise mechanisms deserve further clarification, dietary polyphenols have shown benefits in distinct disorders [18]. Dihydroxylated phenolic acids produced from dietary proanthocyanidins potentially lowered the secretion of cytokines, including TNF-α, IL-1β, and IL-6, from healthy individuals [19]. Supplementation with 0.8% quercetin decreased interferon-γ, IL-1α, and IL-4 in male C57Bl/6j mice [20]. The administration of 10 mg/kg of quercetin also reduced the plasma nitrate plus nitrite (NOx) concentration and TNF-α production in adipose tissue of obese Zucker rats, resulting in an important anti-inflammatory effect [21].

3.3. Antibacterial Properties

Dietary polyphenols and plants rich in polyphenols have been demonstrated to be natural antimicrobials against both Gram-positive and Gram-negative bacteria. Epigallocatechin gallate (EGCG) was able to bind directly to the peptidoglycan from *Staphylococcus aureus*, affecting its cell integrity and destroying the osmotic protection of the cell wall [22]. Other than bacterial cell walls, tea polyphenols also damaged the inner cyto-

plasmic membrane of *Serratia marcescens*, increasing its permeability and releasing small cellular molecules [23]. Moreover, polyphenols can exhibit antibacterial activity via anti-biofilm agents. Cranberry proanthocyanidins limited the motility—particularly swarming motility—and reduced the biofilm formation of *Pseudomonas aeruginosa* [24]. However, due to the structural diversity of polyphenol classes, the mechanisms of their antimicrobial activities have not yet been fully resolved.

3.4. Anti-Adipogenic Properties

Stimulating the development of beige adipocytes (so called 'browning') can reduce adverse obesity effects and help to improve metabolic health [25,26]. Dietary polyphenols have been demonstrated to effectively activate adipose tissue browning and relieve obesity and lipid accumulation through the induction of beige adipocytes. Daily ingestion of a catechin-rich beverage increases brown adipose tissue density in healthy young women, supporting the brown adipogenesis of polyphenols [27]. Also, in mice fed with a high energy diet, vanillic acid could accelerate thermogenesis and mitochondrial synthesis in both classical brown adipose tissue (BAT) and inguinal white adipose tissue (WAT) [28]. Resveratrol decreased triglycerides (TG) accumulation in the liver by suppressing the expression of adipogenesis-related genes, such as acetyl-CoA carboxylase (ACC), peroxisome proliferator-activated receptor (PPAR-γ), and sterol regulatory element binding protein (SREBP-1) [26,29]. Piceatannol treatment suppressed protein levels of the adipogenic transcription factors PPAR-γ, while it increased ACC protein expression [30]. Therefore, a positive relationship may exist between dietary polyphenol and anti-adipogenesis, and the underlying mechanisms are worthy of exploration.

3.5. Neuro-Protective Properties

The neuro-protective effects of dietary polyphenols have received considerable attention in recent years, suggesting that polyphenols may be effective in reversing neurodegenerative pathology and age-related declines in neurocognitive performance. Animal evidence demonstrates that blueberries are effective at reversing age-related deficits in rat spatial working memory, and (−)-epicatechin enhances the retention of mice spatial memory and may relate to their potential to influence the synthesis of neurotrophic factors [31,32]. In addition, curcumin could disrupt existing plaques and partially restore distorted neurites in an Alzheimer mouse model [33]. Resveratrol can activate the phosphorylation of protein kinase C and secretes transthyretin to prevent Aβ aggregation in cultured rat hippocampal cells [34]. However, a direct association between dietary polyphenol and an improvement in neurological health has not been made at present.

4. Impact of Dietary Polyphenols on Gut Microbiota

Emerging evidence demonstrates that gut microbiota plays an important role in maintaining the physiological function of host health and the pathogenesis of various diseases, including obesity, diabetes, inflammatory bowel disease, and even neurodegenerative disorders. Diet can alter the composition of gut microbiota, which in turn affects host metabolism. The alteration of gut microbiota by the administration of probiotics, prebiotics, or fecal microbiota transplantation is already well established. However, the gut microbiota-modulating effects of polyphenol are less clear. Nevertheless, there is growing evidence showing that dietary polyphenol may directly modulate the gut microbiome, i.e., increasing beneficial microbial or decreasing harmful microbial species in the gut microbiota. In this part, we summarize the in vitro and in vivo studies that studied the effects of polyphenol supplementation on the gut microbiota.

4.1. In Vitro Modulation of Dietary Polyphenols on Gut Microbiota

In vitro experiments on polyphenols and polyphenol-rich food sources have been studied through extraction, digestion, and fermentation to demonstrate that they could modulate the resident bacteria. A series of in vitro studies with polyphenol from different

sources have been listed in Table 1, including grapes, berries, tea, pomegranate, and other plants, to demonstrate the regulatory effect of polyphenol supplementation on intestinal micro-organisms.

Table 1. Study on the effect of polyphenols on gut microbiota in vitro.

Polyphenol and Source	Model	Impact on Microbiota	Reference
Flavonoids, Red wine	In vitro feces fermentation	Inhibit *Clostridium histolyticum* group	[35]
Grape polyphenol, Grape seeds	In vitro feces fermentation	Increase *Bifidobacterium* spp. and *Lactobacillus-Enterococcus* group; Inhibit *Clostridium histolyticum* group and the *Bacteroides-Prevotella* group	[36]
Ellagic acid and anthocyanins, Raspberry	In vitro colonic fermentation	Increase the abundance of *Escherichia coli*, butyric acid-producing bacteria, *Lactobacillus* and *Akkermansia*; Decrease *Bacteroides* and *Ruminococcus*.	[37]
Anthocyanins, flavonoids, neochlorogenic acids, tart cherry	The Simulator of the Human Intestinal Microbial Ecosystem	Increase *Bacteroidetes, Firmicutes, Proteobacteria* Decrease *Verrumicrobia*	[38]
Catechins and Flavonol, Black tea	The Simulator of the Human Intestinal Microbial Ecosystem	Increase *Klebsiella, enterococci, Akkermansia.* Reduce *bifidobacteria, B. coccoides, Anaeroglobus, Victivallis*	[39]
Green tea, oolong tea and black tea	In vitro fermentation Intestinal absorption	Increase *Bifidobacterium* spp., *Lactobacillus*/*Enterococcus* spp.; Decrease *Firmicutes*/*Bacteroidetes* ratio and *Clostridium histolyticum*	[40]
Ellagitannins, Pomegranate by-product	In vitro feces fermentation	Enhance *Bifidobacterium* spp. and *Lactobacillus* spp.	[41]
Mango peel	In vitro model of the colon	Enhance *Bifidobacterium* and *Lactobacillus*	[42]
Red fruit	In vitro fermentation	Decrease *B. cereus*, *S. aureus*, *E. coli*	[43]
Olive pomace	In vitro feces fermentation	Increase *Firmicutes* and *Bacteroidetes* groups	[44]
6-gingerols, Ginger	Simulated digestion model in vitro	Increase *Bifidobacterium* and *Enterococcus*	[45]
Proanthocyanidins, Sorghum bran	In vitro model of the colon	Increase *Bifidobacterium* spp., *Lactobacillus–Enterococcus* group; Decrease *Clostridium histolyticum* group, *Bacteroides–Prevotella* group	[46]

Polyphenols can selectively inhibit the growth of pathogenic bacteria. Flavonoids in red wine showed a slight inhibition of the *Clostridium* [35]. Ellagic acid and anthocyanins in raspberry juice may inhibit the growth of *Ruminococcus* [37]. Grape polyphenols can inhibit the growth of *Clostridium histolyticum* [36]. On the other hand, polyphenols can promote the growth of beneficial bacteria in the gut, such as *Bifidobacterium*. Tannin in pomegranate, gingerol in ginger, grape polyphenols, and sorghum polyphenols can promote the growth of *Bifidobacterium* [41,45,46]. Tannin can also promote the growth of *Lactobacillus* [45]. Gingerol and grape polyphenols can promote the growth of *Enterococci* [36,45]. Sorghum polyphenols can cooperate with fructooligosaccharides to enhance the abundance of lactic acid bacteria, *Roseburia*, and *Prevotella* [46]. However, Kemperman's research shows that polyphenols in red wine and black tea can reduce the abundance of *Bifidobacterium* [39]. They conducted in vitro experiments using fluids from the colon and found that catechins and flavonoids in black tea could stimulate *Klebsiella, Enterococci,* and *Akkermansia* and reduce *Bifidobacteria*, *B. coccoids, Anaeroglobus,* and *Victivallis*. Anthocyanins and catechins in red wine can promote the growth of *Klebsiella, Alistipes, Cloacibacillus, Victivallis,* and *Akkermansia*, and reduce the growth of *Bifidobacteria, B. coccoides, Anaeroglobus, Subdoligranulum,* and *Bacteroides* [39]. Mango peel is another high-polyphenol food, with gallates, flavonoids, gallotannins, gallic acid, and so on, and in vitro fermentation of mango peel could increase the growth of *Bifidobacterium* and *Lactobacillus*.

4.2. In Vivo Modulation of Dietary Polyphenols on Gut Microbiota of Animal Models

Similarly, in vivo studies have shown that polyphenol supplementation can modulate gut microbiota in animal models, including the increase of beneficial microbes and the decrease of harmful microbes. Detailed information on the published in vivo studies, from invertebrate *Drosophila* and zebrafish to vertebrate rat, mouse, chick, pig, etc., have been listed in Table 2. Both vertebrate and invertebrate model organisms confirmed that polyphenol supplementation can increase the number of beneficial bacteria in the gut, such as *Bifidobacterium* and *Lactobacillus*. Mango supplementation in mice fed with a high-fat diet can prevent the loss of beneficial intestinal bacteria, especially *Bifidobacteria, Akkermansia,* and *Aldercrutzia* [47]. Orso applied a diet of chestnut shell extract rich in tannin to a zebrafish intestinal inflammation model and found that it promoted the growth of healthy and beneficial bacteria (*Enterobacteriaceae* and *Pseudomonas*) [48]. Supplementation with polyphenols can also change the ratio of *Firmicutes* to *Bacteroides*. Cranberry extract is rich in phenolic acids, flavonoids, anthocyanins, and other polyphenols, which can reduce the ratio of *Firmicutes* to *Bacteroides* in mice induced by a high-fat/high-sugar diet [49]. Moreover, a polyphenol diet intervention can selectively inhibit pathogenic bacteria. Polyphenols from *Smilax china L. rhizome* can reduce the relative abundance of *Desulfovibrionaceae, Lachnospiraceae,* and *Streptococcaceae* [50], and grape pomace reduces potentially pathogenic bacteria to humans, such as *Salmonella, E. coli, Shigella, Yersinia,* and *Proteus* [51]. The combination of quercetin and resveratrol can significantly inhibit the relative abundance of *Desulfovibrionaceae, Acidaminococcaceae, Coriobacteriaceae, Bilophila,* and *Lachnospiraceae*, which may be related to diet-induced obesity [52]. Blueberry polyphenols were used to interfere with ovariectomized rats, with an upregulation of *Bacteroides dorei* and *Lachnoclostridium* and a decrease of *Rickenellaceae* and *Eubacterium* [53].

Table 2. Effect of polyphenols on animal gut microbiota.

Polyphenol and Source	Model	Impact on Microbiota	Reference
	Rat		
Epicatechin and catechin, Commercial	Wistar rats	Decrease *Bacteroides, Clostridium and Staphylococcus*	[54]
Quercetin and Resveratrol, Commercial	HFD (High-fat-diet) rats	Reduce *Firmicutes* and the proportion of *Firmicutes* to *Bacteroidetes*.	[52]
Sinapic acid and resveratrol, Commercial	HFD rats	Increase *Lachaospiraceae*; Decrease *Bacteroides and Desulfovibrionaceaesp*	[55]
Chlorogenic acid, Commercial	Wistar male rats	Increase *Burkholderiales, Bifidobacterium*; Decrease *Desulfovibrionales, Desulfovibrio, Klebsiella,*	[56]
Hesperetin, Commercial	Rats	Increase *Bifidobacterium, Lactobacillales*; Decrease *Clostridium subcluster XIVa*	[57]
Blueberry polyphenols, Blueberry	Rats	Reduce the *Firmicutes* to *Bacteroidetes* ratio; Increase *Proteobacteria Bacteroides dorei* and *Lachnoclostridium*.	[53]
Epicatechin and procyanidin, Cocoa	Male Zucker diabetic fatty rats	Increase acetate-producing bacteria such as *Blautia*; Prevent lactate-producing bacteria (*Enterococcus* and *Lactobacillus genera*)	[58]
Gallic acid	Rats	Increase *Lactobacillus, Bifidobacterium, Enterobacteriaceae*	[59]
Pomegranate peel	HFD rats	Decrease *Firmicutes* to *Bacteroidetes* ratio; Increase *Bacteroidales, Lactobacillus*	[60]
Persimmon tannin	Rats	Decrease *Firmicutes/Bacteroidetes* ratio; Increase *Bifidobacterium* spp., *Lactobacillus* spp	[61]
Seaweed polyphenols	HFD/streptozotocin rats	Increase *Odoribacter, Muribaculum, Parabacteroides*; Decrease *Firmicutes/Bacteroidetes* ratio	[62]
Phenolic acids, flavan-3-ols	A high salt diet fed rats	Increase *Bacteroidetes, Ruminococcaceae*; Decrease *Proteobacteria, Erysipelotrichaceae*	[63]
Ellagic acid, gallic acid, and quercetin-3-rutinoside	Colon cancer rats	Increase *Bacteroidetes*; Decrease *Firmicutes*	[64]

Table 2. *Cont.*

Polyphenol and Source	Model	Impact on Microbiota	Reference
	Mice		
Resveratrol, Commercial	HFD mice	Increase *Bacteroidetes*; Decrease *Firmicutes*	[65]
Chlorogenic acid, Commercial	HFD mice	Increase *Bacteroidaceae, Lactobacillaceae*; Decrease *Desulfovibrionaceae, Ruminococcaceae, Lachnospiraceae*	[66]
Tea polyphenols, Commercial	HFD mice	Increase *Actinobacteria*; Decrease *Proteobacteria*	[67]
Anthocyanins, Commercial	Mice	Increase *Lachnospiraceae*; Decrease *Bacilli, Clostridia*	[3]
Flavonoid apigenin, Commercial	Mice	Increase *Actinobacteria*; Decrease *Firmicutes*	[68]
Phenolic acids, flavonoids, anthocyanins, Cranberry	High fat/sucrose mice	Reduce the *Firmicutes* to *Bacteroidetes* ratio; Stimulate the growth of *Akkermansia* spp.	[49]
Caffeoylquinic acid, Quercetin, *Smilax china L. rhizome*	High fat/high sucrose mice	Decrease ratios of *Firmicutes* to *Bacteroidetes*; Increase *Desulfovibrionaceae, Streptococcaceae, Akkermansiaceae*	[50]
Betacyanins, Red pitayas	HFD mice	Decrease the ratio of *Firmicutes* to *Bacteroidetes*; Increase the relative abundance of *Akkermansia.*	[69]
Flavonoids, Painong-San	Colitis mice	Increase *Romboutsia, Lactobacillus, Bifidobacterium, Akkermansia*; Decrease *Oscillospiraceae, Helicobacter*	[70]
Gallic acid, *Canarium album*	HFD mice	Increase *Firmicutes, Verrucomicrobia, Akkermansia*; Decrease of *Bacteroidetes*	[71]
Gallic acid, anthocyanins, epicatechin, Berry	High-fat/sucrose mice	Increase *Akkermansiaceae*; Decrease *Firmicutes, Lachnospiraceae, Ruminococcaceae, Peptostreptococcaceae*	[72]
Flavonoid, Penthorum chinense pursh	Mice	Increase *Bacteroidetes, Proteobacteria, Verrucomicrobia*; Decrease *Firmicutes, Actinobacteria, Deferribacteres*	[73]
Grape polyphenols, Grape	Mice	Increase *Akkermansia, Lactobacillus*	[74]
Anthocyanins, Lycium ruthenicum Murray	Mice	Increase *Barnesiella, Alistipes, Eisenbergiella, Coprobacter, Odoribacter*	[75]
Camellia japonica bee pollen kaempferol	Oxonate-induced mice	Increase *Firmicutes*; Decrease *Bacteroidetes, Actinobacteria, Proteobacteria*	[76]
Ellagitannins, ellagic acid, anthocyanins, Raspberry	Mice	Increase *Lactobacillus*; Decrease *Blautia, Ruminiclostridium*	[37]
Anthocyanidins, Lycium ruthenicum	Mice	Increase *Verrucomicrobia, Bacteroidetes, Akkermansia, Odoribacter, Bifidobacterium*; Decrease *Firmicutes*	[77]
Tea polyphenol, Kombucha	HFD/streptozotocin mice	Increase *Lactobacillus, Butyricicoccus*; Decrease *Proteobacteria, Desulfovibrio, Escherichia-Shigella, Bacteroidetes*	[78]
3-hydroxybenzylhydrazine, isophorone, Millet shells	HFD mice	Increase *Bacteroidetes*; Decrease *Verrucomicrobia, Actinobacteria*	[79]
Tea polyphenol, Tea extract	Colitis Mice	Increase *Faecalibaculum, Bifidobacterium*; Decrease *Bacteroids, Mucispirillum*	[80]
Mango Polyphenols, Mango pulp	HFD mice	Prevent the loss of beneficial gut bacteria, specifically *Bifidobacteria, Akkermansia*, and *Aldercrutzia.*	[47]
Chlorogenic acid, Chicory root	Mice	Increase *Prevotellaceae, Lachnospiraceae bacterium* A2, *Clostridium* ASF356, Decrease *Oscillospirales, Ruminococcus*, the ratio *Firmicutes/Bacteroidetes*	[81]
	Pig		
Gallic acid, ethyl gallate, Red-osier dogwood	Pig	Increase class *Bacilli, Lactobacillales* and family *lactobacillaceae*	[82]
Proanthocyanidin, Grape seed	Pig	Increase *Lachnospiraceae, Clostridales, Lactobacillus* and *Ruminococcacceae.*	[83]
Chlorogenic acid, Commercial	Pig	Increase *Lactobacillus* spp., *Prevotella* spp., *Anaerovibrio* spp., *and Alloprevotella* spp.; Decrease *Proteobacteria*	[84]

Table 2. *Cont.*

Polyphenol and Source	Model	Impact on Microbiota	Reference
	Chick		
Procyanidins and anthocyanidins, Grape	Broiler chicks	Increase the populations of *Enterococcus*, *Escherichia coli*, *Lactobacillus*; Decrease the counts of *Clostridium*.	[85]
Pentagalloyl glucose, Eucalyptus	Broiler chicks	Increase the *Firmicutes* to *Bacteroidetes* ratio, *Verrucomicrobia*; Decrease *Actinobacteria*, *Proteobacteria*	[86]
Epicatechin and quercetin 3-glucoside, Carioca Bean	Broiler chicks	Increase *Coriobacteriaceae*, *Dehalobacteriaceae*, *Lachnospiraceae*	[87]
	Lamb		
Resveratrol, catechin, epicatechin, procyanidins, Grape pomace	Lambs	Enhance the growth of facultative probiotic bacteria and inhibit the growth of pathogen populations such as *Enterobacteriaceae* and *E. coli*.	[51]
	Zebrafish		
Tannins, Chestnut shells	Inflammation zebrafish	Increase the *Enterobacteriaceae*, *Pseudomonas* spp. and anaerobic bacteria (e.g., *Lactobacilli* and *Bifidobacteria*)	[48]
Dendrobium candidum	Inflammation zebrafish	Increase *Lactobacillus*, *Faecalibacterium*, *Rummeliibacillus*; Decrease *Shewanella*, *Geodermatophilus*	[88]
	Drosophila		
Eigallocatechin-3-gallate (EGCG), commercial	Rotenone-treated flies	Decrease *Proteobacteria*, *Acetobacter*, *Lactobacillus*; Increase the relative abundance of *Firmicutes* and *Bacteroidetes*	[89]

4.3. In Vivo Modulation of Dietary Polyphenols on Gut Microbiota of Humans

Clinical studies further confirmed the regulatory effect of polyphenols on human intestinal micro-organisms (Table 3). Consistent with in vitro and in vivo animal studies, supplementation with polyphenols such as anthocyanins and flavonoids increase the abundance of *Bifidobacterium* and *Lactobacillus*, which are two intestinal protective agents in the human gut [90,91]. Blueberries are rich in anthocyanins, which can increase the number of *Bifidobacteria* and lactic acid bacteria in healthy volunteers [92]. Almonds and almond skins are heavily rich in a range of flavonoids, including catechin, flavonol, and flavanone glycosides, and adding almonds or almond skins to the diet can increase the number of *Bifidobacteria* and *Lactobacillus* in feces [93]; Moreno-Indias found that polyphenols in red wine can increase the number of *Bifidobacteria* and *Lactobacillus* [94]. Besides, a diet rich in polyphenols can regulate the ratio of *Firmicutes* to *Bacteroides* in the human body. Daily consumption of cranberries rich in proanthocyanidins can reduce the number of *Firmicutes* in the body and increase the number of *Bacteroides* [95]; however, Yuan used tea polyphenols in tea to intervene in healthy volunteers and found different results. The diet that intervened with tea polyphenols resulted in an increase in the number of *Firmicutes* in feces, a decrease in the number of *Bacteroides*, and an increase in the ratio of *Firmicutes* to *Bacteroides* [96]. Queipo-Ortu found that the combined action of alcohol and polyphenols could increase the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *Blautia coccoides–Eubacterium*, but had no significant effect on the changes of *Lactobacillus* [97].

Table 3. Effect of polyphenols on human gut microbiota.

Polyphenol and Source	Impact on Microbiota	Reference
Anthocyanins, Blackcurrant	Increase *Lactobacilli, Bifidobacteria*; Decrease *Bacteroides* spp., *Clostridium* spp.	[90]
Flavanols, Cocoa	Increase *Bifidobacterial, Lactobacilli, E. rectale-C. coccoides*; Decrease *Clostridia*; While low–cocoa group: Increase *Clostridia, E. rectale-C. coccoides*	[91]
Proanthocyanins, Blueberry	Increase *Bifidobacterium, Prevotella* spp., *Bacteroides* spp., *Clostridium coccoides*; Decrease *Enterococcus* spp.	[92]
Flavonoid, Almond	Increase *Bifidobacterium* spp. and *Lactobacillus* spp.; Repress *pathogen Clostridum perfringens*	[93]
Red wine polyphenols	Increase *Bifidobacteria, Lactobacillus* and butyrate-producing (*Faecalibacterium prausnitzii* and *Roseburia*); Decrease Lipopolysaccharide (LPS)-producing (*Escherichia coli* and *Enterobacter cloacae*)	[94]
Proanthocyanidins, Cranberry	Increase abundance of *Bacteroidetes, Lachnospira* and *Anaerostipes*.; Decrease abundance of *Firmicutes, Clostridia, Oribacterium*	[95]
Catechins, Green tea	Increase *Firmicutes* and *Actinobacteria, Lachnospiraceae*.; Reduce *Bacteroidetes* and increase the FIR:BAC (*Firmicutes: Bacteroidetes*)	[96]
Red wine polyphenols	Increase the relative abundance of *Enterococcus, Prevotella, Bacteroides, Bifidobacterium, Bacteroides uniformis* groups	[97]
Anthocyanins, Tart cherry	High-Bacteroide: Increase *Lachnospiraceae, Ruminococcus, Collinsella*; Decrease *Bacteroides, Bifidobacterium*. Low-Bacteroides: Increase *Bacteroides* or *Prevotella* and *Bifidobacterium*; Decrease *Lachnospiraceae, Ruminococcus* and *Collinsella.*	[38]
Polyphenolic, *Schisandra chinensis*	Increase *Akkermansia, Roseburia, Bacteroides, Prevotella,* and *Bifidobacterium*	[98]
Increase	Increase *Clostridium, Lactobacillus, Faecalibacterium, Bifidobacterium*	[99]
Cocoa flavanols, Dark chocolate	Increase *Lactobacillus*; Decrease *Bacteroidetes*	[100]
Phenolic acids, Dietary raisin	Increase *Faecalibacterium prausnitzii, Bacteroidetes* spp., *Ruminococcus* spp.; Decrease *Klebsiella* spp., *Prevotella* spp., *Bifidobacterium* spp.	[101]
Apple polyphenol	Increase *Lactobacillus, Streptococcus*; Decrease *lecithinase-positive clostridia, Enterobacteriaceae, Pseudomonas*	[102]
Flavanones, Orange	Increase *Lactobacillus*; Decrease *Blautia coccoides*, *Clostridium leptum*	[103]

The effect of polyphenols on gut microbiota is related to the number of initial microbiota in the intestinal tract. Mayta-Apaza classified them according to the initial number of *Bacteroides* in the body, and different microbial compositions led to different performances after receiving a dietary intervention. After receiving sour cherry juice, the volunteers with high initial *Bacteroides* reduced *Bacteroides* and *Bifidobacterium* and increased the *Lachnospiraceae, Ruminococcus,* and potential polyphenol metabolite *Collinsella*. The volunteers with low *Bacteroides* responded to the increase of *Bacteroides* and *Bifidobacterium* and the decrease in the relative abundance of *Lachnospiraceae, Ruminococcus,* and *Collinsella* [38]. The effect of polyphenols on gut microbiota is related to the intake of polyphenols. Tzounis found that high-dose cocoa flavanone beverages increase the number of *Bifidobacterium*, lactic acid bacteria, and *Enterococci*; increase the number of *E.rectale–C.coccoides*; and reduce the number of *Histolytic Chlamydia*. A low dose of cocoa flavanone beverage will not cause a significant change in the number of *Bifidobacteria,* but will increase *Clostridia* [91].

5. Mechanism of Dietary Polyphenol and Gut Microbiota Affecting Host Health

The gut microbiota and the host maintain normal physiological function and morphology of the intestine by forming a mutually beneficial relationship. Gut microbiota not only play a bridge role between the diet and host in digesting dietary food complexes, but also yields short-chain fatty acids and other metabolites to regulate human health. Studies have shown that only a small portion of polyphenols (5–10% of the total polyphenol intake) are absorbed in the small intestine, while most (90–95% of the total polyphenol intake) are transported to the human large intestine [104]. Diet polyphenol can modulate the gut microbial composition, and, at the same time, gut microbiota also improve the bioavailability of polyphenols by converting them to bioavailable metabolites (Figure 2).

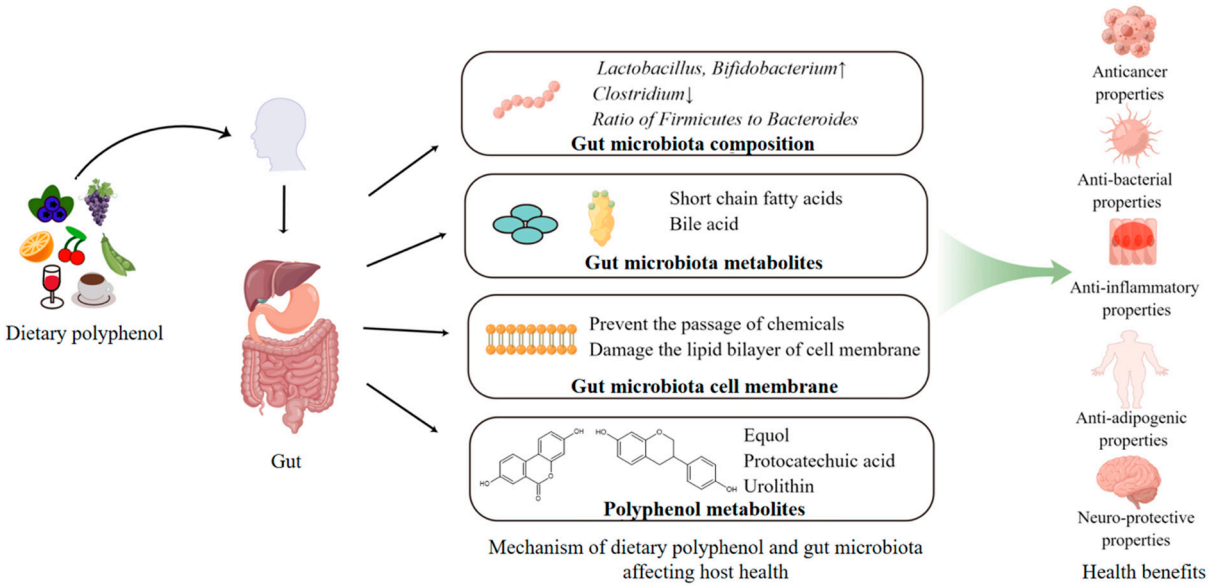

Figure 2. Possible mechanisms among dietary polyphenols, gut microbiota, and host health.

5.1. Dietary Polyphenols Affect the Composition of Gut Microbiota

Dietary polyphenol has a definite role in the composition and functional profile of the gut microbiota. Polyphenols promote the growth of beneficial microbes, such as *Lactobacillus* and *Bifidobacterium*, which are two major health beneficial probiotics and bring benefits to human health, such as improving gastrointestinal disorders, suppressing diarrhea and constipation [105], alleviating lactose intolerance [106], relieving irritable bowel symptoms [107], and preventing inflammatory bowel disease [108]. A systematic review by Ma et al. with a meta-analysis revealed that polyphenol supplementation profoundly increased the abundance of *Lactobacillus* by 220% and *Bifidobacterium* by 56%. On the other hand, polyphenols can inhibit the growth of harmful microbiota, and *Clostridium histolyticum* and *Clostridium perfringens* in *Clostridium* are common pathogenic bacteria. *Clostridium histolyticum* causes inflammatory bowel disease [5] and *Clostridium perfringens* produces many toxins and hydrolytic enzymes, which are related to gastrointestinal disease and necrotizing enteritis [109]. Ma's review system by meta-analysis showed that polyphenols derived from different foods all suppress the abundance of *Clostridium pathogen* species in the human gut microbiota, with tea being the most effective polyphenol food source for reducing *Clostridium* [110]. Dietary polyphenols can also regulate the ratio of *Firmicutes* to *Bacteroides*, which is related to body weight, and the ratio of *Firmicutes* to *Bacteroides* in obese patients is higher [111]. Xue's studies have shown that four dietary polyphenols, rutin, quercetin, chlorogenic acid, and caffeic acid, can reduce the ratio of *Firmicutes* to *Bacteroides* in in vitro gut microbiota experiments [112]. However, due to the different types of polyphenols, polyphenol dosage, and research methods, the results of different studies are different to some extent, resulting in the changes between microbes not being completely consistent.

5.2. Dietary Polyphenols Affect the Metabolites of Gut Microbiota

Short-chain fatty acids (SCFAs) are the most well-studied microbial metabolites so far. SCFAs are a saturated aliphatic organic acid [113] that are produced by the incomplete metabolism of plant-derived carbohydrates by intestinal flora present in an anaerobic environment [114]. Acetate, propionate, and butyrate are the main SCFAs in the gut (accounting for 90% of the total SCFAs) [115]. Wu's studies have shown that EGCG can significantly increase the number of SCFAs-producing bacteria, especially *Akkermansia*, and then promote the production of SCFAs, thereby enhancing anti-inflammatory effects and colon barrier integrity, which reduces enteritis [116]. Previous studies have shown that *Akkermansia muciniphila* can promote the production of acetate and propionate, and the nutritional interaction between *Akkermansia muciniphila* and butyrate-producing bacteria promotes butyrate production [117]. Liu's experiment showed that after a week-long intervention with an Aronia-berry-rich diet, the polyphenol diet extracted by Aronia berry was 57% higher than that in the control group [3]. In the human model intestinal system, the in vitro fermentation of wild cherry juice increased the microbial production of propionate and butyrate [118]. McDougall found that after ingesting anthocyanin-rich raspberry, the concentration of bile acid in an ideal fluid of ileostomy subjects changed significantly, wherein the glycine and taurine derivatives of cholate and deoxycholate increased [119]. Fotschki further described the beneficial effects of raspberry dregs on the bile acid profile of the cecum in a hyperlipidemic mouse model [120]. Studies by Huang have shown that EGCG can significantly reduce the content of intestinal bile acid; increase the excretion of bile acid, cholesterol, and total lipids in feces; and alleviate metabolic abnormalities and fatty liver induced by a high-fat diet in mice [121]. Therefore, after dietary polyphenols reach the gut, microbiota can then further produce metabolites, and, once absorbed and transported to target tissues and organs, contribute to metabolite health.

5.3. Dietary Polyphenols Affect the Bacterial Cell Membrane

Dietary polyphenol can interfere with the bacterial cell function of the cell membrane. For example, flavonols and flavones in the *Staphylococcus* genus can increase membrane cytoplasm permeability. Studies have shown that the antibacterial effect of polyphenols is more effective against Gram-positive bacteria. Inouye pointed out that because of the hydrophilic outer membrane outside the cell wall of Gram-negative bacteria, the passage of chemicals is prevented. Gram-negative bacteria are more resistant to plant secondary metabolites, including phenols [122]. When polyphenols were ingested, the growth of Gram-negative *Salmonella* and *Escherichia* strains was inhibited, but the growth of Gram-positive lactic acid bacteria was not affected [123]. The effect of polyphenols on bacteria depends on the interaction between compounds and the bacterial cell surface, which can inhibit bacterial growth by disturbing the function of the cell membrane [124]. Tea polyphenols, such as tea catechins, have a strong affinity to the lipid bilayers of the cell membrane through hydrogen bonds with the bilayer surface, thus penetrating underneath the surface and giving play to antibacterial, anticancer, and other beneficial effects [125]. EGCG has antibacterial activity against Staphylococcus; possible mechanisms include damaging the lipid bilayer of the cell membrane, reducing mucus production and affecting the formation of biofilm, and binding and neutralizing with enterotoxin B [126]. Therefore, the effect of polyphenols on the bacterial cell membranes is considered to be one of the mechanisms for regulating metabolic health.

5.4. Biotransformation of Polyphenols by Gut Microbiota

With respect to the complicated structures and high molecular weights, dietary polyphenols have low bioavailability and are difficult to be absorbed in the small intestine. About 90% of dietary polyphenols arrive at the colon in an intact form where they are biotransformed and metabolized into bioactive, low-molecular-weight phenolic metabolites through the residing microbiota [127]. Chen discovered that gut bacteria can deconjugate mulberry anthocyanin (cyanidin-3-glucoside, cyanidin-3-rutin, and delphinidin-

3-rutinoside) to lower molecular-weight metabolites, and metabonomic data showed that the first two compounds were decomposed into protocatechuic, vanillic acid, and p-coumaric acids, while the latter was converted to syringic acid and gallic acid [128,129]. The core bacteria that can metabolize anthocyanins are *Bifidobacterium* spp. and *Lactobacillus* spp. [130,131] with probiotic effects to produce antibacterial substances, to compete with pathogens for adhering to the epithelium and for nutrients, to regulate the host immune system, and to inhibit the production of bacterial toxins [132]. The flavonoids (flavonols, flavones, and flavanones) can be biotransformed into p-hydroxyphenylacetic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, hydrocaffeic acid, coumaric acid, 3-(4-hydroxyphenyl) propionic acid, and other aromatic metabolites [133]. Soybean isoflavones can be converted to dihydrodaidzein, dihydrogenistein,6′-OH-*O*-desmethylangolensin, and cis-4-OH-equol by anaerobic bacteria in the distal region of the small intestine and colon [134–136]. The bioavailability of ellagic tannin, which was found in pomegranate and grape, is low, but they can be metabolized by intestinal micro-organisms into urolithins with antioxidant activity and preventive effects for chronic diseases such as cancer, diabetes, and cardiovascular and neurodegenerative diseases [137,138]. Therefore, polyphenol metabolites produced by gut microbiota have potentially beneficial effects on the host.

6. Conclusions

There is increasing evidence in the literature to emphasize that dietary polyphenols have potentially beneficial effects on host health through interactions with gut microbiota. Numerous studies listed in this review, both in vitro and in vivo, demonstrated the relationship between dietary polyphenols and gut microbiota, while the possible mechanism may be through the alteration of gut microbiota composition, the production of gut microbiota metabolites, the modulation of intestinal barrier function, and the biotransformation and metabolism of dietary polyphenols. However, a clear and deep understanding of these mechanisms between polyphenols and gut microbiota is necessitated, especially considering the metabolic pathways, which will allow for new therapeutic targets in the future.

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References

1. Aravind, S.M.; Wichienchot, S.; Tsao, R.; Ramakrishnan, S.; Chakkaravarthi, S. Role of dietary polyphenols on gut microbiota, their metabolites and health benefits. *Food Res. Int.* **2021**, *142*, 110189. [CrossRef] [PubMed]
2. Scalbert, A.; Johnson, I.T.; Saltmarsh, M. Polyphenols: Antioxidants and beyond. *Am. J. Clin. Nutr.* **2005**, *81*, 215S–217S. [CrossRef] [PubMed]
3. Liu, X.; Martin, D.A.; Valdez, J.C.; Sudakaran, S.; Rey, F.; Bolling, B.W. Aronia berry polyphenols have matrix-dependent effects on the gut microbiota. *Food Chem.* **2021**, *359*, 129831. [CrossRef] [PubMed]
4. Aso, T. Equol Improves Menopausal Symptoms in Japanese Women. *J. Nutr.* **2010**, *140*, 1386S–1389S. [CrossRef] [PubMed]
5. Ma, G.; Chen, Y. Polyphenol supplementation benefits human health via gut microbiota: A systematic review via meta-analysis. *J. Funct. Foods* **2020**, *66*, 103829. [CrossRef]
6. Papuc, C.; Goran, G.V.; Predescu, C.N.; Nicorescu, V.; Stefan, G. Plant Polyphenols as Antioxidant and Antibacterial Agents for Shelf-Life Extension of Meat and Meat Products: Classification, Structures, Sources, and Action Mechanisms. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 1243–1268. [CrossRef]
7. Kamiloglu, S.; Tomas, M.; Ozdal, T.; Capanoglu, E. Effect of food matrix on the content and bioavailability of flavonoids. *Trends Food Sci. Technol.* **2021**, *117*, 15–33. [CrossRef]
8. Singla, R.K.; Dubey, A.K.; Garg, A.; Sharma, R.K.; Fiorino, M.; Ameen, S.M.; Haddad, M.A.; Al-Hiary, M. Natural Polyphenols: Chemical Classification, Definition of Classes, Subcategories, and Structures. *J. AOAC Int.* **2019**, *102*, 1397–1400. [CrossRef]
9. Sigurdson, G.T.; Robbins, R.J.; Collins, T.M.; Giusti, M.M. Impact of location, type, and number of glycosidic substitutions on the color expression of o-dihydroxylated anthocyanidins. *Food Chem.* **2018**, *268*, 416–423. [CrossRef]

10. Šamec, D.; Karalija, E.; Šola, I.; Vujčić Bok, V.; Salopek-Sondi, B. The role of polyphenols in abiotic stress response: The influence of molecular structure. *Plants* **2021**, *10*, 118. [CrossRef]
11. Wu, X.; Li, M.; Xiao, Z.; Daglia, M.; Dragan, S.; Delmas, D.; Vong, C.T.; Wang, Y.; Zhao, Y.; Shen, J.; et al. Dietary polyphenols for managing cancers: What have we ignored? *Trends Food Sci. Technol.* **2020**, *101*, 150–164. [CrossRef]
12. Tsao, R. Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients* **2010**, *2*, 1231–1246. [CrossRef] [PubMed]
13. Mi, Y.; Zhang, W.; Tian, H.; Li, R.; Huang, S.; Li, X.; Qi, G.; Liu, X. EGCG evokes Nrf2 nuclear translocation and dampens PTP1B expression to ameliorate metabolic misalignment under insulin resistance condition. *Food Funct.* **2018**, *9*, 1510–1523. [CrossRef] [PubMed]
14. Palsamy, P.; Subramanian, S. Modulatory effects of resveratrol on attenuating the key enzymes activities of carbohydrate metabolism in streptozotocin–nicotinamide-induced diabetic rats. *Chem. Biol. Interact.* **2009**, *179*, 356–362. [CrossRef]
15. Liu, T.; Zhang, L.; Joo, D.; Sun, S.-C. NF-κB signaling in inflammation. *Signal Transduct. Target. Ther.* **2017**, *2*, 17023. [CrossRef]
16. Opal, S.M.; DePalo, V.A. Anti-Inflammatory Cytokines. *Chest* **2000**, *117*, 1162–1172. [CrossRef]
17. Zhang, H.; Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **2016**, *8*, 33–42. [CrossRef]
18. Li, H.; Christman, L.M.; Li, R.; Gu, L. Synergic interactions between polyphenols and gut microbiota in mitigating inflammatory bowel diseases. *Food Funct.* **2020**, *11*, 4878–4891. [CrossRef]
19. Monagas, M.; Khan, N.; Andrés-Lacueva, C.; Urpí-Sardá, M.; Vázquez-Agell, M.; Lamuela-Raventós, R.M.; Estruch, R. Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells. *Br. J. Nutr.* **2009**, *102*, 201–206. [CrossRef]
20. Stewart, L.K.; Soileau, J.L.; Ribnicky, D.; Wang, Z.Q.; Raskin, I.; Poulev, A.; Majewski, M.; Cefalu, W.T.; Gettys, T.W. Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet. *Metabolism* **2008**, *57*, S39–S46. [CrossRef]
21. Rivera, L.; Morón, R.; Sánchez, M.; Zarzuelo, A.; Galisteo, M. Quercetin Ameliorates Metabolic Syndrome and Improves the Inflammatory Status in Obese Zucker Rats. *Obesity* **2008**, *16*, 2081–2087. [CrossRef] [PubMed]
22. Zhao, W.-H.; Hu, Z.-Q.; Hara, Y.; Shimamura, T. Inhibition of Penicillinase by Epigallocatechin Gallate Resulting in Restoration of Antibacterial Activity of Penicillin against Penicillinase-Producing *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **2002**, *46*, 2266–2268. [CrossRef] [PubMed]
23. Yi, S.; Wang, W.; Bai, F.; Zhu, J.; Li, J.; Li, X.; Xu, Y.; Sun, T.; He, Y. Antimicrobial effect and membrane-active mechanism of tea polyphenols against Serratia marcescens. *World J. Microbiol. Biotechnol.* **2014**, *30*, 451–460. [CrossRef] [PubMed]
24. Ulrey, R.K.; Barksdale, S.M.; Zhou, W.; Van Hoek, M.L. Cranberry proanthocyanidins have anti-biofilm properties against Pseudomonas aeruginosa. *BMC Complement. Altern. Med.* **2014**, *14*, 499. [CrossRef]
25. Bartelt, A.; Heeren, J. Adipose tissue browning and metabolic health. *Nat. Rev. Endocrinol.* **2014**, *10*, 24–36. [CrossRef]
26. Hu, J.; Wang, Z.; Tan, B.K.; Christian, M. Dietary polyphenols turn fat "brown": A narrative review of the possible mechanisms. *Trends Food Sci. Technol.* **2020**, *97*, 221–232. [CrossRef]
27. Nirengi, S.; Amagasa, S.; Homma, T.; Yoneshiro, T.; Matsumiya, S.; Kurosawa, Y.; Sakane, N.; Ebi, K.; Saito, M.; Hamaoka, T. Daily ingestion of catechin-rich beverage increases brown adipose tissue density and decreases extramyocellular lipids in healthy young women. *SpringerPlus* **2016**, *5*, 1363. [CrossRef]
28. Han, X.; Guo, J.; You, Y.; Yin, M.; Liang, J.; Ren, C.; Zhan, J.; Huang, W. Vanillic acid activates thermogenesis in brown and white adipose tissue. *Food Funct.* **2018**, *9*, 4366–4375. [CrossRef]
29. Andrade, J.M.O.; Paraíso, A.F.; de Oliveira, M.V.M.; Martins, A.M.; Neto, J.F.; Guimaraes, A.; de Paula, A.M.; Qureshi, M.; Santos, S.H.S. Resveratrol attenuates hepatic steatosis in high-fat fed mice by decreasing lipogenesis and inflammation. *Nutrition* **2014**, *30*, 915–919. [CrossRef]
30. Tung, Y.-C.; Lin, Y.-H.; Chen, H.-J.; Chou, S.-C.; Cheng, A.-C.; Kalyanam, N.; Ho, C.-T.; Pan, M.-H. Piceatannol Exerts Anti-Obesity Effects in C57BL/6 Mice through Modulating Adipogenic Proteins and Gut Microbiota. *Molecules* **2016**, *21*, 1419. [CrossRef]
31. Casadesus, G.; Shukitt-Hale, B.; Stellwagen, H.M.; Zhu, X.; Lee, H.-G.; Smith, M.A.; Joseph, J.A. Modulation of Hippocampal Plasticity and Cognitive Behavior by Short-term Blueberry Supplementation in Aged Rats. *Nutr. Neurosci.* **2004**, *7*, 309–316. [CrossRef] [PubMed]
32. Van Praag, H.; Lucero, M.J.; Yeo, G.W.; Stecker, K.; Heivand, N.; Zhao, C.; Yip, E.; Afanador, M.; Schroeter, H.; Hammerstone, J.; et al. Plant-Derived Flavanol (-)Epicatechin Enhances Angiogenesis and Retention of Spatial Memory in Mice. *J. Neurosci.* **2007**, *27*, 5869–5878. [CrossRef] [PubMed]
33. Garcia-Alloza, M.; Borrelli, L.A.; Rozkalne, A.; Hyman, B.T.; Bacskai, B.J. Curcumin labels amyloid pathology *in vivo*, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. *J. Neurochem.* **2007**, *102*, 1095–1104. [CrossRef] [PubMed]
34. Dasgupta, B.; Milbrandt, J. Resveratrol stimulates AMP kinase activity in neurons. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7217–7222. [CrossRef]
35. Sánchez-Patán, F.; Cueva, C.; Monagas, M.; Walton, G.E.; Gibson, G.R.; Quintanilla-López, J.E.; Lebrón-Aguilar, R.; Martín-Álvarez, P.J.; Moreno-Arribas, M.; Bartolomé, B. In Vitro Fermentation of a Red Wine Extract by Human Gut Microbiota: Changes in Microbial Groups and Formation of Phenolic Metabolites. *J. Agric. Food Chem.* **2012**, *60*, 2136–2147. [CrossRef]

36. Zhou, L.; Wang, W.; Huang, J.; Ding, Y.; Pan, Z.; Zhao, Y.; Zhang, R.; Hu, B.; Zeng, X. In vitro extraction and fermentation of polyphenols from grape seeds (Vitis vinifera) by human intestinal microbiota. *Food Funct.* **2016**, *7*, 1959–1967. [CrossRef]
37. Wu, T.; Chu, X.; Cheng, Y.; Tang, S.; Zogona, D.; Pan, S.; Xu, X. Modulation of Gut Microbiota by *Lactobacillus casei* Fermented Raspberry Juice In Vitro and In Vivo. *Foods* **2021**, *10*, 3055. [CrossRef]
38. Mayta-Apaza, A.C.; Pottgen, E.; De Bodt, J.; Papp, N.; Marasini, D.; Howard, L.; Abranko, L.; Van de Wiele, T.; Lee, S.-O.; Carbonero, F. Impact of tart cherries polyphenols on the human gut microbiota and phenolic metabolites in vitro and in vivo. *J. Nutr. Biochem.* **2018**, *59*, 160–172. [CrossRef]
39. Kemperman, R.A.; Gross, G.; Mondot, S.; Possemiers, S.; Marzorati, M.; Van de Wiele, T.; Doré, J.; Vaughan, E.E. Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. *Food Res. Int.* **2013**, *53*, 659–669. [CrossRef]
40. Xu, M.; Yang, K.; Zhu, J. Monitoring the Diversity and Metabolic Shift of Gut Microbes during Green Tea Feeding in an In Vitro Human Colonic Model. *Molecules* **2020**, *25*, 5101. [CrossRef]
41. Bialonska, D.; Ramnani, P.; Kasimsetty, S.G.; Muntha, K.R.; Gibson, G.R.; Ferreira, D. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *Int. J. Food Microbiol.* **2010**, *140*, 175–182. [CrossRef] [PubMed]
42. Sáyago-Ayerdi, S.G.; Venema, K.; Tabernero, M.; Sarriá, B.; Bravo, L.L.; Mateos, R. Bioconversion by gut microbiota of predigested mango (Mangifera indica L) 'Ataulfo' peel polyphenols assessed in a dynamic (TIM-2) in vitro model of the human colon. *Food Res. Int.* **2021**, *139*, 109963. [CrossRef] [PubMed]
43. Jiao, X.; Wang, Y.; Lin, Y.; Lang, Y.; Li, E.; Zhang, X.; Zhang, Q.; Feng, Y.; Meng, X.; Li, B. Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. *J. Nutr. Biochem.* **2019**, *64*, 88–100. [CrossRef] [PubMed]
44. Coman, M.M.; Oancea, A.M.; Verdenelli, M.C.; Cecchini, C.; Bahrim, G.E.; Orpianesi, C.; Cresci, A.; Silvi, S. Polyphenol content and in vitro evaluation of antioxidant, antimicrobial and prebiotic properties of red fruit extracts. *Eur. Food Res. Technol.* **2017**, *244*, 735–745. [CrossRef]
45. Wang, J.; Chen, Y.; Hu, X.; Feng, F.; Cai, L.; Chen, F. Assessing the Effects of Ginger Extract on Polyphenol Profiles and the Subsequent Impact on the Fecal Microbiota by Simulating Digestion and Fermentation In Vitro. *Nutrients* **2020**, *12*, 3194. [CrossRef]
46. Sost, M.M.; Ahles, S.; Verhoeven, J.; Verbruggen, S.; Stevens, Y.; Venema, K. A Citrus Fruit Extract High in Polyphenols Beneficially Modulates the Gut Microbiota of Healthy Human Volunteers in a Validated In Vitro Model of the Colon. *Nutrients* **2021**, *13*, 3915. [CrossRef]
47. Ojo, B.; El-Rassi, G.D.; Payton, M.E.; Perkins-Veazie, P.; Clarke, S.; Smith, B.J.; Lucas, E.A. Mango Supplementation Modulates Gut Microbial Dysbiosis and Short-Chain Fatty Acid Production Independent of Body Weight Reduction in C57BL/6 Mice Fed a High-Fat Diet. *J. Nutr.* **2016**, *146*, 1483–1491. [CrossRef]
48. Orso, G.; Solovyev, M.M.; Facchiano, S.; Tyrikova, E.; Sateriale, D.; Kashinskaya, E.; Pagliarulo, C.; Hoseinifar, H.S.; Simonov, E.; Varricchio, E.; et al. Chestnut Shell Tannins: Effects on Intestinal Inflammation and Dysbiosis in Zebrafish. *Animals* **2021**, *11*, 1538. [CrossRef]
49. Anhê, F.F.; Roy, D.; Pilon, G.; Dudonné, S.; Matamoros, S.; Varin, T.V.; Garofalo, C.; Moine, Q.; Desjardins, Y.; Levy, E.; et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. *Gut* **2014**, *64*, 872–883. [CrossRef]
50. Li, X.; Yang, L.; Xu, M.; Qiao, G.; Li, C.; Lin, L.; Zheng, G. Smilax china L. polyphenols alleviates obesity and inflammation by modulating gut microbiota in high fat/high sucrose diet-fed C57BL/6J mice. *J. Funct. Foods* **2021**, *77*, 104332. [CrossRef]
51. Kafantaris, I.; Kotsampasi, B.; Christodoulou, V.; Kokka, E.; Kouka, P.; Terzopoulou, Z.; Gerasopoulos, K.; Stagos, D.; Mitsagga, C.; Giavasis, I.; et al. Grape pomace improves antioxidant capacity and faecal microflora of lambs. *J. Anim. Physiol. Anim. Nutr.* **2017**, *101*, e108–e121. [CrossRef] [PubMed]
52. Zhao, L.; Zhang, Q.; Ma, W.; Tian, F.; Shen, H.; Zhou, M. A combination of quercetin and resveratrol reduces obesity in high-fat diet-fed rats by modulation of gut microbiota. *Food Funct.* **2017**, *8*, 4644–4656. [CrossRef] [PubMed]
53. Cladis, D.P.; Simpson, A.M.R.; Cooper, K.J.; Nakatsu, C.H.; Ferruzzi, M.G.; Weaver, C.M. Blueberry polyphenols alter gut microbiota & phenolic metabolism in rats. *Food Funct.* **2021**, *12*, 2442–2456. [CrossRef] [PubMed]
54. Massot-Cladera, M.; Pérez-Berezo, T.; Franch, A.; Castell, M.; Pérez-Cano, F.J. Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk. *Arch. Biochem. Biophys.* **2012**, *527*, 105–112. [CrossRef]
55. Yang, C.; Deng, Q.; Xu, J.; Wang, X.; Hu, C.; Tang, H.; Huang, F. Sinapic acid and resveratrol alleviate oxidative stress with modulation of gut microbiota in high-fat diet-fed rats. *Food Res. Int.* **2019**, *116*, 1202–1211. [CrossRef]
56. Song, J.; Zhou, N.; Ma, W.; Gu, X.; Chen, B.; Zeng, Y.; Yang, L.; Zhou, M. Modulation of gut microbiota by chlorogenic acid pretreatment on rats with adrenocorticotropic hormone induced depression-like behavior. *Food Funct.* **2019**, *10*, 2947–2957. [CrossRef]
57. Unno, T.; Hisada, T.; Takahashi, S. Hesperetin Modifies the Composition of Fecal Microbiota and Increases Cecal Levels of Short-Chain Fatty Acids in Rats. *J. Agric. Food Chem.* **2015**, *63*, 7952–7957. [CrossRef]
58. Álvarez-Cilleros, D.; Ramos, S.; López-Oliva, M.E.; Escrivá, F.; Álvarez, C.; Fernández-Millán, E.; Martín, M.Á. Cocoa diet modulates gut microbiota composition and improves intestinal health in Zucker diabetic rats. *Food Res. Int.* **2020**, *132*, 109058. [CrossRef]

59. da Silva-Maia, J.K.; Batista, A.G.; Correa, L.C.; Lima, G.C.; Bogusz, S.B., Jr.; Marostica, M.R., Jr. Aqueous extract of berry (*Plinia jaboticaba*) byproduct modulates gut microbiota and maintains the balance on antioxidant defense system in rats. *J. Food Biochem.* **2019**, *43*, e12705. [CrossRef]
60. Zhao, R.; Long, X.; Yang, J.; Du, L.; Zhang, X.; Li, J.; Hou, C. Pomegranate peel polyphenols reduce chronic low-grade inflammatory responses by modulating gut microbiota and decreasing colonic tissue damage in rats fed a high-fat diet. *Food Funct.* **2019**, *10*, 8273–8285. [CrossRef]
61. Zhu, W.; Lin, K.; Li, K.; Deng, X.; Li, C. Reshaped fecal gut microbiota composition by the intake of high molecular weight persimmon tannin in normal and high-cholesterol diet-fed rats. *Food Funct.* **2018**, *9*, 541–551. [CrossRef] [PubMed]
62. Yuan, Y.; Zheng, Y.; Zhou, J.; Geng, Y.; Zou, P.; Li, Y.; Zhang, C. Polyphenol-Rich Extracts from Brown Macroalgae *Lessonia trabeculate* Attenuate Hyperglycemia and Modulate Gut Microbiota in High-Fat Diet and Streptozotocin-Induced Diabetic Rats. *J. Agric. Food Chem.* **2019**, *67*, 12472–12480. [CrossRef] [PubMed]
63. Gomes, A.; Oudot, C.; Macià, A.; Foito, A.; Carregosa, D.; Stewart, D.; Van De Wiele, T.; Berry, D.; Motilva, M.-J.; Brenner, C.; et al. Berry-Enriched Diet in Salt-Sensitive Hypertensive Rats: Metabolic Fate of (Poly)Phenols and the Role of Gut Microbiota. *Nutrients* **2019**, *11*, 2634. [CrossRef]
64. Fidelis, M.; Santos, J.S.; Escher, G.B.; Rocha, R.S.; Cruz, A.G.; Cruz, T.M.; Marques, M.B.; Nunes, J.B.; do Carmo, M.A.V.D.; de Almeida, L.A.; et al. Polyphenols of jabuticaba [Myrciaria jaboticaba (Vell.) O.Berg] seeds incorporated in a yogurt model exert antioxidant activity and modulate gut microbiota of 1,2-dimethylhydrazine-induced colon cancer in rats. *Food Chem.* **2021**, *334*, 127565. [CrossRef] [PubMed]
65. Wang, P.; Li, D.; Ke, W.; Liang, D.; Hu, X.; Chen, F. Resveratrol-induced gut microbiota reduces obesity in high-fat diet-fed mice. *Int. J. Obes.* **2020**, *44*, 213–225. [CrossRef]
66. Wang, Z.; Lam, K.-L.; Hu, J.; Ge, S.; Zhou, A.; Zhou, A.; Zheng, B.; Zeng, S.; Lin, S. Chlorogenic acid alleviates obesity and modulates gut microbiota in high-fat-fed mice. *Food Sci. Nutr.* **2019**, *7*, 579–588. [CrossRef]
67. Ma, H.; Zhang, B.; Hu, Y.; Wang, J.; Liu, J.-M.; Qin, R.; Lv, S.; Wang, S. Correlation Analysis of Intestinal Redox State with the Gut Microbiota Reveals the Positive Intervention of Tea Polyphenols on Hyperlipidemia in High Fat Diet Fed Mice. *J. Agric. Food Chem.* **2019**, *67*, 7325–7335. [CrossRef]
68. Bian, S.; Wan, H.; Liao, X.; Wang, W. Inhibitory Effects of Apigenin on Tumor Carcinogenesis by Altering the Gut Microbiota. *Mediat. Inflamm.* **2020**, *2020*, 7141970. [CrossRef]
69. Song, H.; Chu, Q.; Yan, F.; Yang, Y.; Han, W.; Zheng, X. Red pitaya betacyanins protects from diet-induced obesity, liver steatosis and insulin resistance in association with modulation of gut microbiota in mice. *J. Gastroenterol. Hepatol.* **2016**, *31*, 1462–1469. [CrossRef]
70. Wang, K.; Guo, J.; Chang, X.; Gui, S. Painong-San extract alleviates dextran sulfate sodium-induced colitis in mice by modulating gut microbiota, restoring intestinal barrier function and attenuating TLR4/NF-κB signaling cascades. *J. Pharm. Biomed. Anal.* **2022**, *209*, 114529. [CrossRef]
71. Zhang, N.-N.; Guo, W.-H.; Hu, H.; Zhou, A.-R.; Liu, Q.-P.; Zheng, B.-D.; Zeng, S.-X. Effect of A Polyphenol-Rich Canarium album Extract on the Composition of the Gut Microbiota of Mice Fed a High-Fat Diet. *Molecules* **2018**, *23*, 2188. [CrossRef] [PubMed]
72. Rodríguez-Daza, M.-C.; Roquim, M.; Dudonné, S.; Pilon, G.; Levy, E.; Marette, A.; Roy, D.; Desjardins, Y. Berry Polyphenols and Fibers Modulate Distinct Microbial Metabolic Functions and Gut Microbiota Enterotype-Like Clustering in Obese Mice. *Front. Microbiol.* **2020**, *11*, 2032. [CrossRef] [PubMed]
73. Yin, J.; Ren, W.; Wei, B.; Huang, H.; Li, M.; Wu, X.; Wang, A.; Xiao, Z.; Shen, J.; Zhao, Y.; et al. Characterization of chemical composition and prebiotic effect of a dietary medicinal plant Penthorum chinense Pursh. *Food Chem.* **2020**, *319*, 126568. [CrossRef]
74. Lu, F.; Li, Y.; Wang, X.; Hu, X.; Liao, X.; Zhang, Y. Early-life polyphenol intake promotes Akkermansia growth and increase of host goblet cells in association with the potential synergistic effect of Lactobacillus. *Food Res. Int.* **2021**, *149*, 110648. [CrossRef] [PubMed]
75. Peng, Y.; Yan, Y.; Wan, P.; Dong, W.; Huang, K.; Ran, L.; Mi, J.; Lu, L.; Zeng, X.; Cao, Y. Effects of long-term intake of anthocyanins from Lycium ruthenicum Murray on the organism health and gut microbiota in vivo. *Food Res. Int.* **2020**, *130*, 108952. [CrossRef] [PubMed]
76. Xu, Y.; Cao, X.; Zhao, H.; Yang, E.; Wang, Y.; Cheng, N.; Cao, W. Impact of *Camellia japonica* Bee Pollen Polyphenols on Hyperuricemia and Gut Microbiota in Potassium Oxonate-Induced Mice. *Nutrients* **2021**, *13*, 2665. [CrossRef]
77. Tian, B.; Zhao, J.; An, W.; Zhang, J.; Cao, X.; Mi, J.; Zhao, J.; Zhang, Y.; Li, J. Lycium ruthenicum diet alters the gut microbiota and partially enhances gut barrier function in male C57BL/6 mice. *J. Funct. Foods* **2019**, *52*, 516–528. [CrossRef]
78. Xu, S.; Wang, Y.; Wang, J.; Geng, W. Kombucha Reduces Hyperglycemia in Type 2 Diabetes of Mice by Regulating Gut Microbiota and Its Metabolites. *Foods* **2022**, *11*, 754. [CrossRef]
79. Liu, F.; Shan, S.; Li, H.; Shi, J.; Hao, R.; Yang, R.; Li, Z. Millet shell polyphenols prevent atherosclerosis by protecting the gut barrier and remodeling the gut microbiota in ApoE$^{-/-}$ mice. *Food Funct.* **2021**, *12*, 7298–7309. [CrossRef]
80. Liu, Y.; Wang, X.; Chen, Q.; Luo, L.; Ma, M.; Xiao, B.; Zeng, L. *Camellia sinensis* and *Litsea coreana* Ameliorate Intestinal Inflammation and Modulate Gut Microbiota in Dextran Sulfate Sodium-Induced Colitis Mice. *Mol. Nutr. Food Res.* **2020**, *64*, e1900943. [CrossRef]
81. Pouille, C.L.; Ouaza, S.; Roels, E.; Behra, J.; Tourret, M.; Molinié, R.; Fontaine, J.-X.; Mathiron, D.; Gagneul, D.; Taminiau, B.; et al. Chicory: Understanding the Effects and Effectors of This Functional Food. *Nutrients* **2022**, *14*, 957. [CrossRef] [PubMed]

82. Zheng, S.; Song, J.; Qin, X.; Yang, K.; Liu, M.; Yang, C.; Nyachoti, C.M. Dietary supplementation of red-osier dogwood polyphenol extract changes the ileal microbiota structure and increases Lactobacillus in a pig model. *AMB Express* **2021**, *11*, 145. [CrossRef] [PubMed]
83. Choy, Y.Y.; Quifer-Rada, P.; Holstege, D.M.; Frese, S.A.; Calvert, C.C.; Mills, D.A.; Lamuela-Raventos, R.M.; Waterhouse, A.L. Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins. *Food Funct.* **2014**, *5*, 2298–2308. [CrossRef]
84. Chen, J.; Yu, B.; Chen, D.; Zheng, P.; Luo, Y.; Huang, Z.; Luo, J.; Mao, X.; Yu, J.; He, J. Changes of porcine gut microbiota in response to dietary chlorogenic acid supplementation. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 8157–8168. [CrossRef] [PubMed]
85. Viveros, A.; Chamorro, S.; Pizarro, M.; Arija, I.; Centeno, C.; Brenes, A. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. *Poult. Sci.* **2011**, *90*, 566–578. [CrossRef]
86. Li, W.; Zhang, X.; He, Z.; Chen, Y.; Li, Z.; Meng, T.; Li, Y.; Cao, Y. In vitro and in vivo antioxidant activity of eucalyptus leaf polyphenols extract and its effect on chicken meat quality and cecum microbiota. *Food Res. Int.* **2020**, *136*, 109302. [CrossRef]
87. Dias, D.M.; Kolba, N.; Binyamin, D.; Ziv, O.; Regini Nutti, M.; Martino, H.S.D.; Glahn, R.P.; Koren, O.; Tako, E. Iron Biofortified Carioca Bean (*Phaseolus vulgaris* L.)—Based Brazilian Diet Delivers More Absorbable Iron and Affects the Gut Microbiota In Vivo (Gallus gallus). *Nutrients* **2018**, *10*, 1970. [CrossRef]
88. Gong, X.; Jiang, S.; Tian, H.; Xiang, D.; Zhang, J. Polyphenols in the Fermentation Liquid of Dendrobium candidum Relieve Intestinal Inflammation in Zebrafish Through the Intestinal Microbiome-Mediated Immune Response. *Front. Immunol.* **2020**, *11*, 1542. [CrossRef]
89. Xu, Y.; Xie, M.; Xue, J.; Xiang, L.; Li, Y.; Xiao, J.; Xiao, G.; Wang, H. EGCG ameliorates neuronal and behavioral defects by remodeling gut microbiota and TotM expression in *Drosophila* models of Parkinson's disease. *FASEB J.* **2020**, *34*, 5931–5950. [CrossRef]
90. Molan, A.-L.; Liu, Z.; Plimmer, G. Evaluation of the Effect of Blackcurrant Products on Gut Microbiota and on Markers of Risk for Colon Cancer in Humans. *Phytotherapy Res.* **2014**, *28*, 416–422. [CrossRef]
91. Tzounis, X.; Rodriguez-Mateos, A.; Vulevic, J.; Gibson, G.R.; Kwik-Uribe, C.; Spencer, J.P. Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *Am. J. Clin. Nutr.* **2011**, *93*, 62–72. [CrossRef] [PubMed]
92. Vendrame, S.; Guglielmetti, S.; Riso, P.; Arioli, S.; Klimis-Zacas, D.; Porrini, M. Six-Week Consumption of a Wild Blueberry Powder Drink Increases Bifidobacteria in the Human Gut. *J. Agric. Food Chem.* **2011**, *59*, 12815–12820. [CrossRef] [PubMed]
93. Liu, Z.; Lin, X.; Huang, G.; Zhang, W.; Rao, P.; Ni, L. Prebiotic effects of almonds and almond skins on intestinal microbiota in healthy adult humans. *Anaerobe* **2014**, *26*, 1–6. [CrossRef] [PubMed]
94. Moreno-Indias, I.; Sánchez-Alcoholado, L.; Pérez-Martínez, P.; Andrés-Lacueva, C.; Cardona, F.; Tinahones, F.J.; Queipo-Ortuño, M.I. Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients. *Food Funct.* **2016**, *7*, 1775–1787. [CrossRef] [PubMed]
95. Rodríguez-Morató, J.; Matthan, N.R.; Liu, J.; de la Torre, R.; Chen, C.-Y.O. Cranberries attenuate animal-based diet-induced changes in microbiota composition and functionality: A randomized crossover controlled feeding trial. *J. Nutr. Biochem.* **2018**, *62*, 76–86. [CrossRef] [PubMed]
96. Yuan, X.; Long, Y.; Ji, Z.; Gao, J.; Fu, T.; Yan, M.; Zhang, L.; Su, H.; Zhang, W.; Wen, X.; et al. Green Tea Liquid Consumption Alters the Human Intestinal and Oral Microbiome. *Mol. Nutr. Food Res.* **2018**, *62*, e1800178. [CrossRef]
97. Queipo-Ortuño, M.I.; Boto-Ordóñez, M.; Murri, M.; Gomez-Zumaquero, J.M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andrés-Lacueva, C.; Tinahones, F.J. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am. J. Clin. Nutr.* **2012**, *95*, 1323–1334. [CrossRef]
98. Song, M.-Y.; Wang, J.-H.; Eom, T.; Kim, H. Schisandra chinensis fruit modulates the gut microbiota composition in association with metabolic markers in obese women: A randomized, double-blind placebo-controlled study. *Nutr. Res.* **2015**, *35*, 655–663. [CrossRef]
99. Clavel, T.; Fallani, M.; Lepage, P.; Levenez, F.; Mathey, J.; Rochet, V.; Sérézat, M.; Sutren, M.; Henderson, G.; Bennetau-Pelissero, C.; et al. Isoflavones and Functional Foods Alter the Dominant Intestinal Microbiota in Postmenopausal Women. *J. Nutr.* **2005**, *135*, 2786–2792. [CrossRef]
100. Wiese, M.; Bashmakov, Y.; Chalyk, N.; Nielsen, D.S.; Krych, L.; Kot, W.; Klochkov, V.; Pristensky, D.; Bandaletova, T.; Chernyshova, M.; et al. Prebiotic Effect of Lycopene and Dark Chocolate on Gut Microbiome with Systemic Changes in Liver Metabolism, Skeletal Muscles and Skin in Moderately Obese Persons. *BioMed Res. Int.* **2019**, *2019*, 4625279. [CrossRef]
101. Wijayabahu, A.T.; Waugh, S.G.; Ukhanova, M.; Mai, V. Dietary raisin intake has limited effect on gut microbiota composition in adult volunteers. *Nutr. J.* **2019**, *18*, 14. [CrossRef] [PubMed]
102. Shinohara, K.; Ohashi, Y.; Kawasumi, K.; Terada, A.; Fujisawa, T. Effect of apple intake on fecal microbiota and metabolites in humans. *Anaerobe* **2010**, *16*, 510–515. [CrossRef]
103. Cuervo, A.; Valdés, L.; Salazar, N.; de los Reyes-Gavilán, C.G.; Ruas-Madiedo, P.; Gueimonde, M.; González, S. Pilot Study of Diet and Microbiota: Interactive Associations of Fibers and Polyphenols with Human Intestinal Bacteria. *J. Agric. Food Chem.* **2014**, *62*, 5330–5336. [CrossRef] [PubMed]

104. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef] [PubMed]
105. Lye, H.-S.; Kuan, C.-Y.; Ewe, J.-A.; Fung, W.-Y.; Liong, M.-T. The Improvement of Hypertension by Probiotics: Effects on Cholesterol, Diabetes, Renin, and Phytoestrogens. *Int. J. Mol. Sci.* **2009**, *10*, 3755–3775. [CrossRef] [PubMed]
106. Pelletier, X.; Laure-Boussuge, S.; Donazzolo, Y. Hydrogen excretion upon ingestion of dairy products in lactose-intolerant male subjects: Importance of the live flora. *Eur. J. Clin. Nutr.* **2001**, *55*, 509–512. [CrossRef]
107. Hoveyda, N.; Heneghan, C.; Mahtani, K.R.; Perera, R.; Roberts, N.W.; Glasziou, P. A systematic review and meta-analysis: Probiotics in the treatment of irritable bowel syndrome. *BMC Gastroenterol.* **2009**, *9*, 15. [CrossRef]
108. Golowczyc, M.A.; Mobili, P.; Garrote, G.L.; Abraham, A.; De Antoni, G.L. Protective action of Lactobacillus kefir carrying S-layer protein against Salmonella enterica serovar Enteritidis. *Int. J. Food Microbiol.* **2007**, *118*, 264–273. [CrossRef]
109. Kleessen, B.; Kroesen, A.J.; Buhr, H.J.; Blaut, M. Mucosal and Invading Bacteria in Patients with Inflammatory Bowel Disease Compared with Controls. *Scand. J. Gastroenterol.* **2002**, *37*, 1034–1041. [CrossRef]
110. Petit, L.; Gibert, M.; Popoff, M.R. Clostridium perfringens: Toxinotype and genotype. *Trends Microbiol.* **1999**, *7*, 104–110. [CrossRef]
111. Guo, X.; Xia, X.; Tang, R.; Zhou, J.; Zhao, H.; Wang, K. Development of a real-time PCR method for *Firmicutes* and *Bacteroidetes* in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* **2008**, *47*, 367–373. [CrossRef]
112. Parkar, S.G.; Trower, T.M.; Stevenson, D.E. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe* **2013**, *23*, 12–19. [CrossRef] [PubMed]
113. Cait, A.; Hughes, M.R.; Antignano, F.; Cait, J.; Dimitriu, P.A.; Maas, K.R.; Reynolds, L.A.; Hacker, L.; Mohr, J.; Finlay, B.B.; et al. Microbiome-driven allergic lung inflammation is ameliorated by short-chain fatty acids. *Mucosal Immunol.* **2018**, *11*, 785–795. [CrossRef] [PubMed]
114. Rauf, A.; Khalil, A.A.; Rahman, U.-U.; Khalid, A.; Naz, S.; Shariati, M.A.; Rebezov, M.; Urtecho, E.Z.; de Albuquerque, R.D.D.G.; Anwar, S.; et al. Recent advances in the therapeutic application of short-chain fatty acids (SCFAs): An updated review. *Crit. Rev. Food Sci. Nutr.* **2021**, 1–21. [CrossRef] [PubMed]
115. Den Besten, G.; van Eunen, K.; Groen, A.K.; Venema, K.; Reijngoud, D.-J.; Bakker, B.M. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* **2013**, *54*, 2325–2340. [CrossRef]
116. Wu, Z.; Huang, S.; Li, T.; Li, N.; Han, D.; Zhang, B.; Xu, Z.Z.; Zhang, S.; Pang, J.; Wang, S.; et al. Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. *Microbiome* **2021**, *9*, 184. [CrossRef]
117. Zhai, Q.; Feng, S.; Arjan, N.; Chen, W. A next generation probiotic, *Akkermansia muciniphila*. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 3227–3236. [CrossRef]
118. Wu, T.; Grootaert, C.; Pitart, J.; Vidovic, N.K.; Kamiloglu, S.; Possemiers, S.; Glibetic, M.; Smagghe, G.; Raes, K.; Van de Wiele, T.; et al. Aronia (*Aronia melanocarpa*) Polyphenols Modulate the Microbial Community in a Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and Decrease Secretion of Proinflammatory Markers in a Caco-2/endothelial Cell Coculture Model. *Mol. Nutr. Food Res.* **2018**, *62*, e1800607. [CrossRef]
119. McDougall, G.J.; Allwood, J.W.; Pereira-Caro, G.; Brown, E.M.; Ternan, N.; Verrall, S.; Stewart, D.; Lawther, R.; O'Connor, G.; Rowland, I.; et al. Nontargeted LC-MSn Profiling of Compounds in Ileal Fluids That Decrease after Raspberry Intake Identifies Consistent Alterations in Bile Acid Composition. *J. Nat. Prod.* **2016**, *79*, 2606–2615. [CrossRef]
120. Fotschki, B.; Juskiewicz, J.; Jurgoński, A.; Rigby, N.; Sójka, M.; Kołodziejczyk, K.; Mackie, A.; Zdunczyk, Z. Raspberry pomace alters cecal microbial activity and reduces secondary bile acids in rats fed a high-fat diet. *J. Nutr. Biochem.* **2017**, *46*, 13–20. [CrossRef]
121. Huang, J.; Feng, S.; Liu, A.; Dai, Z.; Wang, H.; Reuhl, K.; Lu, W.; Yang, C.S. Green Tea Polyphenol EGCG Alleviates Metabolic Abnormality and Fatty Liver by Decreasing Bile Acid and Lipid Absorption in Mice. *Mol. Nutr. Food Res.* **2018**, *62*. [CrossRef] [PubMed]
122. Inouye, S.; Yamaguchi, H.; Takizawa, T. Screening of the antibacterial effects of a variety of essential oils on respiratory tract pathogens, using a modified dilution assay method. *J. Infect. Chemother.* **2001**, *7*, 251–254. [CrossRef]
123. Puupponen-Pimia, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kahkonen, M.; Heinonen, M.; Maatta-Riihinen, K.; Oksman-Caldentey, K.-M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991–1000. [CrossRef]
124. Bouarab-Chibane, L.; Forquet, V.; Lantéri, P.; Clément, Y.; Léonard-Akkari, L.; Oulahal, N.; Degraeve, P.; Bordes, C. Antibacterial Properties of Polyphenols: Characterization and QSAR (Quantitative Structure–Activity Relationship) Models. *Front. Microbiol.* **2019**, *10*, 829. [CrossRef] [PubMed]
125. Sirk, T.W.; Friedman, M.; Brown, E.F. Molecular Binding of Black Tea Theaflavins to Biological Membranes: Relationship to Bioactivities. *J. Agric. Food Chem.* **2011**, *59*, 3780–3787. [CrossRef]
126. Marín, L.; Miguélez, E.M.; Villar, C.J.; Lombó, F. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *BioMed Res. Int.* **2015**, *2015*, 905215. [CrossRef] [PubMed]
127. Gowd, V.; Karim, N.; Shishir, M.R.I.; Xie, L.; Chen, W. Dietary polyphenols to combat the metabolic diseases via altering gut microbiota. *Trends Food Sci. Technol.* **2019**, *93*, 81–93. [CrossRef]

128. Chen, Y.; Li, Q.; Zhao, T.; Zhang, Z.; Mao, G.; Feng, W.; Wu, X.; Yang, L. Biotransformation and metabolism of three mulberry anthocyanin monomers by rat gut microflora. *Food Chem.* **2017**, *237*, 887–894. [CrossRef]
129. Hanske, L.; Engst, W.; Loh, G.; Sczesny, S.; Blaut, M.; Braune, A. Contribution of gut bacteria to the metabolism of cyanidin 3-glucoside in human microbiota-associated rats. *Br. J. Nutr.* **2013**, *109*, 1433–1441. [CrossRef] [PubMed]
130. Verediano, T.A.; Stampini Duarte Martino, H.; Dias Paes, M.C.; Tako, E. Effects of Anthocyanin on Intestinal Health: A Systematic Review. *Nutrients* **2021**, *13*, 1331. [CrossRef]
131. Faria, A.; Fernandes, I.; Norberto, S.; Mateus, N.; Calhau, C. Interplay between Anthocyanins and Gut Microbiota. *J. Agric. Food Chem.* **2014**, *62*, 6898–6902. [CrossRef] [PubMed]
132. Markowiak, P.; Śliżewska, K. Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. *Nutrients* **2017**, *9*, 1021. [CrossRef]
133. Lin, W.; Wang, W.; Yang, H.; Wang, D.; Ling, W. Influence of Intestinal Microbiota on the Catabolism of Flavonoids in Mice. *J. Food Sci.* **2016**, *81*, H3026–H3034. [CrossRef] [PubMed]
134. Decroos, K.; Eeckhaut, E.; Possemiers, S.; Verstraete, W. Administration of Equol-Producing Bacteria Alters the Equol Production Status in the Simulator of the Gastrointestinal Microbial Ecosystem (SHIME). *J. Nutr.* **2006**, *136*, 946–952. [CrossRef] [PubMed]
135. Heinonenab, S.; Wähälä, K.; Adlercreutz, H. Identification of Isoflavone Metabolites Dihydrodaidzein, Dihydrogenistein, 6′-OH-O-dma, and cis-4-OH-equol in Human Urine by Gas Chromatography–Mass Spectroscopy Using Authentic Reference Compounds. *Anal. Biochem.* **1999**, *274*, 211–219. [CrossRef]
136. Mayo, B.; Vázquez, L.; Flórez, A.B. Equol: A Bacterial Metabolite from The Daidzein Isoflavone and Its Presumed Beneficial Health Effects. *Nutrients* **2019**, *11*, 2231. [CrossRef]
137. Espin, J.C.; Larrosa, M.; Garcia-Conesa, M.T.; Tomas-Barberan, F. Biological significance of urolithins, the gut microbial ellagic Acid-derived metabolites: The evidence so far. Evid. Based Complement. *Alternat. Med.* **2013**, *2013*, 2704. [CrossRef]
138. Cerdá, B.; Periago, P.; Espín, A.J.C.; Tomás-Barberán, F.A. Identification of Urolithin A as a Metabolite Produced by Human Colon Microflora from Ellagic Acid and Related Compounds. *J. Agric. Food Chem.* **2005**, *53*, 5571–5576. [CrossRef]

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Effect of Red-Beetroot-Supplemented Diet on Gut Microbiota Composition and Metabolite Profile of Weaned Pigs—A Pilot Study

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Simple Summary: Weaning causes gut microbiota disruption that results in dysbiosis and post-weaning diarrhea. The recent ban on pharmacological doses of in-feed zinc oxide in the weaned pig diet has made exploration of alternative dietary supplements to improve the post-weaning condition of pigs imperative. Plants (e.g., red beetroot) containing bioactive compounds have shown great potential in this regard, favorably abating gut microbiota dysbiosis and promoting gut metabolite production and health.

Abstract: Red beetroot is a well-recognized and established source of bioactive compounds (e.g., betalains and polyphenols) with anti-inflammatory and antimicrobial properties. It is proposed as a potential alternative to zinc oxide with a focus on gut microbiota modulation and metabolite production. In this study, weaned pigs aged 28 days were fed either a control diet, a diet supplemented with zinc oxide (3000 mg/kg), or 2% and 4% pulverized whole red beetroot (CON, ZNO, RB2, and RB4; respectively) for 14 days. After pigs were euthanized, blood and digesta samples were collected for microbial composition and metabolite analyses. The results showed that the diet supplemented with red beetroot at 2% improved the gut microbial richness relative to other diets but marginally influenced the cecal microbial diversity compared to a zinc-oxide-supplemented diet. A further increase in red beetroot levels (4%—RB4) led to loss in cecal diversity and decreased short chain fatty acids and secondary bile acid concentrations. Also, an increased Proteobacteria abundance, presumably due to increased lactate/lactic-acid-producing bacteria was observed. In summary, red beetroot contains several components conceived to improve the gut microbiota and metabolite output of weaned pigs. Future studies investigating individual components of red beetroot will better elucidate their contributions to gut microbiota modulation and pig health.

Keywords: weaned pig; gut microbiota; red beetroot; short chain fatty acids; bile acids

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1. Introduction

Weaning is a stressful phase in pig production characterized by reduced feed intake, poor growth rate, gut microbiota disruption, and diarrhea [1,2]. It is a transitionary phase in the pig life associated with compositional and functional alterations of the gut microbiota, resulting in enteric infections. In pig production, several measures to prevent dire economic losses are currently being explored. Diets provided to weaned pigs have been demonstrated to modulate significant gut microbiota changes leading to increased population of beneficial bacteria species, with remarkable changes observed 10 to 14 days post-weaning [3,4].

Pathogenic colonization of the gut, a leading cause of diarrhea and death in young pigs at weaning, is thus avoidable via dietary modulation of a healthy gut microbial compo-

sition [5,6]. Notably, alteration in the composition and diversity of the gut microbiota by the source and level of protein and fiber can increase or deplete gut microbiota metabolite production and corresponding biological responses [7–9]. The gut microbiota and metabolite levels in return enhance or inhibit the growth of certain bacteria phyla (e.g., Bacteroidetes, Firmicutes, and Proteobacteria) in the gastrointestinal tract [10,11].

Similarly, in-feed antibiotics and zinc oxide (ZnO) have been reported to reduce piglet mortality during weaning [12,13]. Their capacity to suppress post-weaning diarrhea, alter host-gut microbiota metabolism, and improve feed intake and energy production for growth is well known [14–16]. However, despite these advantages, they have been found to destabilize the gut microbial diversity, alter short chain fatty acid (SCFA) levels, and support the emergence of harmful and antibiotic-resistant bacteria species [17–19].

As is evident from past literature, in-feed ZnO induces gut microbiota changes characterized by increased coliforms [20,21], reduced anaerobic and lactic acid bacteria [22,23], and reduced commensal bacteria population [24,25]. Consequently, the functional potential of the gut microbiota and its ability to produce health-promoting metabolites (e.g., short chain fatty acids and bile acids) may be compromised.

Additionally, there are concerns about severe environmental pollution from high fecal excretions of zinc [26] linked to in-feed pharmacological doses of zinc oxide coupled with increasing trends of multidrug-resistant *E. coli* [27,28]. This presents a risk to the animal–environment food chain, which necessitated the ban on therapeutic doses of in-feed zinc oxide in weaned pigs across the European Union in June 2022. Thus, it highlights the urgent and continued search for alternatives to in-feed ZnO. There has been an increased research interest in plants containing bioactive compounds; e.g., red beetroot, with health-promoting properties as possible replacements for in-feed ZnO, with emphasis on modulating a healthy gut microbiota and prevention of pathogenic colonization post-weaning [29,30].

Red beetroot (*Beta vulgaris* subsp. *vulgaris conditiva*) contains bioactive compounds such as betalains, polyphenols, inorganic nitrate (NO_3), fiber, and minerals (e.g., potassium, sodium, phosphorus, calcium, magnesium, copper, iron, zinc, and manganese) [31–33]. These bioactives (i.e., betalains, polyphenols, nitrate, and fiber) contribute to the potential prebiotic effect of red beetroot, driving gut microbiota modulation and metabolite production with impacts on host metabolism, physiology, and immune functions [34–36].

Red beetroot is one of the top 10 plants with high antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic, and hepatoprotective characteristics [37,38]. Currently, it is being considered as a therapeutic ingredient in the treatment of conditions caused by oxidative stress, inflammation, and metabolic disorders (e.g., hypertension, diabetes, insulin resistance, and kidney dysfunction) [39–41]. The health benefits of red beetroot in humans, rodents [42,43], and rainbow trout [44,45] have been widely studied and reported in the literature, but studies using pigs have not been considered, and research demonstrating the potential of red beetroot supplementation of the gut microbiota is still lacking. This study therefore examined the effect of red beetroot on the gut microbiota composition and metabolite output of weaned pigs.

2. Materials and Methods

The animal trial was conducted at the National Pig Centre, UK, under an ethical approval granted by the University of Leeds Animal Welfare and Ethical Review Committee (AWERC) under the approval number 070510HM. All husbandry practices were set by the farm in accordance with the Welfare of Farmed Animals (England) Regulations 2007, and all procedures followed the amended Animals (Scientific Procedures) Act 1986. For ethical reasons, the number of piglets per treatment was reduced and determined based on previous studies [46–48] that focused on gut microbiota diversity.

The basal diet was provided by Primary Diets, UK, and whole red beetroot powder was purchased from Buy Wholefoods online Ltd. (Ramsgate, UK). Reference bile salts for bile acid quantification were from Sigma-Aldrich (Steinheim, Germany) and Cayman (Cambridge, UK). The mixed short chain fatty acid standard solution containing acetate,

propionate, butyrate, valerate, isobutyrate, and isovalerate was from Supelco–Merck Life Science Ltd. (Dorset, UK). All chemicals, solvents, and other reagents used were purchased from Sigma-Aldrich (Germany) and Fischer Scientific (Loughborough, UK) accordingly.

2.1. Experimental Animals and Experimental Design

A total of 48 piglets (Large White × Landrace × Duroc) weaned on day 28 (average body weight: 7.58 ± 0.69 kg) were randomly allocated to one of four diets (n = 12) while balancing for body weight, sex, and litter origin for a 14-day feeding experiment. The pigs were housed in a temperature-controlled flat deck with open feed troughs and nipple drinkers for easy access to feed and water ad libitum. The experimental diets comprised a basal control diet (CON) and a diet supplemented with 3000 mg/kg zinc oxide (ZNO), both formulated according to the National Research Council (2012) recommendations (Table 1). Red-beetroot-supplemented diets (RB2 and RB4) were obtained by adding 2% (20 g/kg) and 4% (40 g/kg) pulverized whole red beetroot to the basal diet, respectively, and thoroughly mixed with an electric mixer on-site.

Table 1. Composition of experimental diets and results for analyzed nutrients.

Ingredients (%)	Control (CON)	Diet with ZnO (ZNO)	2% Red-Beetroot-Supplemented Diet (RB2)	4% Red-Beetroot-Supplemented Diet (RB4)
Barley	15.00	15.00	14.70	14.40
Wheat	28.17	28.17	27.51	26.95
Micronized maize bulk	2.50	2.50	2.45	2.40
Micronized oats	5.00	5.00	4.90	4.80
Fishmeal bulk	6.00	6.00	5.88	5.76
Soya hypro	18.16	18.16	17.80	17.43
Full fat soybean	2.50	2.50	2.45	2.40
Pig weaner premix	0.50	0.50	0.49	0.48
Whey powder bulk	13.89	13.89	13.61	13.33
Potato protein	1.60	1.60	1.57	1.54
Sugar/sucrose	0.63	0.63	0.61	0.60
L-Lysine HCl	0.28	0.28	0.28	0.27
DL-Methionine	0.19	0.19	0.19	0.19
L-Threonine	0.15	0.15	0.15	0.15
L-Tryptophan	0.02	0.02	0.02	0.02
L-Valine	0.04	0.04	0.04	0.04
Vitamin E	0.04	0.04	0.04	0.04
Pan-tek robust	0.02	0.02	0.02	0.02
Sucram	0.01	0.01	0.01	0.01
Benzoic acid	0.50	0.50	0.49	0.48
Pigzin (zinc oxide)	0.00	0.31	0.00	0.00
Di-calcium phosphate	1.13	1.13	1.11	1.08
Sodium carbonate	0.05	0.05	0.05	0.05
* Sipernat 50	0.31	0.00	0.30	0.30
Red beetroot	0.00	0.00	2.00	4.00
Soya oil	3.40	3.40	3.33	3.26
Total (%)	100	100	100	100
Dry matter (%)	89.93	89.65	89.47	89.01
Analyzed nutrient				
Ash (%)	6.80	7.50	6.70	6.60
Ether extract (%)	6.73	6.99	6.62	5.92
Crude protein (%)	21.30	21.30	20.70	20.40
Crude fibre (%)	1.90	1.50	1.80	2.20
Zinc (mg/kg)	422.00	2252.00	193.00	187.00

* An inert ingredient made from silica added as a filler with respect to the zinc-oxide-containing diet.

During the trial, pigs were weighted individually on days 0, 7, and 14, and feed intake was estimated per diet group for the calculation of the average daily feed intake (ADFI), average daily weight gain (ADG), and feed conversion ratio (FCR). Pig feces on the pen floor was assessed visually by the same personnel and scored on a scale of 1 to 5 (where 1: firm feces; 2: soft feces; 3: mild diarrhea; 4: severe diarrhea; and 5: scour).

2.2. Sample Collection

At the end of the experimental period, eight animals per diet (n = 8) were euthanized via captive bolt and exsanguination. Blood samples were collected from the jugular vein into heparinized tubes, from which plasma was obtained after centrifugation at 2000× g and 4 °C for 10 min. Fecal samples were collected from the rectum into designated tubes. The abdominal cavity was immediately opened, each intestinal segment (duodenum, jejunum, ileum, cecum, and colon) was identified, separately cut, and emptied into a sterile beaker. Digesta from each segment was mixed and aliquoted into sterile 2 mL Eppendorf tubes. All samples were snap frozen in liquid nitrogen prior to storage at −80 °C for gut bacterial composition, short chain fatty acid, and bile acid analyses.

2.3. Gut Microbiota Analyses and Bioinformatics

Pig gut microbial composition was examined using digesta samples from the jejunum, ileum, and cecum. Genomic DNA was extracted from (approx. 1.0 g) the digesta samples with a QIAamp Power fecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the DNA samples were measured spectrophotometrically with a Nano Drop® ND-1000 (Nano Drop Technologies Inc., Dover, DE, USA) using an absorbance ratio at 260/280 nm; those observed were within the range of 1.8–2.0. DNA samples were submitted to the University of Leeds Next Generation Sequencing Facility, St. James Hospital Leeds, UK, for quality screening, 16S rRNA gene library preparation, and sequencing. According to a previous study [49], the V4 hypervariable region of the 16S rRNA gene was amplified in a two-step polymerase chain reaction (PCR) with specific primers (564F and 806R) and an Illumina adaptor overhang. Following the Illumina 16S metagenomics sequencing library preparation protocol, the final libraries were pooled and pair-end sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Sequence reads were processed in Mothur v.1.43.0 with the MiSeq standard operation procedure developed by the Schloss group [50,51]. The chimera-free and unique sequences identified were aligned to the SILVA (v.138) database, and sequences with 97% similarity were clustered into operational taxonomic unit. The "Biome" file generated by Mothur was transferred to the R (v 3.6.2 and 4.0.0) environment for further analyses, including the alpha and beta diversity indices.

The alpha diversity of the gut microbial community was evaluated using the Chao1, Shannon, and Simpson indices, and variables were compared using ANOVA to evaluate the effect of diet, gut location, and their interaction with the lmerTest (linear mixed effects). Differences between gut samples (beta diversity) were determined using a permutational multivariate analysis of variance (PERMANOVA) of the non-phylogenetic distance matrix (Bray–Curtis), which was then visualized on a non-metric multidimensional scale (NMDS) plot. The diet effect on each gut location was computed via a paired comparison of the distance matrices with a pairwise Adonis function (adonis2) in the vegan package (v. 2.6.4).

Differentially (distinct) abundant taxa between gut locations per diet were identified in a two-sided Welch's t-test and Benjamin Hochberg false discovery ratio (FDR) correction in Statistical Analysis of Metagenomics and other Profiles (STAMP) software [52]. Further analysis employed DESeq2 (v. 1.27.32) in R [53] with Wald hypothesis testing for distinct genera in each gut location comparing multiple diet groups. The differences between diets were estimated as the fold change (Log2-fold change) and FDR-corrected p-values.

2.4. Predicted Functions of Pig Gut Microbiota

To predict the functional pathways mediated by the gut microbiota, OTU abundance and representative sequences processed in Mothur were submitted to Piphillin (https://piphillin.secondgenome.com/ accessed on 12 August 2020). Gene sequences were matched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as described in Iwai et al. [54] using USEARCH version 8.0.1623 with the global alignment setting for sequence identification fixed to a 90% cut-off (a level significantly associated with PICRUSt—phylogenetic investigation of communities by reconstruction of unobserved states) [55].

Pathways differentially mediated by diet in the different gut locations were computed with DESeq2 in R using the Wald test and *p*-values adjusted for multiple inter-diet comparisons.

2.5. Quantification of Short Chain Fatty Acids (SCFA) and Bile Acids

Short chain fatty acids (acetate, propionate, butyrate, valerate, isobutyrate, and isovalerate) in plasma, jejunum, ileum, cecum, colon digesta, and fecal samples were determined with gas chromatography (Varian 3400; Varian Ltd., Oxford, UK). The method used was as described in Taylor et al. [56] with slight modification. Briefly, a 1.0 g or 1 mL sample was mixed with an equal volume of distilled water in an Eppendorf tube and centrifuged at 12,000× *g* and 4 °C for 10 min. Phosphoric acid (50 µL, 85% *v*/*v*) was added to the supernatant (500 µL) collected alongside 150 µL of caproic acid (150 mM/L) as the internal standard. The mixture was topped up to 1 mL with distilled water and centrifuged at 14,000× *g* for 20 min, after which the supernatant was collected for SCFA analyses. Individual short chain fatty acids in processed samples were quantified using a standard curve obtained from a mixed volatile fatty acid prepared in the concentration range of 0 to 125 mM.

Bile acids in samples were determined as described in Zhang et al. [57]. Briefly, 0.3 g of digesta was mixed with acetonitrile (final conc. 80% *v*/*w*), incubated for 20 min at room temperature, and centrifuged at 15,000× *g* and 4 °C for 20 min. The supernatant collected was passed through Strata-X 33 µm polymer-based solid phase extraction cartridges (Phenomenex, Torrance, CA, USA) after the cartridges had been conditioned with methanol and water. Subsequently, bile acids were eluted in 1.5 mL of methanol, concentrated, dried using a solvent evaporator (SP Genevac EZ-2 Series, Stone Ridge, NY, USA), and then reconstituted in 150 µL of methanol before subjecting them to HPLC-MS (Shimadzu, Kyoto, Japan). Regarding the mobile phase, A and B were a mixture of 5 mM of ammonium acetate in water and methanol, respectively, both acidified with 0.012% formic acid. A mixed standard reference (0–0.1 mM) containing taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid was prepared for quantification of bile salts.

2.6. Statistical Analyses

SCFA and bile acid concentrations were analyzed in the R environment (v. 4.2.2); zero-inflated data were analyzed using a negative binomial with the square root link function, and the multiple comparison of means was computed using Tukey's post hoc test with a significance level of $p < 0.05$. Results expressed as the mean and standard error of mean (SEM) are presented in tables. A Spearman correlation analysis was conducted between the SCFA levels, bile acids, and the mean relative abundance of the top 25 genera in each gut location per diet using the "Psych" [58] and "Pheatmap" [59] packages in R (v. 3.31).

3. Results

3.1. Effect of Diets on Gut Microbial Diversity and Taxonomic Composition

In the present study, there was no difference in the growth performance or fecal score of the pigs, which is documented in Table S1. However, the diets ($p = 0.01$) significantly influenced the species richness and diversity of the gut microbiota with respect to the gut locations ($p < 0.001$; jejunum, ileum, and cecum) examined. Diet RB2 increased the jejunal species richness ($p = 0.02$) compared to other diets according to the Chao1 index, but the cecal species abundance was comparable for all the diets (Figure 1a). According to the Shannon index of alpha diversity, the gut microbial community was diverse, albeit not influenced by the diets ($p = 0.07$). A pairwise diet comparison showed that the ZNO diet was different from CON and RB4 in the cecum (Figure 1b). No significant species abundance, divergence, or evenness (i.e., dominance) was observed between the diets or in the gut locations for the Simpson index.

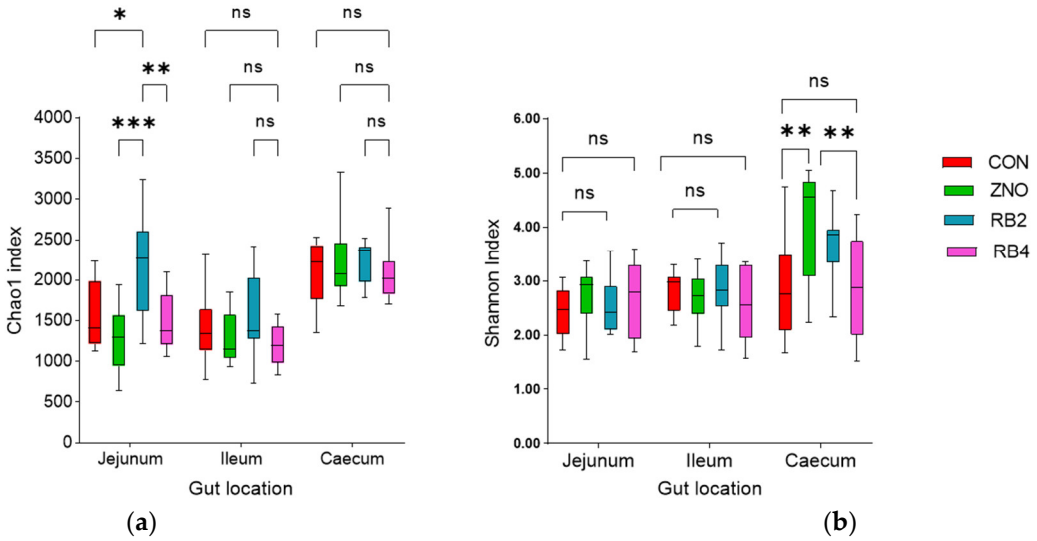
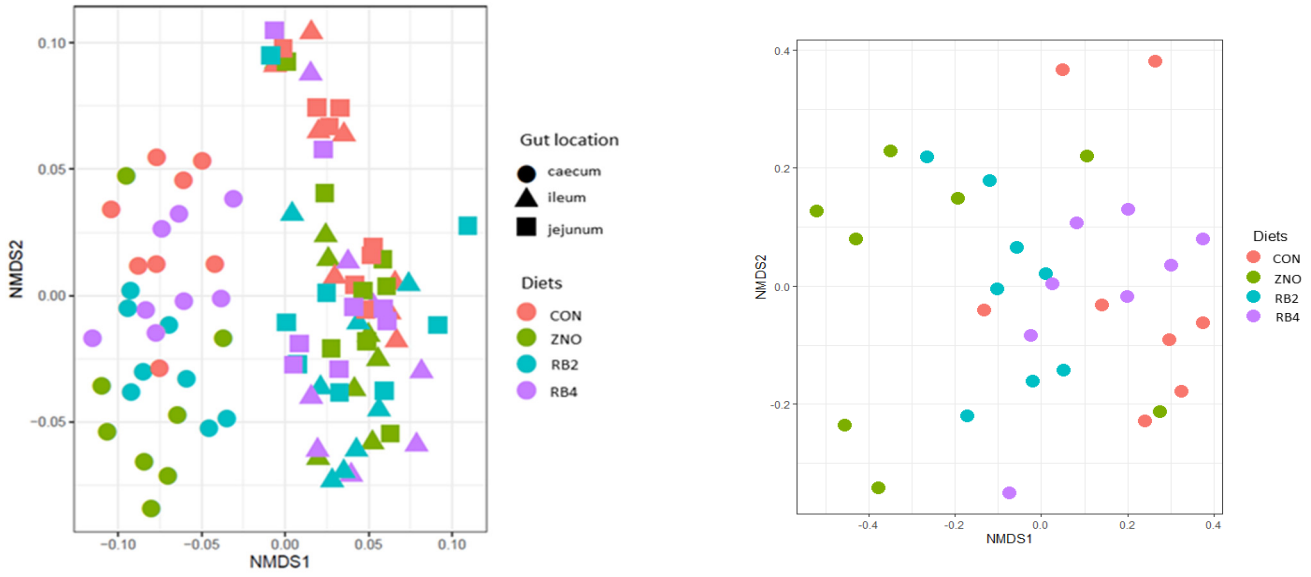


Figure 1. Alpha diversity indices of pig gut microbiota. (**a**) Chao1 index and (**b**) Shannon index showing diet effect on gut species richness and/or diversity. Boxplot represents mean (minimum to maximum) species richness and or evenness from each diet in the gut locations evaluated. A significant difference between the diets linked by a line is indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (ns—not significant). CON, ZNO, RB2, and RB4 represent the control diet and diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.

The beta diversity was as shown in the non-metric multidimensional scaling (NMDS) plots (Figure 2a,b). Samples from the cecum clustered distinctively away from the ileum and jejunum, depicting that the cecum had a significantly different ($p = 0.013$) microbial composition from the ileum and jejunum, while the ileum and jejunum microbial communities were marginally different ($p = 0.051$). A subset analysis of the cecal biome with inter-diet comparisons indicated the CON pigs had more similar species in the cecum than the ZNO ($p = 0.03$) and RB2 ($p = 0.05$) pigs but were related to RB4 pigs ($p = 0.35$). Hence, the ZNO pigs contained more dissimilar species than the RB4 pigs, whereas the RB2 and RB4 pigs were not different ($p = 0.09$).

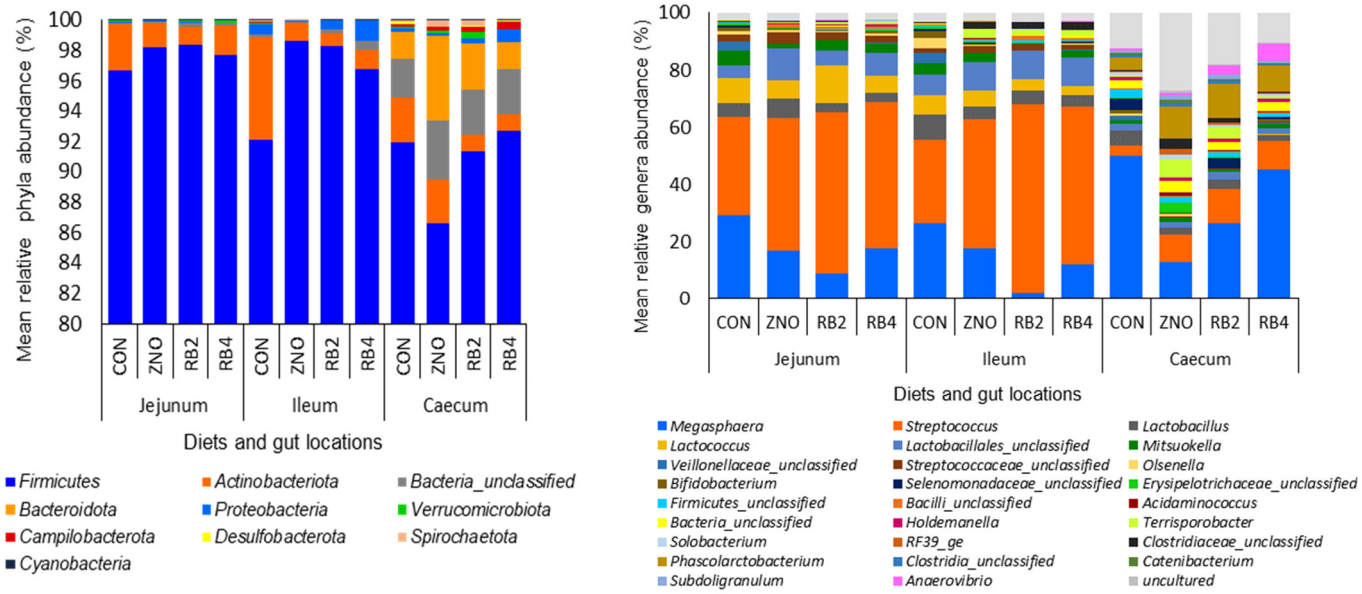
In the digesta samples analyzed, 15 phyla and 310 genera were observed with approximately 99% of total sequences (17,573,278) assigned. The mean relative phyla and genera abundance are as presented in Figure 3a,b. The dominant bacteria phyla with mean relative abundance > 1% were Firmicutes, Actinobacteriota, Bacteria unclassified, and Bacteroidota. The mean relative phyla and abundant genera in the gut were compared across the diet groups and are presented in Table 2 and Table S2, respectively. The gut locations mainly influenced ($p < 0.05$) the relative mean phyla distribution with an increase in the cecum compared to other regions examined; however, Firmicutes' abundance was reduced. Also,

an increase in phylum Actinobacteriota and Proteobacteria abundance in the CON and RB4 pigs was observed.



(**a**) (**b**)

Figure 2. Non-metric multidimensional scaling (NMDS) plots of Bray-Curtis non-phylogenetic distance matrices of gut microbial community of weaned pigs fed different diets: (**a**) distribution of samples by diet and gut location; (**b**) distribution of samples from the cecum. Diets: CON, ZNO, RB2, and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.



(**a**) (**b**)

Figure 3. Mean relative abundance of (**a**) phyla and (**b**) genera in pig gut locations with respective diets. CON, ZNO, RB2 and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.

Table 2. Comparative analyses of mean relative phyla abundance between diet and gut locations.

Phylum	Diets				Gut Locations			[c] SEM	*p*-Value		
	CON	ZNO	RB2	RB4	Jejunum	Ileum	Cecum		[d] L	[e] D	[f] L × D
Firmicutes	93.58 [a]	94.48 [a]	96.01 [a]	95.72 [a]	97.74 [a]	96.46 [a]	90.64 [b]	0.617	0.000	0.312	0.062
Actinobacteriota	4.25 [a]	1.88 [ab]	1.06 [b]	1.42 [b]	1.93 [a]	2.51 [a]	2.02 [a]	0.347	0.733	0.004	0.224
Bacteria unclassified	0.95 [a]	1.37 [a]	1.13 [a]	1.26 [a]	0.17 [b]	0.28 [b]	3.09 [a]	0.206	0.000	0.796	0.800
Bacteroidota	0.61 [a]	1.86 [a]	1.03 [a]	0.59 [a]	0.003 [b]	0.005 [b]	3.06 [a]	0.248	0.000	0.067	0.029
Proteobacteria	0.31 [ab]	0.11 [b]	0.31 [ab]	0.71 [a]	0.03 [b]	0.66 [a]	0.39 [a]	0.068	0.000	0.005	0.126
Verrucomicrobiota	0.09 [a]	0.06 [a]	0.18 [a]	0.08 [a]	0.11 [a]	0.04 [a]	0.16 [a]	0.026	0.187	0.389	0.132
Campilobacterota	0.09 [a]	0.08 [a]	0.13 [a]	0.16 [a]	0.01 [b]	0.01 [b]	0.32 [a]	0.041	0.002	0.916	0.921
Desulfobacterota	0.07 [a]	0.01 [a]	0.03 [a]	0.04 [a]	0.004 [b]	0.001 [b]	0.11 [a]	0.010	0.000	0.230	0.213
Spirochaetota	0.05 [a]	0.13 [a]	0.11 [a]	0.01 [a]	0.001 [b]	0.03 [b]	0.19 [a]	0.026	0.003	0.259	0.058

Data represent the mean phyla abundance in each gut location with the different experimental diets; different superscripts ([a,b]) between diet groups and gut location indicate significant differences at $p < 0.05$. [c] Standard error of the group mean; [e] *p*-value for gut location; [d] *p*-value for diet; [f] *p*-value for interaction between the gut location and diet. CON, ZNO, RB2 and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.

In the top genera (Table S2), *Megasphaera*, *Streptococcus*, *Anaerovibrio*, *Rumminococcaceae_unclassified*, *Erysipelotrichaceae_unclassified*, *Bacilli_unclassified*, *Terrisporobacter*, and *Clostridiaceae* unclassified abundance were significantly influenced by the diets ($p < 0.05$) and the gut locations ($p < 0.02$) examined. The mean relative abundance of 11 genera (e.g., *Megasphaera*, *Selenomonadaceae_unclassified*, *Phascolarctobacterium*, *Firmicutes_unclassified*, *Bacteria_unclassified*, *Erysipelotrichaceae_unclassified*, *Negativibacillus*, *Anaerovibrio*) functionally recognized as lactate-utilizing bacteria (LUB) were higher ($p < 0.05$) in the cecum but comparable in the jejunum and ileum. Likewise, lower ($p < 0.05$) *Streptococcus*, *Lactococcus*, *Lactobacillales_unclassified*, *Streptococcaceae_unclassified*, and *Bacilli_unclassified* abundance (mostly lactic-acid-producing bacteria—LAB) was observed in the cecum compared to the jejunum and ileum.

3.2. Differential Abundant Genera Modulated by the Diets

Differential abundance at the genus level and distribution per diet were computed using Welch's *t*-test and FDR-corrected in STAMP. The results indicated an increased ($p < 0.05$) *Megasphaera*, *Selenomonadaceae_unclassified*, and *Veillonellaceae_unclassified* abundance in pigs fed the CON diet along with *Erysipelotrichaceae_unclassified*, *Clostridiaceae_unclassified* and *Rumminococcaceae_unclassified* in the ZNO pigs, while *Bacilli_unclassified* and *Anaerovibrio* increased in the RB2 and RB4 pigs, respectively. Further analyses of each gut location with inter-diet comparisons as shown in Figure 4 presented a decrease in *Veillonellaceae_unclassified* and *Selenomonadaceae_unclassified* abundance in the jejunum of the RB2 and RB4 pigs compared to the CON and ZNO pigs, whereas in the ileum, only the RB2 pigs showed an increased *Terrisporobacter* abundance relative to the CON pigs. The cecum was enriched with nine genera (e.g., *Romboutsia*, *Clostridiaceae_unclassified*, *Terrisporobacter*, *Candidatus_Soleaferrea*, *Muribaculaceae_ge*, and *Clostridium_sensu_stricto_1*) in the ZNO pigs compared to the CON pigs but diminished in genus *Selenomonadaceae_unclassified*. Pigs fed red beetroot diets (RB2 and RB4) had increased cecal *Selenomonadaceae_unclassified* and/or *Anaerovibrio* abundance relative to the ZNO pigs.

3.3. Metabolite Profile and Association with Gut Microbial Composition

The short chain fatty acid (SCFA) profile followed the expected pattern of increased levels in the lower gut (cecum and colon), including the fecal samples. Nonetheless, the experimental diets influenced SCFA levels observed in these locations (Table 3). Concomitant with the species richness in the gut locations, SCFA levels increased significantly in the jejunum of RB2 pigs and were reduced in the ileum of ZNO pigs but comparable in the cecum across the diet groups. Overall, the total SCFA levels were reduced significantly

(p = 0.01) in the RB4 and ZNO pigs, as were most SCFAs (e.g., acetate, propionate, and butyrate).

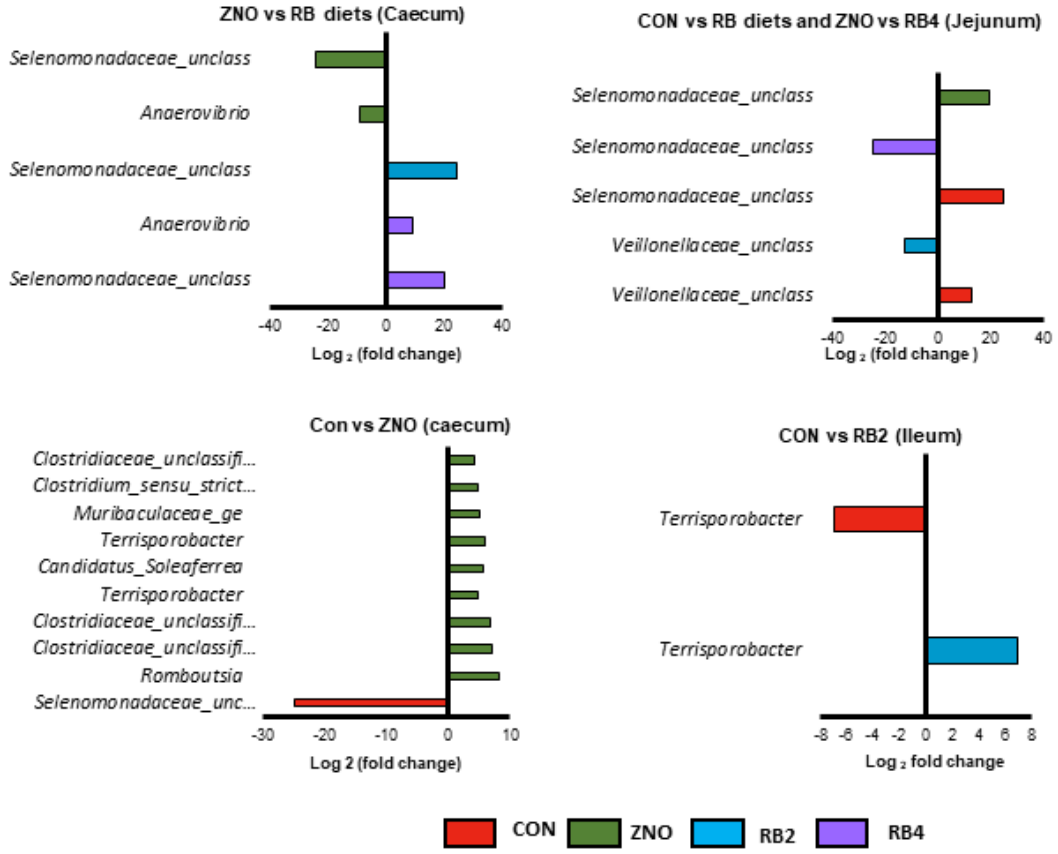


Figure 4. Differentially abundant genera from comparisons between diets in the gut locations with significant (p < 0.001) log2-fold changes presented. RB represents red beetroot; CON, ZNO, RB2, and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.

Similarly, the trend of high jejunal bile acid concentration (approx. 1- to 3-fold) and levels observed in other locations examined was not biologically relevant (Table 4). Pigs fed the RB2 diet had higher (p < 0.05) TCA, GCDCA, CA, GLTCA, CDCA, and DCA compared to other diets but had equivalent total and unconjugated bile acids (CA, DCA, CDCA, and LCA) levels with CON pigs. Conversely, the bile acid concentration was reduced in the ZNO (TCDCA, TDCA, GCDCA, GDCA, and CDCA) and RB4 (TCA, GLTCA, DCA, and CA) pigs relative to the CON pigs, which cumulatively ensued lower total and unconjugated bile acid levels.

Although the total SCFAs in the CON and RB2 pigs was higher, the cecal SCFA levels were comparable across the diet. Acetate and propionate levels correlated significantly with cecal bacteria (e.g., *Firmicutes_unclassified*, *Mitsuokella*, *Megasphaera*, *Streptococcus*, *Streptococcceae_unclassified*, *Anaerovibrio*, *Lactobacillus*, and *Selenomonadaceae_unclassified*) abundance for the CON and RB pigs but were closely associated with the jejunum and ileum (e.g., *Phascolarctobacterium* and *Bacteria_unclassified*) genera abundance in the ZNO and RB4 pigs. Across the gut locations, *Faecalibacterium*, *Blautia*, *Clostridia_unclassified*, *Clostridiaceae_unclassified*, *Dialister*, *Olsenella*, *Selenomonadaceae_unclassified*, *Veillonellaceae_unclassified*, and *Firmicutes_unclassified* were examples of genera that were significantly associated with butyrate in the ZNO and RB4 pigs (Figure 5a,b), most of which were associated with ileal butyrate in the RB2 pigs, although not significant (Figure S1b).

Table 3. Short chain fatty acid (mM) profile of diet groups and locations evaluated.

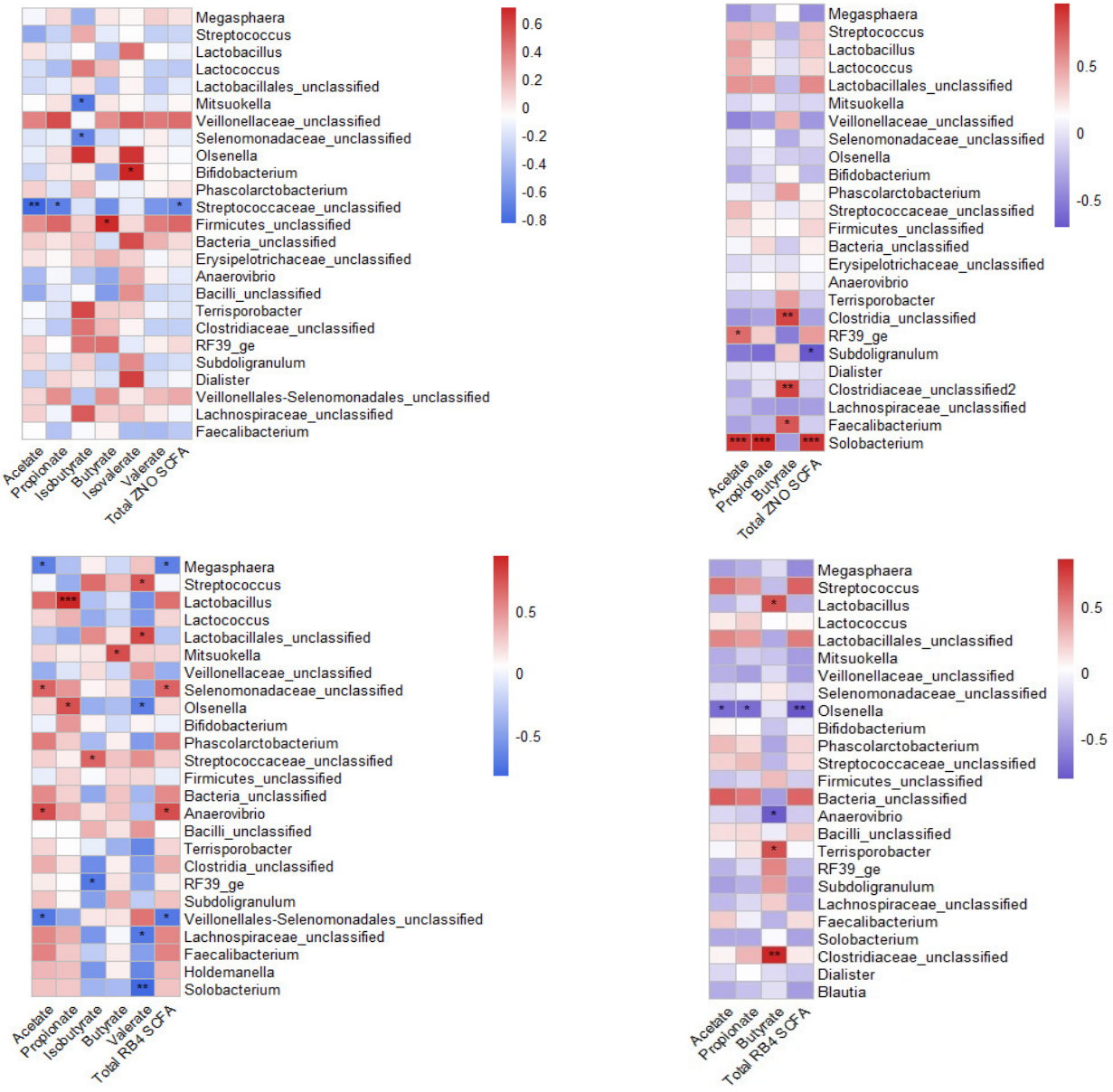
SFCA	Diets					*p*-Value		
	CON	ZNO	RB2	RB4	SEM	* L	# D	+ L × D
Acetate	69.14 [a]	54.76 [b]	63.81 [ab]	56.64 [b]	3.32	<0.01	<0.05	>0.05
Propionate	23.83 [a]	17.04 [d]	20.34 [b]	19.16 [c]	1.42	<0.05	<0.01	<0.01
Isobutyrate	2.59 [a]	1.60 [b]	1.79 [b]	0.86 [c]	0.36	<0.02	<0.02	<0.02
Butyrate	9.48 [a]	6.76 [b]	8.08 [ab]	7.09 [b]	0.61	<0.05	<0.05	>0.05
Isovalerate	1.11 [a]	0.71 [b]	0.67 [b]	0.44 [c]	0.14	<0.02	<0.02	<0.05
Valerate	1.55 [a]	0.82 [b]	0.58 [c]	0.63 [c]	0.22	<0.02	<0.02	<0.05
Location								
[1] Plasma	1.99	1.87	2.31	1.61	0.15	<0.01	>0.05	<0.05
[1] Jejunum	7.40 [b]	7.88 [b]	11.28 [a]	7.08 [b]	0.97	<0.01	<0.05	<0.05
[1] Ileum	9.21 [a]	7.91 [b]	9.82 [a]	8.45 [a]	0.42	<0.01	<0.05	<0.05
Cecum	24.91	22.36	23.71	22.12	0.65	<0.05	>0.05	<0.05
Colon	24.57 [a]	19.25 [b]	17.32 [b]	22.29 [ab]	1.61	<0.05	<0.05	<0.05
Feces	39.61 [a]	22.43 [c]	30.84 [b]	23.27 [c]	4.00	<0.05	<0.05	<0.05
Total SCFA	107.70 [a]	81.69 [b]	95.28 [a]	84.82 [b]	5.876	<0.05	<0.05	<0.05

Data represent the mean SCFA for each diet group and gut location; different superscripts across the rows indicate significant differences at $p < 0.05$. CON, ZNO, RB2, and RB4 represent the control diet and diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively. [1] SCFA levels in these locations were significantly different from levels in the cecum; * *p*-value for effect of location on SCFA levels; # *p*-value for significant effect of diets; + *p*-value interaction between location and diet.

Table 4. Bile acid profile (nmol/g digesta/feces) from diet groups and locations evaluated.

Bile Acids	Diets					*p*-Value		
	CON	ZNO	RB2	RB4	SEM	* L	# D	+ L × D
THCA	40.74 [a]	27.29 [b]	16.88 [c]	26.86 [b]	4.90	<0.01	<0.01	0.75
GHDCA	33.78 [b]	46.08 [a]	25.76 [c]	33.45 [b]	4.20	<0.01	0.05	0.62
TCA	1.50 [b]	1.56 [b]	1.96 [a]	1.30 [b]	0.14	<0.01	<0.01	<0.01
GCA	0.40 [b]	0.47 [b]	0.45 [b]	1.37 [a]	0.23	>0.05	0.01	>0.05
TCDCA	21.90 [a]	8.89 [c]	8.30 [c]	15.09 [b]	3.18	<0.01	<0.01	<0.01
TDCA	5.11 [a]	3.42 [b]	4.07 [ab]	3.90 [b]	0.36	0.02	0.05	0.93
GCDCA	15.42 [c]	13.88 [c]	35.91 [a]	25.40 [b]	5.10	<0.01	<0.01	0.25
GDCA	3.57 [b]	4.91 [a]	2.95 [c]	3.26 [b]	0.43	<0.01	<0.01	0.05
GLTCA	2.11 [ab]	1.99 [b]	3.60 [a]	1.96 [b]	0.40	<0.05	<0.01	0.18
CA	3.80 [b]	2.59 [c]	4.23 [a]	1.63 [d]	0.59	<0.01	<0.01	<0.01
CDCA	121.16 [b]	72.28 [c]	147.09 [a]	74.32 [c]	18.34	<0.01	<0.01	<0.01
DCA	0.10 [b]	0.14 [b]	0.31 [a]	0.12 [b]	0.05	<0.02	<0.02	0.20
LCA	134.25 [a]	108.90 [b]	118.20 [b]	107.66 [b]	6.13	<0.01	0.05	0.42
Location								
[1] Jejunum	138.20 [b]	135.03 [b]	210.83 [a]	128.68 [c]	19.32	<0.01	<0.05	<0.05
Ileum	95.20 [a]	40.21 [c]	29.11 [d]	50.09 [b]	14.40	<0.01	<0.01	<0.05
Cecum	23.95 [a]	17.37 [b]	15.65 [b]	12.84 [c]	2.35	<0.01	<0.05	<0.05
Colon	59.88 [a]	28.45 [c]	46.40 [b]	21.10 [d]	8.77	<0.01	<0.05	<0.05
Feces	66.59 [c]	71.33 [b]	67.72 [c]	83.62 [a]	3.90	<0.01	<0.01	<0.05
Total unconjugated	259.30 [a]	183.91 [b]	269.83 [a]	183.74 [b]	23.41	<0.05	<0.05	<0.05
Total conjugated	124.52 [a]	108.48 [b]	99.88 [b]	112.60 [b]	5.12	<0.05	<0.05	<0.05
Total bile acids	**383.82 [a]**	**292.39 [b]**	**369.71 [a]**	**296.33 [b]**	**23.98**	<0.001	<0.05	<0.01

Data represent the mean bile acid levels for each diet and location; significant differences are indicated by superscripts across the table for the diet groups. CON, ZNO, RB2 and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively. [1] Jejunal bile acid levels were higher than in other locations examined. * *p*–value for effect of location; # *p*-value for significant effect of diets; + *p*-value interaction between location and diet. The bile acids included taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid.



(**a**) (**b**)

Figure 5. Spearman correlation analyses between top abundant bacteria genera and (**a**) cecal short chain fatty acids and (**b**) ileal short chain fatty acids. Fatty acids omitted were not detected in the corresponding gut locations. The color depth depicts the correlation between the genera and gut metabolite, where a red color denotes a positive correlation and a blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity and *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$. CON, ZNO, RB2, and RB4 represent the control diet and the diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively.

Associations between the gut genera abundance and bile acid levels were as presented in Figure S2. In focusing on the unconjugated (CA, CDCA, and LCA) and conjugated (GCA, TCA, GDCA, GCDCA, TCDCA, TDCA, GLTCA, THCA, and GHDCA) bile acids, in the jejunum, the CA, CDCA, and total bile acid levels were strongly associated with most genera in the RB2 pigs, unlike the ZNO and RB4 pigs (Figure 6). Conjugated bile acids were significantly associated with the ilea genera (e.g., *Bacteria_unclassified*, *Selenomonadaceae_unclassified*, and *Veillonellaceae_unclassified*) abundance in the CON and RB pigs; however, most bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Streptococcaceae_unclassified*, *Firmicutes_unclassified*, *Terrisporobacter*, and *RF39_ge*) were associated with unconjugated,

conjugated, and total bile acids in the ileum of the ZNO pigs. In the CON, RB2, and RB4 pigs, there was a significant difference between the bacteria genera (*Dialister*, *Streptococcus*, *Lactobacillaes_unclassified*, *Streptococcaceae_unclassified*, *Lactococcus*, and *Selenomonadaceae_unclassified)* and unconjugated bile acids (CA and CDCA) in the cecum (but not in ZNO pigs).

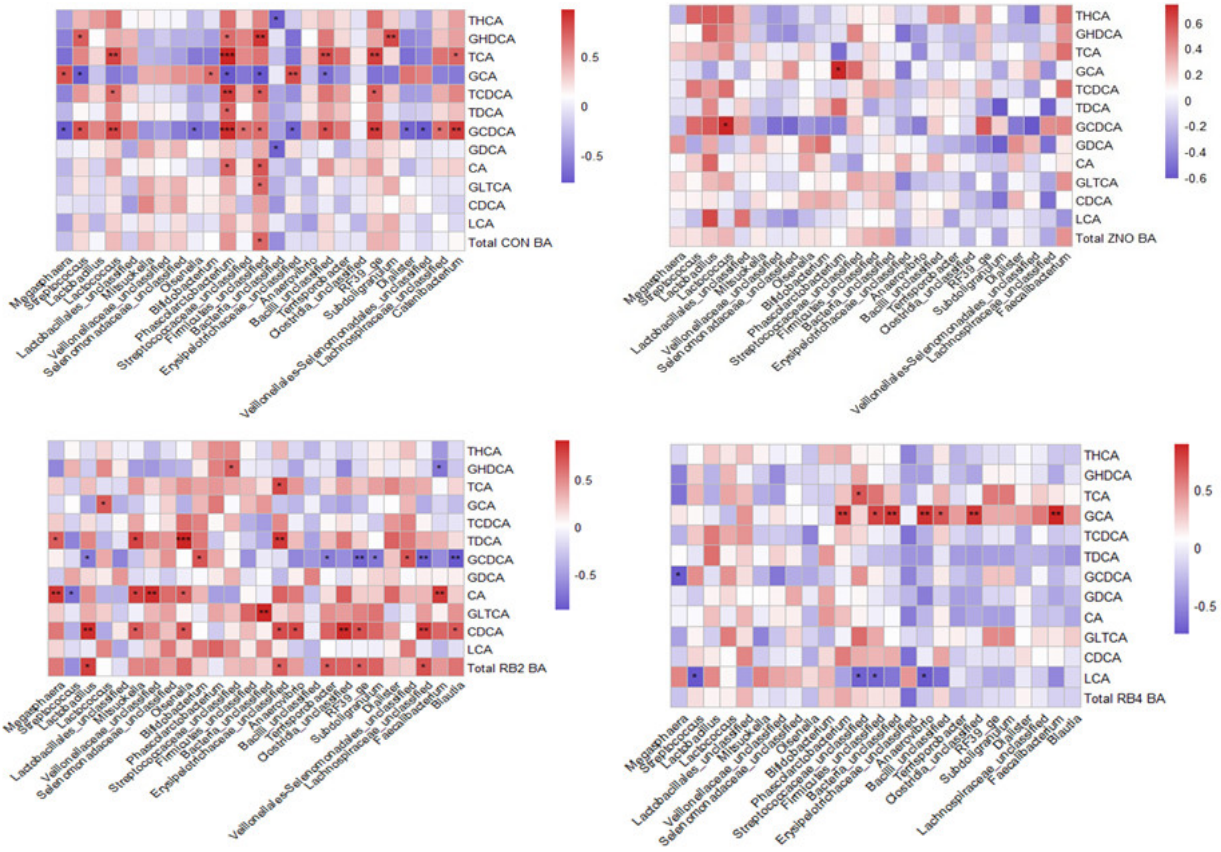


Figure 6. Spearman correlation matrices between jejunal genera abundance and bile acid levels. The bile acid omitted (deoxycholic acid—DCA) was not detected in the jejunum for the pigs and hence is not shown. Correlation depicted by color depth, where a red color denotes a positive and a blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity and *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$. CON, ZNO, RB2, and RB4 represent the control and diets supplemented with zinc oxide and 2% and 4% red beetroot, respectively. The bile acids (BA) included taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), and lithocholic acid.

3.4. Predicted Gut Microbiota Functions

Compared to the ZNO pigs, the pathways enabling bacteria response and adaptation to environmental changes (e.g., biofilm formation, flagella assembly, and a two-component system) were upregulated in the cecum of pigs on the CON diet. Pigs fed the RB4 diet had pathways influencing lipid metabolism (inositol phosphate, glycerol-phospholipid, fatty acid degradation, chloroalkane, and chloroalkene degradation) enhanced. Aside from these, there were no variations between the diets in the functional pathway predictions from the jejunal and ilea microbiota.

4. Discussion

Current reports on the health potential of red beetroot have necessitated evaluations of its probable effect on the gut microbiota and as an alternative to zinc oxide in the weaned pig diet. Red beetroot is a rich source of nutrients, fiber, and bioactive compounds and is recognized for its anti-inflammatory, antioxidant, and antimicrobial properties as well as its prebiotic effect in the gut. Given these benefits, adding red beetroot to a weaned pig diet could promote beneficial microbiota modulation of the gut, thus preventing gut dysbiosis and diarrhea post-weaning.

Diet remains an uncontestable factor shaping and modulating the gut microbiota toward the achievement of gut health and overall wellbeing [60]. In a controlled clinical trial with healthy humans consuming whole cooked red beetroot, Capper et al. [61] showed gut microbiota modulation with reduced Bacteroidetes and increased alpha diversity and short chain fatty acid (SCFA) levels combined with a normal fecal score. The weaning phase in pig production is the focus here due to the attending economic impact, more so due to the recent ban on in-feed ZnO, which has further exacerbated the health implications for weaned pigs.

Studies of the pig gut microbiome have long established that early modulation of the gut microbiota of young pigs is vital to the prevention of post-weaning diarrhea, maturation of the immune system, and improvements in growth performance [2,62,63]. Adaptation of the weaned pig gut to a new diet and achievement of a relatively stable gut microbiota 7 to 10 days post-weaning is essential for early attainment of a richly diverse gut microbial composition and gut health [64].

In the present study, 14-day supplementation of a weaned pig diet with 2% red beetroot (RB2) influenced the alpha diversity, increasing the species richness of the jejunum compared to other diets. However, a comparable number of species was observed in the cecum of all the pigs. Reduced bacteria species in the jejunum [65,66] or ilea digesta [67] of weaned pigs fed a diet with ZnO have been linked to the antimicrobial and growth-promoting ability of in-feed ZnO. According to Bonetti, Tugnoli, Piva, and Grilli [25], in-feed ZnO reduces gut bacteria activity, making more energy available for growth and metabolism.

The cecum had a rich and more diverse bacteria as supposed, whereas the jejunal and ileal microbiota were closely related. This certified the existence of more unique taxa in the cecum compared to the jejunum and the ileum [68]. However, when comparing the RB2 diet with the ZNO diet, the latter clearly modulated a diverse cecal microbiota with more distinct bacteria than the CON and RB4 diets. This was possibly driven by decreased cecal Firmicutes abundance, causing increased relative mean abundance of other phyla (e.g., Bacteroidota). To the best of our knowledge, this is the first report on the supplementation of red beetroot in a weaned pig diet; however, observations of the ZNO diet resonated with previous findings on a pharmacological dose of ZnO in a weaned pig diet [66], while the RB2 diet improved the species richness of the gut.

Meanwhile, increased RB levels did not translate to a diverse cecal microbiota despite increased fiber levels, depicting that the gut microbiota acted differently toward the fiber. In addition, the functions of dietary fiber in the gut are largely determined by its source and physicochemical characteristics (e.g., solubility, viscosity, and fermentability), which subsequently affects the gut microbial composition and metabolite output [69]. Red beetroot contains mainly soluble fiber, which may account for the significant increase in Proteobacteria and reduced SCFAs and secondary bile acids. Bacteria in this phylum tend to increase during weaning stress and in pigs on a diet rich in protein, fat, and fiber, consequently depleting beneficial bacteria like *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* [70].

Firmicutes is consistently the most dominant phylum, accounting for <95% of all phyla observed in the gut [71–73]. However, the reduced cecal Firmicutes abundance observed in this current study negated reports of higher populations in the pig cecum [68,74]. Similarly, Actinobacteriota was the second predominant phylum compared to Bacteroidota reported in most studies. While these phyla are important commensals of the gut, the differences

in management, experimental diets, sampling age, and location used in these studies may explain the observed disparity [4,75]. However, such gut microbiota alterations were recently attributed to lactate accumulation in the gut.

Wang et al. [76] confirmed gut microbiota variation from lactate accumulation, where Actinobacteria and Proteobacteria replaced the phyla Bacteroidetes and Firmicutes with a concomitant reduction in butyrate and propionate production. Proteobacteria (e.g., *Campylobacter* and *Salmonella* species), which utilize lactate under microaerophilic conditions to produce carbon dioxide and water [77], have predominantly been linked to gut perturbations mostly associated with diarrhea.

The small intestine, which is dominated by lactic acid bacteria—LAB (e.g., *Lactobacilli*, *Lactococcus*, *Streptococcus*, *Bifidobacterium*, etc.), is responsible for lactate production through various biochemical pathways [78]. Lactate prevents the growth of pathogenic organisms by lowering the gut pH value, but increased levels can be harmful and cause alterations in the gut microbiota, toxicity, and pathogenic colonization of the gut. To corroborate this claim, high ilea lactate levels (mM) were observed in the CON (96.76) and RB4 (76.85) pigs, but levels in the ZNO (44.72) and RB2 pigs (48.83) were similar (unpublished).

It is noteworthy that the gut microbiota employs lactate-utilizing bacteria—LUB (from the phylum Firmicutes) with remarkable SCFA-producing ability to avert the detrimental effect of lactate accumulation, thereby stabilizing the gut microbiota [79,80]. Hence, a balance between the LAB and LUB (functional groups) in terms of the production and utilization of lactate is necessary for gut health [77]. Prominent LUB (e.g., *Megasphaera*, *Phascolarctobacterium*, *Negativibacillus*, and *Veillonellaceae*) and LAB (e.g., *Streptococcus*, *Lactococcus*, *Lactobacillales_unclassified*, and *Streptococcaceae_unclassified*) were identified in this study. Importantly, a higher cecal relative LUB abundance but a reduced LAB were observed, while the abundance of both bacteria groups was comparable in the small intestine. These genera were associated with SCFA (acetate, propionate, and butyrate) levels across the gut locations (jejunum, ileum, and cecum) as shown by the correlation matrices.

Though the small intestine is not the major site for microbiota fermentation and SCFA production, significant SCFA levels and correlations with the jejunal and ilea microbiota were observed in the RB diets. The nutritional functions of the jejunum with capacity for energy metabolism and fiber fermentation have been confirmed in many studies, while the gut microbiota metabolite impact the jejunal immune system, barrier function, and cell proliferation [81,82]. In addition, the host immune system is regulated by continuous interaction between the gut microbiota and dietary metabolites; hence, the reduced gut microbiota association with butyrate levels observed in this study may partly be due to host immune responses as well as lactate accumulation in the gut [83]. Moreover, a decline in bacteria sensitivity to metabolite production may have doused a strong correlation between the gut microbiota and butyrate levels in the RB2 pigs, unlike in the ZNO pigs. Overall, inter-individual variability in response to diet as well as variations in gut microbial composition and function cannot be ruled out.

Bile acids have also been linked to host physiology and immunity via gut microbial metabolism. Diet influences the gut microbiota composition and bile acid levels through bile-salt-hydrolyzing bacteria (BSHB) species (e.g., *Clostridium* spp., *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*) that possess inducible genes responsible for the conversion of primary bile acids to secondary bile acids [84,85]. However, interactions between the bile acid and the gut microbiota can be severely impaired in the event of gut dysbiosis at weaning. Song et al. [86] observed that dietary supplementation with CDCA, a natural primary bile acid in animal bile, improved growth performance and reduced diarrheal incidence in weaned pigs.

Generally, the RB2 diet increased individual primary and secondary (CA, CDCA, and DCA) unconjugated bile acid levels compared to the CON diet. DCA (deoxycholic acid) was observed in the colon and feces and hence was not correlated with the gut microbiota abundance. Another study by Tian et al. [87] confirmed a higher and potent antibacterial activity in unconjugated bile acids compared to their other counterpart, and the sensitivity

of bile acids to Gram-positive bacteria compared to Gram-negative was also demonstrated. The jejunal bile acid profile was strongly associated with the jejunal microbiota of RB2 pigs (unlike the ZNO and RB4 pigs) but was the same as observed in the ileum for the ZNO pigs. Most bacteria in this region (small intestine) are usually resistant to bile acids, offering protection against pathogenic invasion [88]. Reduced bile acid levels in the gut have been implicated in bacterial overgrowth and inflammation [11]. Conversely, across the diet groups in the cecum, very few genera (e.g., *Streptococcus*, *Lactobacillales_unclassified*, *Lactococcus*, *Selenomonadaceae_unclassified*, and *Erysipelotrichaceae_unclassified*) were involved in bacterial metabolism of the unconjugated bile acids (CA and CDCA). The reasons for the reduced bile acid levels with increased red beetroot is not clear. Usually, a high fat diet increases bile acid discharge, increasing circulating bile acid levels. Alteration of secondary bile acids with dietary fiber and an increased Proteobacteria abundance with the RB4 diet are some possible causes of this trend.

Regarding differentially abundant genera in the pigs, lactate utilizers (e.g., *Veillonellaceae_ unclassified* and *Selenomonadaceae_unclassified*) increased in the jejunum of the CON pigs relative to those fed RB diets, which signified an increased abundance of lactic-acid-producing bacteria (LAB) and a potential for lactate accumulation in the CON pigs. *Terrisporobacter*, an anaerobic Gram-positive bacterium in the family Peptostreptococcaceae, increased in the ileum of the RB2 pigs compared to the CON pigs but was associated with butyrate, GDCA, and GLTCA. Other compositional differences observed in the cecum include increased gut fermenters (e.g., *Romboutsia*, *Muribauculaceae_ge*, *Terrisporobacter*, and *Clostridiaceae_unclassified*) and decreased *Selenomonadaceae_unclassified* in the ZNO pigs compared to the control. Except for increased *Selenomonadaceae_unclassified*, RB2 was not different from ZNO, while RB4 had increased *Anaerovibrio* inclusive.

Generally, *Clostridiaceae_unclassified*, *Rumminococcaceae_unclassified*, and *Erysipelotrichaceae_unclassified* were significantly higher in pigs fed the ZNO diet, which coincided with results of [20] in pigs fed 2425 mg/kg of dietary zinc. The presence of these strict anaerobes demonstrated a rapid transition of the pig gut microbiota from a (milk-based diet) suckling microbiota to a post-weaning (solid-based diet containing complex compounds) microbiota. The preceding genera are linked to bile acid and SCFA production; however, increased *Erysipelotrichaceae* abundance has been implicated in dysbiosis-related disorders of the gut [89] and in mice post-treated with broad spectrum antibiotics (e.g., gentamicin) [90].

Similarly, many studies have confirmed associations between bacteria belonging to this genus and host lipidemic profiles [91–93] and cholesterol metabolism [94,95]. This characteristic may be connected to the high systemic and hepatic lipid peroxidation observed in the plasma and liver tissue of pigs in this group (unpublished data) coupled with an increased tendencies for hepatic toxicity and oxidative stress in ZnO-fed pigs. This additionally coincides with the downregulation of pathways facilitating lipid metabolism for pigs fed the ZNO diet compared to those fed the RB4 diet.

Moreover, dietary supplementation with quercetin was reported to inhibit *Erysipelotrichaceae* [96]. Quercetin is a flavonoid (polyphenol) found in fruits and vegetables that is recognized for its health benefits and potential therapeutic effects. Polyphenol and bioactive pigments (betalains) in red beetroot may have been responsible for a decreased abundance of *Erysipelotrichaceae_unclassified* in the RB pigs.

Indeed, red beetroot contains betalains and polyphenols known for effective lipid peroxidation in membrane, thus decreasing oxidative damage [97]. This resonates with observations of upregulated pathways for lipid-metabolism pathways (i.e., inositol and glycerol-phospholipid metabolism) in the cecum of pigs in this group relative to the ZNO pigs. Also, an increased abundance of *Anaerovibrio*, a strictly lipolytic bacteria known for the hydrolysis of triglycerides to fatty acids, in pigs fed a red-beetroot-supplemented diet further confirmed these inferences. Overall, the predicted functional profile from the microbiota of each gut location (jejunum and ileum) did not differ from each diet; aside from what has been earlier described (the cecal microbiota of the CON pigs' enhanced response to bacteria adaptation to environmental changes).

This study showed the benefit of red beetroot supplementation of the diet of weaned pigs. The ability to modulate the metabolism and function of the gut microbiota demonstrates its potential as an alternative to therapeutic doses of in-feed ZnO to prevent gut dysbiosis and diarrhea that ensues post-weaning. The use of whole red beetroot represents a cost-effective way to provide bioactive compounds (e.g., betalains, nitrate, polyphenols, and fiber) to the pigs at a critical stage in their development. Hence, the effect observed can only be inferred. Future studies utilizing individual components could improve the understanding of red beetroot functionality in modulating the gut microbiota and pig health.

5. Conclusions

Diet remains a viable strategy to modulate the gut microbiota of weaned pigs, and red beetroot supplementation provides an avenue to explore its bioactives for pig gut health. In this study, a weaned pig diet supplemented with red beetroot at 2% increased the species richness of the gut microbiota. However, inclinations of lactate accumulation were observed with an increase in RB to 4% (RB4), which was characterized by potential decline in butyrate and propionate and an increased Proteobacteria abundance. The jejunum and ileum microbial compositions were similar across the diet groups, but the cecum was diverse with the ZNO diet relative to the RB2 diet, while the CON and RB4 diets were comparable. The RB2 diet also increased the gut microbiota metabolite (SCFAs and unconjugated bile acids) production in the jejunum and ileum, depicting fore gut fiber fermentation, but butyrate levels were not significantly associated with the gut microbiota as observed in the ZNO and RB4 pigs. The functional pathway predictions from cecal microbiota were closely associated with the distinct bacteria present in the cecum of the pigs across the diets. Altogether, red beetroot has the potential to modulate the gut microbiota of weaned pigs with increased species richness and enhanced lipid metabolism and metabolite production. Future work focused on purified red beetroot components and dosage in weaned pigs is warranted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13132196/s1, Table S1: Growth performance and feed intake of weaned pigs. Table S2: Comparison of mean relative abundance of top bacteria genera between diet groups and gut location. Figure S1: Spearman correlation matrices between top abundant bacteria genera and short chain fatty acids in the (a) jejunum and (b) ileum. Figure S2: Spearman correlation matrices between top abundant bacteria genera and bile acid levels in the (a) ileum and (b) cecum.

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Data Availability Statement: The 16S rRNA sequences (fastq files) generated in this study were deposited in the NCBI Sequence Read Archive (BioProject ID: PRJNA798387). All data analyzed are described in the manuscript, and other statistical outputs are available in the Supplementary Materials.

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References

1. Kim, H.B.; Isaacson, R.E. The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Vet. Microbiol.* **2015**, *177*, 242–251. [CrossRef] [PubMed]
2. Guevarra, R.B.; Lee, J.H.; Lee, S.H.; Seok, M.J.; Kim, D.W.; Kang, B.N.; Johnson, T.J.; Isaacson, R.E.; Kim, H.B. Piglet gut microbial shifts early in life: Causes and effects. *J. Anim. Sci. Biotechnol.* **2019**, *10*, 1. [CrossRef] [PubMed]
3. Bian, G.; Ma, S.; Zhu, Z.; Su, Y.; Zoetendal, E.G.; Mackie, R.; Liu, J.; Mu, C.; Huang, R.; Smidt, H. Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environ. Microbiol.* **2016**, *18*, 1566–1577. [CrossRef] [PubMed]
4. Guevarra, R.B.; Hong, S.H.; Cho, J.H.; Kim, B.R.; Shin, J.; Lee, J.H.; Kang, B.N.; Kim, Y.H.; Wattanaphansak, S.; Isaacson, R.E.; et al. The dynamics of the piglet gut microbiome during the weaning transition in association with health and nutrition. *J. Anim. Sci. Biotechnol.* **2018**, *9*, 54. [CrossRef]
5. Valeriano, V.D.; Balolong, M.P.; Kang, D.K. Probiotic roles of Lactobacillus sp. in swine: Insights from gut microbiota. *J. Appl. Microbiol.* **2017**, *122*, 554–567. [CrossRef] [PubMed]
6. Wang, T.; Yao, W.; Li, J.; Shao, Y.; He, Q.; Xia, J.; Huang, F. Dietary garcinol supplementation improves diarrhea and intestinal barrier function associated with its modulation of gut microbiota in weaned piglets. *J. Anim. Sci. Biotechnol.* **2020**, *11*, 12. [CrossRef] [PubMed]
7. Gao, J.; Liu, Z.; Wang, C.; Ma, L.; Chen, Y.; Li, T. Effects of dietary protein level on the microbial composition and metabolomic profile in postweaning piglets. *Oxid. Med. Cell Longev.* **2022**, *2022*, 3355687. [CrossRef]
8. Huting, A.M.S.; Middelkoop, A.; Guan, X.; Molist, F. Using nutritional strategies to shape the gastro-intestinal tracts of suckling and weaned piglets. *Animals* **2021**, *11*, 402. [CrossRef]
9. Xiong, X.; Tan, B.; Song, M.; Ji, P.; Kim, K.; Yin, Y.; Liu, Y. Nutritional intervention for the intestinal development and health of weaned pigs. *Front. Vet. Sci.* **2019**, *6*, 46. [CrossRef]
10. Vasquez, R.; Oh, J.K.; Song, J.H.; Kang, D.K. Gut microbiome-produced metabolites in pigs: A review on their biological functions and the influence of probiotics. *J. Anim. Sci. Technol.* **2022**, *64*, 671–695. [CrossRef]
11. Ridlon, J.M.; Kang, D.J.; Hylemon, P.B.; Bajaj, J.S. Bile acids and the gut microbiome. *Curr. Opin. Gastroenterol.* **2014**, *30*, 332–338. [CrossRef] [PubMed]
12. Seal, B.S.; Lillehoj, H.S.; Donovan, D.M.; Gay, C.G. Alternatives to antibiotics: A symposium on the challenges and solutions for animal production. *Anim. Health Res. Rev.* **2013**, *14*, 78–87. [CrossRef] [PubMed]
13. Slade, R.; Kyriazakis, I.; Carroll, S.; Reynolds, F.; Wellock, I.; Broom, L.; Miller, H. Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic escherichia coli o149 challenge. *Animal* **2011**, *5*, 1170–1178. [CrossRef] [PubMed]
14. Becattini, S.; Taur, Y.; Pamer, E.G. Antibiotic-induced changes in the intestinal microbiota and disease. *Trends. Mol. Med.* **2016**, *22*, 458–478. [CrossRef] [PubMed]
15. Hu, C.H.; Xiao, K.; Song, J.; Luan, Z.S. Effects of zinc oxide supported on zeolite on growth performance, intestinal microflora and permeability, and cytokines expression of weaned pigs. *Anim. Feed. Sci. Technol.* **2013**, *181*, 65–71. [CrossRef]
16. Dong, X.; Xu, Q.; Wang, C.; Zou, X.; Lu, J. Supplemental-coated zinc oxide relieves diarrhoea by decreasing intestinal permeability in weanling pigs. *Appl. Anim. Res.* **2019**, *47*, 362–368. [CrossRef]
17. Zhao, Y.; Su, J.Q.; An, X.L.; Huang, F.Y.; Rensing, C.; Brandt, K.K.; Zhu, Y.G. Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci. Total Environ.* **2018**, *621*, 1224–1232. [CrossRef]
18. Holman, D.B.; Chenier, M.R. Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. *Canad. J. Microbiol.* **2015**, *61*, 785–798. [CrossRef]
19. Pi, Y.; Gao, K.; Peng, Y.; Mu, C.; Zhu, W. Antibiotic-induced alterations of the gut microbiota and microbial fermentation in protein parallel the changes in host nitrogen metabolism of growing pigs. *Animal* **2018**, *13*, 262–272. [CrossRef]
20. Starke, I.C.; Pieper, R.; Neumann, K.; Zentek, J.; Vahjen, W. The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets. *FEMS Microbiol. Ecol.* **2014**, *87*, 416–427. [CrossRef]
21. Pieper, R.; Vahjen, W.; Neumann, K.; Van Kessel, A.G.; Zentek, J. Dose-dependent effects of dietary zinc oxide on bacterial communities and metabolic profiles in the ileum of weaned pigs. *J. Anim. Physiol. Anim. Nutr.* **2012**, *96*, 825–833. [CrossRef] [PubMed]
22. Ciesinski, L.; Guenther, S.; Pieper, R.; Kalisch, M.; Bednorz, C.; Wieler, L.H. High dietary zinc feeding promotes persistence of multi-resistant e. Coli in the swine gut. *PLoS ONE* **2018**, *13*, e0191660. [CrossRef] [PubMed]
23. Vahjen, W.; Pieper, R.; Zentek, J. Increased dietary zinc oxide changes the bacterial core and enterobacterial composition in the ileum of piglets. *J. Anim. Sci.* **2011**, *89*, 2430–2439. [CrossRef]
24. Schokker, D.; Zhang, J.; Vastenhouw, S.A.; Heilig, H.G.; Smidt, H.; Rebel, J.M.; Smits, M.A. Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. *PLoS ONE* **2015**, *10*, e0116523. [CrossRef]
25. Bonetti, A.; Tugnoli, B.; Piva, A.; Grilli, E. Towards zero zinc oxide: Feeding strategies to manage post-weaning diarrhea in piglets. *Animals* **2021**, *11*, 642. [CrossRef] [PubMed]

26. Jensen, J.; Kyvsgaard, N.C.; Battisti, A.; Baptiste, K.E. Environmental and public health related risk of veterinary zinc in pig production-using denmark as an example. *Environ. Int.* **2018**, *114*, 181–190. [CrossRef] [PubMed]
27. Yazdankhah, S.; Rudi, K.; Bernhoft, A. Zinc and copper in animal feed—Development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microb. Ecol. Health Dis.* **2014**, *25*, 25862. [CrossRef]
28. Bednorz, C.; Oelgeschlager, K.; Kinnemann, B.; Hartmann, S.; Neumann, K.; Pieper, R.; Bethe, A.; Semmler, T.; Tedin, K.; Schierack, P.; et al. The broader context of antibiotic resistance: Zinc feed supplementation of piglets increases the proportion of multi-resistant escherichia coli in vivo. *Int. J. Med. Microbiol.* **2013**, *303*, 396–403. [CrossRef]
29. De Oliveira, S.P.A.; do Nascimento, H.M.A.; Sampaio, K.B.; de Souza, E.L. A review on bioactive compounds of beet (beta vulgaris l. Subsp. Vulgaris) with special emphasis on their beneficial effects on gut microbiota and gastrointestinal health. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 2022–2033. [CrossRef]
30. Dos, S. Baião, D.; da Silva, D.V.; Paschoalin, V.M. Beetroot, a remarkable vegetable: Its nitrate and phytochemical contents can be adjusted in novel formulations to benefit health and support cardiovascular disease therapies. *Antioxidants* **2020**, *9*, 960. [CrossRef]
31. Ninfali, P.; Angelino, D. Nutritional and functional potential of beta vulgaris cicla and rubra. *Fitoterapia* **2013**, *89*, 188–199. [CrossRef] [PubMed]
32. Nemzer, B.; Pietrzkowski, Z.; Spórna, A.; Stalica, P.; Thresher, W.; Michałowski, T.; Wybraniec, S. Betalainic and nutritional profiles of pigment-enriched red beet root (beta vulgaris l.) dried extracts. *Food Chem.* **2011**, *127*, 42–53. [CrossRef]
33. Chhikara, N.; Kushwaha, K.; Sharma, P.; Gat, Y.; Panghal, A. Bioactive compounds of beetroot and utilization in food processing industry: A critical review. *Food Chem.* **2019**, *272*, 192–200. [CrossRef] [PubMed]
34. Gómez, B.; Peláez, C.; Martínez-Cuesta, M.C.; Parajó, J.C.; Alonso, J.L.; Requena, T. Emerging prebiotics obtained from lemon and sugar beet byproducts: Evaluation of their in vitro fermentability by probiotic bacteria. *LWT* **2019**, *109*, 17–25. [CrossRef]
35. Danneskiold-Samsøe, N.B.; Barros, H.D.d.F.Q.; Santos, R.; Bicas, J.L.; Cazarin, C.B.B.; Madsen, L.; Kristiansen, K.; Pastore, G.M.; Brix, S.; Júnior, M.R.M. Interplay between food and gut microbiota in health and disease. *Food Res. Int.* **2019**, *115*, 23–31. [CrossRef] [PubMed]
36. Van der Merwe, M. Gut microbiome changes induced by a diet rich in fruits and vegetables. *Int. J. Food Sci. Nutr.* **2021**, *72*, 665–669. [CrossRef]
37. Clifford, T.; Howatson, G.; West, D.J.; Stevenson, E.J. The potential benefits of red beetroot supplementation in health and disease. *Nutrients* **2015**, *7*, 2801–2822. [CrossRef]
38. Singh, B.; Hathan, B.S. Chemical composition, functional properties and processing of beetroot—A review. *Int. J. Sci. Eng. Res.* **2014**, *5*, 679–684.
39. Klewicka, E.; Zduńczyk, Z.; Juśkiewicz, J.; Klewicki, R. Effects of lactofermented beetroot juice alone or with n-nitroso-n-methylurea on selected metabolic parameters, composition of the microbiota adhering to the gut epithelium and antioxidant status of rats. *Nutrients* **2015**, *7*, 5905–5915. [CrossRef]
40. Butt, M.S.; Randhawa, M.A.; Shahid, M. Nephroprotective effects of red beetroot-based beverages against gentamicin-induced renal stress. *Food Biochem.* **2019**, *43*, e12873.
41. Mirmiran, P.; Houshialsadat, Z.; Gaeini, Z.; Bahadoran, Z.; Azizi, F. Functional properties of beetroot (beta vulgaris) in management of cardio-metabolic diseases. *Nutr. Metab.* **2020**, *17*, 3. [CrossRef] [PubMed]
42. Wootton-Beard, P.C.; Brandt, K.; Fell, D.; Warner, S.; Ryan, L. Effects of a beetroot juice with high neobetanin content on the early-phase insulin response in healthy volunteers. *J. Nutr. Sci.* **2014**, *3*, e9. [CrossRef] [PubMed]
43. da Silva, D.V.T.; Pereira, A.D.; Boaventura, G.T.; Ribeiro, R.S.A.; Vericimo, M.A.; Carvalho-Pinto, C.E.; Baiao, D.D.S.; Del Aguila, E.M.; Paschoalin, V.M.F. Short-term betanin intake reduces oxidative stress in wistar rats. *Nutrients* **2019**, *11*, 1978. [CrossRef] [PubMed]
44. Pinedo-Gil, J.; Tomás-Vidal, A.; Jover-Cerdá, M.; Tomás-Almenar, C.; Sanz-Calvo, M.Á.; Martín-Diana, A.B. Red beet and betaine as ingredients in diets of rainbow trout (oncorhynchus mykiss): Effects on growth performance, nutrient retention and flesh quality. *Arch. Anim. Nutr.* **2017**, *71*, 486–505. [CrossRef]
45. Pinedo-Gil, J.; Martin-Diana, A.B.; Bertotto, D.; Sanz-Calvo, M.A.; Jover-Cerda, M.; Tomas-Vidal, A. Effects of dietary inclusions of red beet and betaine on the acute stress response and muscle lipid peroxidation in rainbow trout. *Fish Physiol. Biochem.* **2018**, *44*, 939–948. [CrossRef]
46. Tian, G.; Wu, X.; Chen, D.; Yu, B.; He, J. Adaptation of gut microbiome to different dietary nonstarch polysaccharide fractions in a porcine model. *Mol. Nutr. Food Res.* **2017**, *61*, 1700012. [CrossRef]
47. Gu, C.; Howell, K.; Padayachee, A.; Comino, T.; Chhan, R.; Zhang, P.; Ng, K.; Cottrell, J.J.; Dunshea, F.R. Effect of a polyphenol-rich plant matrix on colonic digestion and plasma antioxidant capacity in a porcine model. *J. Funct. Foods* **2019**, *57*, 211–221. [CrossRef]
48. Li, H.; Li, H.; Xie, P.; Li, Z.; Yin, Y.; Blachier, F.; Kong, X. Dietary supplementation with fermented mao-tai lees beneficially affects gut microbiota structure and function in pigs. *AMB Express* **2019**, *9*, 26. [CrossRef]
49. Sutton, T.; O'Neill, H.M.; Bedford, M.; McDermott, K.; Miller, H. Effect of xylanase and xylo-oligosaccharide supplementation on growth performance and faecal bacterial community composition in growing pigs. *Anim. Feed Sci. Technol.* **2021**, *274*, 114822. [CrossRef]

50. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.; et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef] [PubMed]
51. Kozich, J.J.; Westcott, S.L.; Baxter, N.T.; Highlander, S.K.; Schloss, P.D. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl. Environ. Microbiol.* **2013**, *79*, 5112–5120. [CrossRef]
52. Parks, D.H.; Tyson, G.W.; Hugenholtz, P.; Beiko, R.G. Stamp: Statistical analysis of taxonomic and functional profiles. *Bioinformatics* **2014**, *30*, 3123–3124. [CrossRef] [PubMed]
53. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for rna-seq data with deseq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]
54. Iwai, S.; Weinmaier, T.; Schmidt, B.L.; Albertson, D.G.; Poloso, N.J.; Dabbagh, K.; DeSantis, T.Z. Piphillin: Improved prediction of metagenomic content by direct inference from human microbiomes. *PLoS ONE* **2016**, *11*, e0166104. [CrossRef] [PubMed]
55. Langille, M.G.; Zaneveld, J.; Caporaso, J.G.; McDonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Vega Thurber, R.L.; Knight, R.; et al. Predictive functional profiling of microbial communities using 16s rrna marker gene sequences. *Nat. Biotechnol.* **2013**, *31*, 814–821. [CrossRef]
56. Taylor, A.E.; Bedford, M.R.; Miller, H.M. The effects of xylanase on grower pig performance, concentrations of volatile fatty acids and peptide yy in portal and peripheral blood. *Animal* **2018**, *12*, 2499–2504. [CrossRef] [PubMed]
57. Zhang, L.; Wu, W.; Lee, Y.K.; Xie, J.; Zhang, H. Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. *Front. Microbiol.* **2018**, *9*, 48. [CrossRef]
58. Revelle, W. *How to: Use the Psych Package for Factor Analysis and Data Reduction*; Northwestern University, Department of Psychology: Evanston, IL, USA, 2016.
59. Gu, Z.; Eils, R.; Schlesner, M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **2016**, *32*, 2847–2849. [CrossRef]
60. Alou, M.T.; Lagier, J.-C.; Raoult, D. Diet influence on the gut microbiota and dysbiosis related to nutritional disorders. *Hum. Microbiome* **2016**, *1*, 3–11. [CrossRef]
61. Capper, T.E.; Houghton, D.; Stewart, C.J.; Blain, A.P.; McMahon, N.; Siervo, M.; West, D.J.; Stevenson, E.J. Whole beetroot consumption reduces systolic blood pressure and modulates diversity and composition of the gut microbiota in older participants. *NFS J.* **2020**, *21*, 28–37. [CrossRef]
62. Argüello, H.; Estellé, J.; Leonard, F.C.; Crispie, F.; Cotter, P.D.; O'Sullivan, O.; Lynch, H.; Walia, K.; Duffy, G.; Lawlor, P.G. Influence of the intestinal microbiota on colonization resistance to salmonella and the shedding pattern of naturally exposed pigs. *mSystems* **2019**, *4*, e00019–e00021. [CrossRef] [PubMed]
63. De Vries, H.; Smidt, H. Microbiota Development in Piglets. In *The Suckling Weaned Piglet*; Wageningen Academic Publishers: Wageningen, The Netherlands, 2020; Volume 7, pp. 179–205.
64. Chen, L.; Xu, Y.; Chen, X.; Fang, C.; Zhao, L.; Chen, F. The maturing development of gut microbiota in commercial piglets during the weaning transition. *Front. Microbiol.* **2017**, *8*, 1688. [CrossRef]
65. Shen, J.; Chen, Y.; Wang, Z.; Zhou, A.; He, M.; Mao, L.; Zou, H.; Peng, Q.; Xue, B.; Wang, L.; et al. Coated zinc oxide improves intestinal immunity function and regulates microbiota composition in weaned piglets. *Br. J. Nutr.* **2014**, *111*, 2123–2134. [CrossRef]
66. Yu, T.; Zhu, C.; Chen, S.; Gao, L.; Lv, H.; Feng, R.; Zhu, Q.; Xu, J.; Chen, Z.; Jiang, Z. Dietary high zinc oxide modulates the microbiome of ileum and colon in weaned piglets. *Front. Microbiol.* **2017**, *8*, 825. [CrossRef]
67. Namkung, H.; Gong, J.; Yu, H.; De Lange, C. Effect of pharmacological intakes of zinc and copper on growth performance, circulating cytokines and gut microbiota of newly weaned piglets challenged with coliform lipopolysaccharides. *Canad. J. Anim. Sci.* **2006**, *86*, 511–522. [CrossRef]
68. Gao, J.; Yin, J.; Xu, K.; Li, T.J.; Yin, Y.L. What is the impact of diet on nutritional diarrhea associated with gut microbiota in weaning piglets: A system review. *Biomed. Res. Int.* **2019**, *2019*, 6916189. [CrossRef]
69. Awati, A.; Williams, B.A.; Bosch, M.W.; Gerrits, W.J.; Verstegen, M.W. Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in feces of weanling piglets. *J. Anim. Sci.* **2006**, *84*, 2133–2140. [CrossRef] [PubMed]
70. Zijlmans, M.A.C.; Korpela, K.; Riksen-Walraven, J.M.; de Vos, W.M.; de Weerth, C. Maternal prenatal stress is associated with the infant intestinal microbiota. *Psychoneuroendocrinology* **2015**, *53*, 233–245. [CrossRef] [PubMed]
71. Kim, H.B.; Borewicz, K.; White, B.A.; Singer, R.S.; Sreevatsan, S.; Tu, Z.J.; Isaacson, R.E. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet. Microbiol.* **2011**, *153*, 124–133. [CrossRef]
72. Looft, T.; Johnson, T.A.; Allen, H.K.; Bayles, D.O.; Alt, D.P.; Stedtfeld, R.D.; Sul, W.J.; Stedtfeld, T.M.; Chai, B.; Cole, J.R.; et al. In-feed antibiotic effects on the swine intestinal microbiome. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 1691–1696. [CrossRef] [PubMed]
73. Hu, J.; Nie, Y.; Chen, J.; Zhang, Y.; Wang, Z.; Fan, Q.; Yan, X. Gradual changes of gut microbiota in weaned miniature piglets. *Front. Microbiol.* **2016**, *7*, 1727. [CrossRef] [PubMed]
74. Li, K.; Xiao, Y.; Chen, J.; Chen, J.; He, X.; Yang, H. Microbial composition in different gut locations of weaning piglets receiving antibiotics. *Asian-Australas. J. Anim. Sci.* **2017**, *30*, 78–84. [CrossRef] [PubMed]
75. Pluske, J.R. Feed-and feed additives-related aspects of gut health and development in weanling pigs. *Anim. Sci. Biotechnol.* **2013**, *4*, 1–7. [CrossRef] [PubMed]

76. Wang, S.P.; Rubio, L.A.; Duncan, S.H.; Donachie, G.E.; Holtrop, G.; Lo, G.; Farquharson, F.M.; Wagner, J.; Parkhill, J.; Louis, P.; et al. Pivotal roles for ph, lactate, and lactate-utilizing bacteria in the stability of a human colonic microbial ecosystem. *mSystems* **2020**, *5*, e00620–e00645. [CrossRef] [PubMed]
77. Louis, P.; Duncan, S.H.; Sheridan, P.O.; Walker, A.W.; Flint, H.J. Microbial lactate utilisation and the stability of the gut microbiome. *Gut Microbiome* **2022**, *3*, e3. [CrossRef]
78. Louis, P.; Flint, H.J. Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* **2017**, *19*, 29–41. [CrossRef] [PubMed]
79. Reichardt, N.; Duncan, S.H.; Young, P.; Belenguer, A.; McWilliam Leitch, C.; Scott, K.P.; Flint, H.J.; Louis, P. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *ISME J.* **2014**, *8*, 1323–1335. [CrossRef]
80. Brestenský, M.; Nitrayová, S.; Bomba, A.; Patráš, P.; Strojný, L.; Szabadošová, V.; Pramuková, B.; Bertková, I. The content of short chain fatty acids in the jejunal digesta, cecal digesta and faeces of growing pigs. *Livest. Sci.* **2017**, *205*, 106–110. [CrossRef]
81. Duarte, M.E.; Kim, S.W. Intestinal microbiota and its interaction to intestinal health in nursery pigs. *Anim. Nutr.* **2022**, *8*, 169–184. [CrossRef] [PubMed]
82. Chen, X.; Zheng, R.; Liu, R.; Li, L. Goat milk fermented by lactic acid bacteria modulates small intestinal microbiota and immune responses. *J. Funct. Foods* **2020**, *65*, 103744. [CrossRef]
83. Ivanov, I.I.; Honda, K. Intestinal commensal microbes as immune modulators. *Cell Host Microbe* **2012**, *12*, 496–508. [CrossRef]
84. Jia, W.; Xie, G.X.; Jia, W.P. Bile acid-microbiota crosstalk in gastrointestinal inflammation and carcinogenesis. *Nat. Rev. Gastro. Hepat.* **2018**, *15*, 111–128. [CrossRef]
85. Winston, J.A.; Theriot, C.M. Diversification of host bile acids by members of the gut microbiota. *Gut Microbes* **2020**, *11*, 158–171. [CrossRef]
86. Song, M.; Zhang, F.; Chen, L.; Yang, Q.; Su, H.; Yang, X.; He, H.; Ling, M.; Zheng, J.; Duan, C. Dietary chenodeoxycholic acid improves growth performance and intestinal health by altering serum metabolic profiles and gut bacteria in weaned piglets. *Anim. Nutr.* **2021**, *7*, 365–375. [CrossRef]
87. Tian, Y.; Gui, W.; Koo, I.; Smith, P.B.; Allman, E.L.; Nichols, R.G.; Rimal, B.; Cai, J.; Liu, Q.; Patterson, A.D. The microbiome modulating activity of bile acids. *Gut Microbes* **2020**, *11*, 979–996. [CrossRef] [PubMed]
88. Wahlström, A.; Sayin, S.I.; Marschall, H.-U.; Bäckhed, F. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metabol.* **2016**, *24*, 41–50. [CrossRef] [PubMed]
89. Kaakoush, N.O. Insights into the role of erysipelotrichaceae in the human host. *Front. Cell. Infect. Microbiol.* **2015**, *5*, 84. [CrossRef]
90. Palm, N.W.; De Zoete, M.R.; Cullen, T.W.; Barry, N.A.; Stefanowski, J.; Hao, L.; Degnan, P.H.; Hu, J.; Peter, I.; Zhang, W. Immunoglobulin a coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* **2014**, *158*, 1000–1010. [CrossRef]
91. Finlayson-Trick, E.C.; Getz, L.J.; Slaine, P.D.; Thornbury, M.; Lamoureux, E.; Cook, J.; Langille, M.G.; Murray, L.E.; McCormick, C.; Rohde, J.R. Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals. *PLoS ONE* **2017**, *12*, e0189404. [CrossRef]
92. Cuevas-Tena, M.; del Pulgar, E.M.G.; Benitez-Paez, A.; Sanz, Y.; Alegria, A.; Lagarda, M.J. Plant sterols and human gut microbiota relationship: An in vitro colonic fermentation study. *J. Funct. Foods* **2018**, *44*, 322–329. [CrossRef]
93. Pereira, A.C.; Bandeira, V.; Fonseca, C.; Cunha, M.V. Egyptian mongoose (herpestes ichneumon) gut microbiota: Taxonomical and functional differences across sex and age classes. *Microorganisms* **2020**, *8*, 392. [CrossRef] [PubMed]
94. Martínez, I.; Perdicaro, D.J.; Brown, A.W.; Hammons, S.; Carden, T.J.; Carr, T.P.; Eskridge, K.M.; Walter, J. Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters. *Appl. Environ. Microbiol.* **2013**, *79*, 516–524. [CrossRef] [PubMed]
95. Martínez, I.; Wallace, G.; Zhang, C.; Legge, R.; Benson, A.K.; Carr, T.P.; Moriyama, E.N.; Walter, J. Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. *Appl. Environ. Microbiol.* **2009**, *75*, 4175–4184. [CrossRef] [PubMed]
96. Etxeberria, U.; Arias, N.; Boque, N.; Macarulla, M.T.; Portillo, M.P.; Martinez, J.A.; Milagro, F.I. Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. *J. Nutr. Biochem.* **2015**, *26*, 651–660. [CrossRef] [PubMed]
97. Wang, C.-Q.; Yang, G.-Q. Betacyanins from portulaca oleracea l. Ameliorate cognition deficits and attenuate oxidative damage induced by d-galactose in the brains of senescent mice. *Phytomedicine* **2010**, *17*, 527–532. [CrossRef]

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Impact of Pomegranate on Probiotic Growth, Viability, Transcriptome and Metabolism

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Abstract: Despite rising interest in understanding intestinal bacterial survival in situ, relatively little attention has been devoted to deciphering the interaction between bacteria and functional food ingredients. Here, we examined the interplay between diverse beneficial *Lactobacillaceae* species and a pomegranate (POM) extract and determined the impact of this functional ingredient on bacterial growth, cell survival, transcription and target metabolite genesis. Three commercially available probiotic strains (*Lactobacillus acidophilus* NCFM, *Lacticaseibacillus rhamnosus* GG and *Lactiplantibacillus plantarum* Lp-115) were used in growth assays and flow cytometry analysis, indicating differential responses to the presence of POM extract across the three strains. The inclusion of POM extract in the growth medium had the greatest impact on *L. acidophilus* cell counts. LIVE/DEAD staining determined significantly fewer dead cells when *L. acidophilus* was grown with POM extract compared to the control with no POM (1.23% versus 7.23%). Whole-transcriptome analysis following exposure to POM extract showed markedly different global transcriptome responses, with 15.88% of the *L. acidophilus* transcriptome, 19.32% of the *L. rhamnosus* transcriptome and only 2.37% of the *L. plantarum* transcriptome differentially expressed. We also noted strain-dependent metabolite concentrations in the medium with POM extract compared to the control medium for punicalagin, ellagic acid and gallic acid. Overall, the results show that POM extract triggers species-specific responses by probiotic strains and substantiates the rising interest in using POM as a prebiotic compound.

Keywords: probiotic; prebiotic; pomegranate; lactobacilli; transcriptomic

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1. Introduction

Many foods encompass active ingredients, and functional foods have been known for centuries to exert health benefits, although the molecular basis of their modes of action remains undeciphered [1]. Functional foods can provide health benefits beyond their nutritional value and are an ever-increasing research area, as the industry was reported to be worth USD 173.26 billion in 2019 and is estimated to grow 7.5% annually, increasing to USD 309 billion by 2027 [2]. In addition, the growth of the functional food industry is fueled by more awareness of the food we consume and reported health benefits that can be achieved through natural means. Indeed, there has been an explosion of studies of the microbiome in the gastrointestinal tract (GIT) and increased examination of the importance of this microbial community on both health and disease [3–5]. Therefore, understanding the interaction and relationship between functional foods and beneficial bacteria is imperative to decipher their function in the GIT [6]. In fact, the National Institute of Health launched a strategic plan in 2021 entitled "Mapping a pathway to research on whole person health", which includes multisystem approaches to studying human health, including a nutritional approach encompassing probiotics, prebiotics, phytochemicals, dietary plants, and food and microbiome metabolites, amongst others [7].

Pomegranate (POM), a fruit consumed for centuries [8], is considered a functional food given its wide range of documented beneficial effects, including antibacterial and

anticarcinogenic, as well as skin protection from UV photodamage [9–12]. In addition to its nutritional attributes, pomegranate is a rich source of plant metabolites, including anthocyanins and polyphenols such as ellagic acid and punicalagins, which are further metabolized by the microbiota in the GIT into bioactive metabolites [12]. Studies have shown that many of the health benefits of POM are due to ellagitannins, particularly punicalagins that are hydrolyzed into ellagic acid [11]. This hydrolysis initially occurs in the small intestine, where ellagic acid is absorbed, and the bacteria in the large intestine metabolize any remaining ellagitannins and ellagic acid into urolithins. In recent studies, punicalagin, the most abundant polyphenol in POM, has demonstrated antiviral [13,14], antibacterial [15] and anticancer properties and has been revealed to play a potential role in preventing cardiovascular disease [11]. The activity of urolithins, which are GIT metabolites, includes anticancer and anti-inflammatory properties [16,17]. The antibacterial effects of POM have been shown against various pathogenic bacteria, while intriguingly enhancing the cell numbers of some beneficial bacteria, including probiotics [18–21]. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [22]. Probiotic bacteria have shown numerous and wide-ranging beneficial effects on the host, particularly in the GIT, including the exclusion of pathogens, immunomodulation, nutrient turnover and the modulation of bile acids [23]. For consideration as a probiotic, strains should meet certain criteria and are often members of genera associated with the human gut microbiome, such as *Bifidobacterium* and Lactobacillaceae [9]. Previous studies have shown a prebiotic effect of POM extract and POM juice on lactobacilli in vitro, with an increase in cell counts and the utilization of ellagic acid [20]. In addition, the consumption of POM extract by healthy volunteers over a 4-week period led to changes in the gut microbiome and levels of fecal urolithin A [24]. In the urolithin A-producer group, there was in increase in the detection of *Lactobacillus* after 4 weeks compared to the baseline [24].

Considering the potential health benefits of POM extract, we studied the growth of *Lactobacillus* species in the presence of POM extract and used RNA sequencing to determine the whole-transcriptome response of three established commercial probiotic strains. We also performed targeted metabolite analysis to further consider the prebiotic potential of POM extract on commercial probiotic strains.

2. Materials and Methods

2.1. Preparation of Pomegranate Extract and Growth Media

In this study, the starting material was pomegranate polyphenol extract dietary supplement (POMx) powder from POM Wonderful, Inc., Los Angeles, CA. Details of the preparation of the pomegranate polyphenol extract dietary supplement (POMx) are described by Herber et al., 2007 [25]. Briefly, after pressing to obtain juice, the aqueous portion is extracted from the fruit residue and seeds are removed, and then a powder is produced by solid-phase extraction of the liquid concentrate [25]. This group also reported the polyphenol content of POMx to be 61% gallic acid equivalents, of which 77% are oligomers of gallic acid, ellagic acid and glucose, 19% are ellagitannins (punicalagins and punicalins) and 4% are free ellagic acid [25]. In addition, they reported that POMx contains 3.3% moisture, 2.2% ash, 2.9% sugars, 1.9% organic acids and 0.7% nitrogen. The preparation of the pomegranate (POM) extract that was used in these studies from POMx was followed as described previously [26,27]. The POM extract was dissolved in ultrapure water (Life Technologies, Carlsbad, CA, USA) at a concentration of 7 mg/mL and vortexed for 10 min. The solution was then centrifuged at 1717× *g* for 10 min and filter-sterilized through a 0.45µm filter. Aliquots of the stock solution were stored at −20 °C. A semi-defined medium (SDM; composition details can be found in Supplementary Materials) was used as the base medium [28], with the addition of glucose (1% (for transfers) or 0.5% (for experiments)). In this study, "transfers" refers to introducing the strains to the growth medium prior to experiments. The stock POM extract was diluted in SDM to 400 µg/mL for transfers and 400 µg/mL (P400) or 800 µg/mL (P800) for growth assays. All subsequent experiments

(Figure S1) were performed with a concentration of P400, given that the initial results of growth assays indicated that the higher concentration resulted in potential strain inhibition.

2.2. Growth Assays

L. acidophilus NCFM (Lac), *L. rhamnosus* GG (ATCC 53103, Lrh) and *L. plantarum* Lp-115 (Lpl) were grown from −80 °C glycerol stocks and transferred (1% inoculum) in SDM medium (1% glucose, with or without P400) for two overnight transfers at 37 °C prior to growth assays (1% inoculum) in test tubes with growth medium without POM (control) or with P400 or P800 to determine the OD and CFU/mL over a 24 h time period. OD 600 mm values were determined in cuvettes using a spectrometer (Genesys 20, ThermoSpectronic) at T0, T4, T6, T8, T12 and T24 h. The CFU/mL values were determined at T0, T8 and T24 h by performing serial dilutions and plating on MRS agar plates.

2.3. Flow Cytometry Analysis

Flow cytometry was performed as described previously [29]. *L. acidophilus* NCFM, *L. plantarum* Lp-115 and *L. rhamnosus* GG were transferred twice in SDM medium and grown for 24 h in SDM medium (control) or transferred twice in SDM medium with P400 and grown for 24 h in SDM medium with P400 (test) at 37 °C prior to flow cytometry analysis (Figure S1). Bacterial cultures were then centrifuged at 1717× *g* for 10 min, washed and resuspended in phosphate-buffered saline (Life Technologies, Carlsbad, CA, USA). A CytoFLEX Flow Cytometer instrument (Beckman Coulter, Brea, CA, USA) located at the College of Veterinary Medicine Flow Cytometry and Cell Sorting core facility (North Carolina State University) was used to determine the scattering patterns. LIVE/DEAD cell viability staining was performed using propidium iodide as detailed by the manufacturer (Fisher Scientific, Waltham, MA, USA). Data analysis was performed with the CytExpert software (Beckman Coulter, Brea, CA, USA).

2.4. RNA Isolation and mRNA Sequencing and Data Analysis

L. acidophilus NCFM, *L. plantarum* Lp-115 and *L. rhamnosus* GG (two biological replicate cultures) were transferred twice in SDM (1% glucose no POM) or SDM (1% glucose and P400) and then grown to the mid-log phase (OD600 = 0.5–0.7) in SDM (0.5% glucose) broth or SDM (0.5% glucose and P400) at 37 °C under ambient atmospheric conditions. Cells were pelleted and flash-frozen, and pellets were stored at −80 °C. Methods for RNA isolation and RNA sequence analysis were as described previously [30]. Total RNA was extracted using the Zymo Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). DNA was removed by incubating samples with Turbo DNase as described by the manufacturer (Ambion Inc., Austin, TX, USA), purified using the RNA clean and concentrator kit (Zymo Research) and checked for integrity by capillary electrophoresis on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Library preparation and sequencing were performed at The High-Throughput Sequencing and Genotyping Unit of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The Ribozero Bacteria kit (Illumina, San Diego, CA, USA) was used to remove rRNA, followed by library preparation with the TruSeq Stranded mRNAseq Sample Prep kit (Illumina). Libraries were then quantitated via qPCR and sequenced on one lane for 151 cycles from each end of the fragments on a NovaSeq 6000 using a NovaSeq SP reagent kit (Illumina); paired-end reads were 150 nts in length. Fastq files were generated and de-multiplexed with the bcl2fastq v2.20 Conversion Software (Illumina). Subsequent processes were performed with Geneious Prime [31]. For each strain, differential gene expression analysis was performed to compare gene expression between cells grown in the control (SDM) and test media (SDM with POM extract). Genes were considered significantly differentially expressed with a Log2 fold change of ±2 and a p value ≤ 0.05.

2.5. Preparation of Samples for Targeted Metabolite Detection

L. acidophilus NCFM, *L. plantarum* Lp-115 and *L. rhamnosus* GG were transferred twice in SDM (1% glucose), and cells were inoculated (1%) into SDM (0.5% glucose, control) or SDM (0.5% glucose with P400) and grown for 24 h at 37 °C. After 24 h, cultures were centrifuged at 2152× *g* for 3 min, and the cell-free supernatant was transferred to tubes for storage at −80 °C. Triplicate samples were prepared for each condition, including the control media at T0 and T24. Targeted detection of the metabolites punicalagin (Sigma, St Louis, MO, USA), ellagic acid (Sigma), gallic acid (Toronto Research Chemicals, North York, ON, CA), urolithin A (Sigma), urolithins C and D (Toronto Research Chemicals) was performed at the Molecular Education, Technology and Research Innovation Center (METRIC) core facility at NCSU.

Cell-free supernatants were centrifuged (10,000× *g*, 5 min, 4 °C) and diluted (25 µL supernatant, 225 µL H_2O) prior to analysis using an ultra-performance liquid chromatography–tandem mass spectrometer (UPLC-MS/MS). The analysis was performed using a Thermo Vanquish LC instrument (Thermo Fisher Scientific, San Jose, CA) coupled to a Thermo TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific) with a heated electrospray ionization (HESI) source. Chromatographic separation was achieved on a Restek Raptor C18 column (2.1 × 100 mm, 1.8 mM) maintained at 30 °C. The following linear gradient of mobile phase A (H_2O + 0.1% FA) and mobile phase B (MeCN + 0.1% FA) was used: 0–0.25 min (5%B, 0.2 mL/min), 0.25–6 min (5–30%B, 0.2 mL/min), 6–10 min (30–95%B, 0.2 mL/min), 10–12.5 min (95%B, 0.2 mL/min) and 12.51–15 min (5%B, 0.2 mL/min). Stock solutions (1 mg/mL) were prepared in DMSO or MeOH, and then individual stocks were combined and diluted with water to provide calibration curves in the linear range for each analyte (12.5–5000 ng/mL for gallic acid and ellagic acid; 250 to 100,000 ng/mL for punicalagin). Both samples and standards were analyzed (10 mL injections) in negative ion mode (spray voltage 2.5 kV, ion transfer tube temperature 325 °C, vaporizer temperature 350 °C, sheath gas 50 a.u., aux gas 10 a.u., sweep gas 1 a.u.) using a Q1 resolution of 0.7 m/z and a Q3 resolution of 1.2 m/z. The following multiple reaction monitoring (MRM) transitions and collision energies were used: 168.9→79.1 (gallic acid quantifier, CE 23.6), 168.9→125.1 (gallic acid qualifier, CE 14.7), 300.9→145.1 (ellagic acid quantifier, CE 38.4), 300.9→283.9 (ellagic acid qualifier, CE 29.2), 541.1→275.1 (punicalagin quantifier, CE 25.9) and 541.1→301.0 (punicalagin qualifier, CE 27.5).

Peak integration and quantification were performed in Skyline [32]. Individual standard curves for each analyte were constructed using peak areas from the quantifier transitions. The concentrations of each analyte in the study samples were calculated in an identical manner relative to the regression line. Calibration curves had R2 values ranging from 0.9936 to 0.9997 with a weighting of 1/(x*x). The peak area of the qualifier transition was compared with the peak area of the quantifier transition to generate an ion ratio for compound validation. The comparison of the ion ratios for the standards to the ion ratio of the unknowns (study samples) was carried out with a threshold of 30%.

2.6. Statistical Analyses

The error bars in the bar graphs represent the ± standard deviation of the mean. Differences between the control and experimental groups were tested by Student's *t*-test with significance set at a p value ≤ 0.05. Gene expression levels were calculated based on the normalized transcripts per million, and differential expression analysis was performed with the DESeq2 package within Geneious [31]. Genes were considered differentially expressed when they had a Log2 ratio ≤ -2 or ≥ 2 and a p value ≤ 0.05.

3. Results

3.1. Growth of Lactobacilli in the Presence of Pomegranate Extract

Three commercial probiotic strains were selected for closer evaluation: *L. acidophilus* NCFM (Lac), *L. rhamnosus* GG (Lrh) and *L. plantarum* Lp-115 (Lpl). These strains all grew well in the presence of POM in our initial screen (Figure S1 and Table S1). Preliminary

growth assays showed that pomegranate (POM) extract did not support the growth of lactobacilli strains in the absence of an additional sugar source. Therefore, we used a semi-defined medium (SDM) with 0.5% glucose for growth assays. Growth curves over a 24 h period showed that *L. acidophilus* reached the highest OD 600 nm value after growth in P400, whereas the growth profiles were similar for *L. rhamnosus* and *L. plantarum* when grown with and without P400 (Figure 1A). In contrast, OD values were lower for all three strains after 6 h when grown in the higher concentration of P800 (Figure 1A), indicating an inhibitory effect on growth with P800. In the case of *L. acidophilus*, after two transfers in media with P400 and subsequent growth in the control media (P-G), the OD values were higher than those of the control (G-G) (Figure 1A), suggesting that preadaptation to POM in the transfer medium provided, while relatively minor, a growth advantage in the medium without POM (P-G). Next, we enumerated cell counts at T8 and T24 h. These data indicated an increase in colony-forming units of 55% and 81% after 8 and 24 h, respectively, for *L. acidophilus* when grown in P400 compared to the control (G-G) (Figure 1B). For *L. acidophilus*, when the cells were preadapted with two transfers in media containing P400, there was an increase in CFU/mL of 34% at eight hours compared to G-P400. We note that these percent increases in CFU correspond to relatively minor increases in relation to the total viable cell count. Neither *L. rhamnosus* nor *L. plantarum* showed significant differences in cell counts at 24 h across all conditions tested. We performed all subsequent experiments with a concentration of P400, given that these results indicated potential strain inhibition in growth curves with P800.

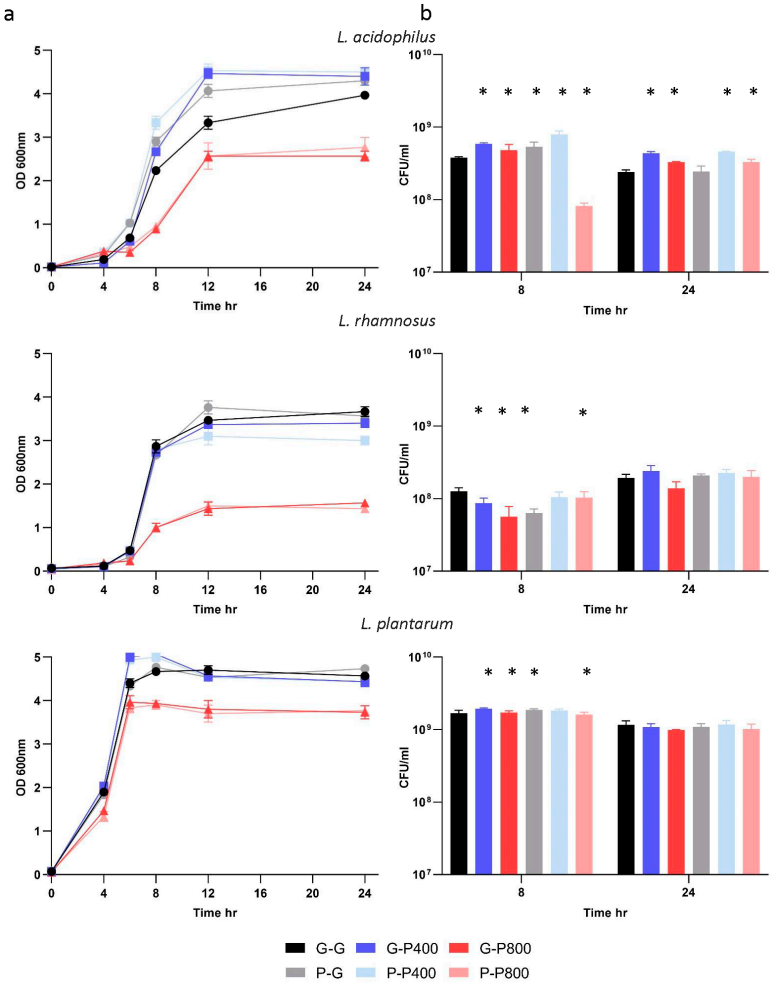
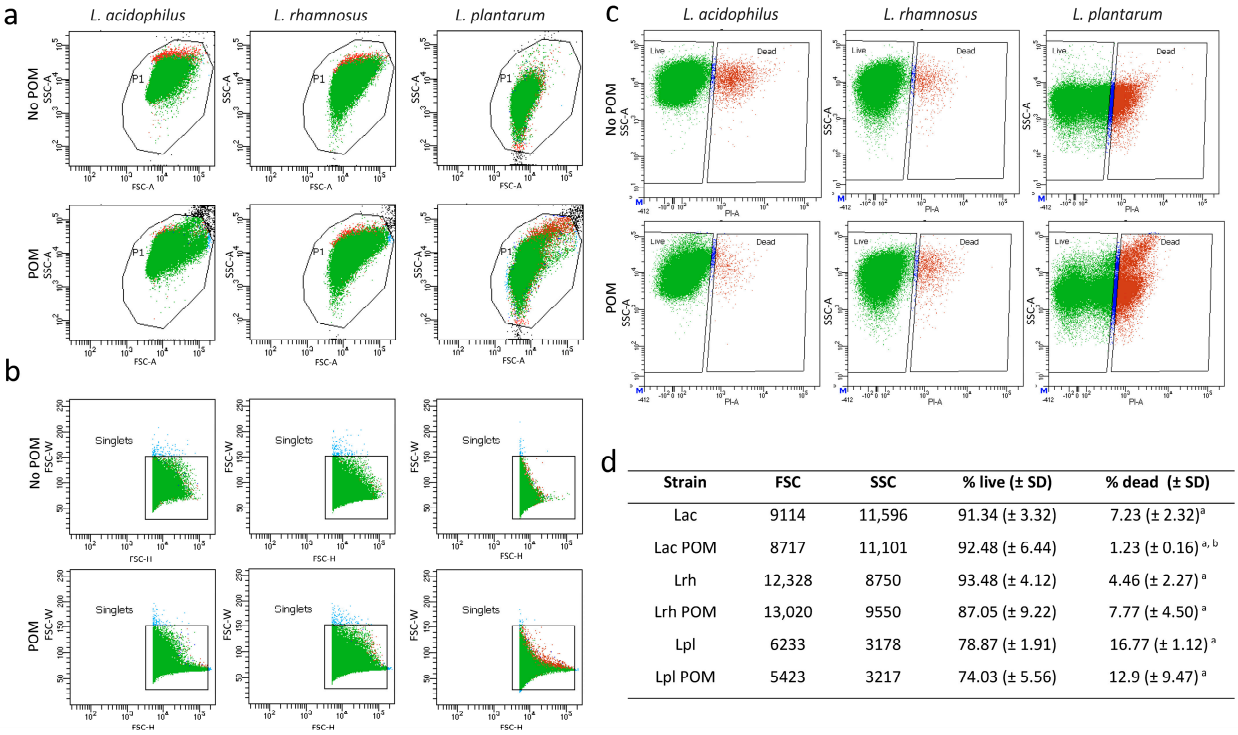


Figure 1. Growth assays of three probiotic strains in the presence of POM extract. (**a**) OD 600 nm values for *L. acidophilus*, *L. rhamnosus* and *L. plantarum* over 24 h at 37 °C. (**b**) CFU/mL counts for each

strain at 8 and 24 h. Error bars show the standard deviation of the mean, and * indicates a p value ≤ 0.05 when compared to the control G-G. G-G: transfer in SDM with no POM extract and growth in SDM with no POM extract; G-P400: transfer in SDM with no POM extract and growth in SDM with POM 400 µg/mL extract; G-P800: transfer in SDM with no POM extract and growth in SDM with POM 800 µg/mL extract; P-G: transfer in SDM with POM 400 µg/mL extract and growth in SDM with no POM extract; P-P400: transfer in SDM with POM 400 µg/mL extract and growth in SDM with POM 400 µg/mL extract; P-P800: transfer in SDM with POM 400 µg/mL extract and growth in SDM with POM 800 µg/mL extract.

3.2. Flow Cytometry Analysis of Lactobacilli after Growth in Pomegranate

We next used flow cytometry to determine the effect of POM in the growth medium for the three strains compared to the control medium with no POM on cell viability, granularity and size. The side scatter and forward scatter plots were similar after growth in POM for *L. acidophilus* and *L. rhamnosus* (Figure 2a,b, respectively), indicating no effect on the cell size or granularity of these strains when POM was present in the growth medium. However, the scatter plots for *L. plantarum* were different between growth conditions, indicating that for this probiotic, POM altered the cell granularity. Using LIVE/DEAD staining, we determined there were significantly fewer dead cells when *L. acidophilus* was grown in P400 compared to the control with no POM (1.23% versus 7.23%, Figure 2c,d). This was not observed with the other two strains. Under both conditions tested, *L. acidophilus* had the lowest number of dead cells, followed by *L. rhamnosus* (4.46% and 7.77%), while close to one-fifth of *L. plantarum* cells were dead (16.77% and 20.9%, Figure 2d). For *L. plantarum*, we observed a higher percentage of dead cells in the presence of POM (20.9%) compared to the control (16.77%); however, this difference was not statistically different (p value = 0.07).



d

Strain	FSC	SSC	% live (± SD)	% dead (± SD)
Lac	9114	11,596	91.34 (± 3.32)	7.23 (± 2.32)[a]
Lac POM	8717	11,101	92.48 (± 6.44)	1.23 (± 0.16)[a, b]
Lrh	12,328	8750	93.48 (± 4.12)	4.46 (± 2.27)[a]
Lrh POM	13,020	9550	87.05 (± 9.22)	7.77 (± 4.50)[a]
Lpl	6233	3178	78.87 (± 1.91)	16.77 (± 1.12)[a]
Lpl POM	5423	3217	74.03 (± 5.56)	12.9 (± 9.47)[a]

Figure 2. Flow cytometry analysis of probiotic strains grown in the presence of POM extract. Side (**a**) and forward (**b**) plots for each of the three strains. LIVE/DEAD staining with propidium iodide (**c**,**d**). Representative plots with corresponding forward scatter (FSC) and side scatter (SSC) values are shown. In panel (**d**), superscript a denotes a p value of ≤ 0.05 when comparing % live and % dead cells, and superscript b denotes a p value ≤ 0.05 when comparing % live or % dead values for each strain after growth in POM versus the control with no POM.

3.3. Whole-Transcriptome Response of Lactobacilli to Pomegranate

Given the phenotypic differences after growth with POM extract on the three probiotic strains of interest, we investigated the whole-transcriptome response for each strain to POM extract. As noted with the phenotypes above, the whole-transcriptome response was different for each strain, with 15.88% of the *L. acidophilus* transcriptome, 19.32% of the *L. rhamnosus* transcriptome and only 2.37% of the *L. plantarum* transcriptome differentially expressed (Figure 3a). In addition, of the differentially expressed genes, 240 were upregulated and 51 were downregulated for *L. acidophilus*, 305 were upregulated and 238 were downregulated for *L. rhamnosus*, and only 2 were upregulated and 72 were downregulated for *L. plantarum* (Figure 3a,b and Table S2). Next, the significantly differentially expressed genes were mapped to their chromosomal locations to determine whether there were hotspots of differential gene expression (Figure 3b). These data clearly indicated a region of clustered downregulated genes for *L. acidophilus*, including an ABC transporter gene, a transcriptional regulator gene and a bacteriocin accessory protein-encoding gene (Figure 3b, blue oval). In the case of *L. plantarum*, two notable hotspots of downregulated genes were identified that both include numerous prophage-related genes (Figure 3B, blue ovals), indicating that exposure to POM extract resulted in the potential induction of these phages.

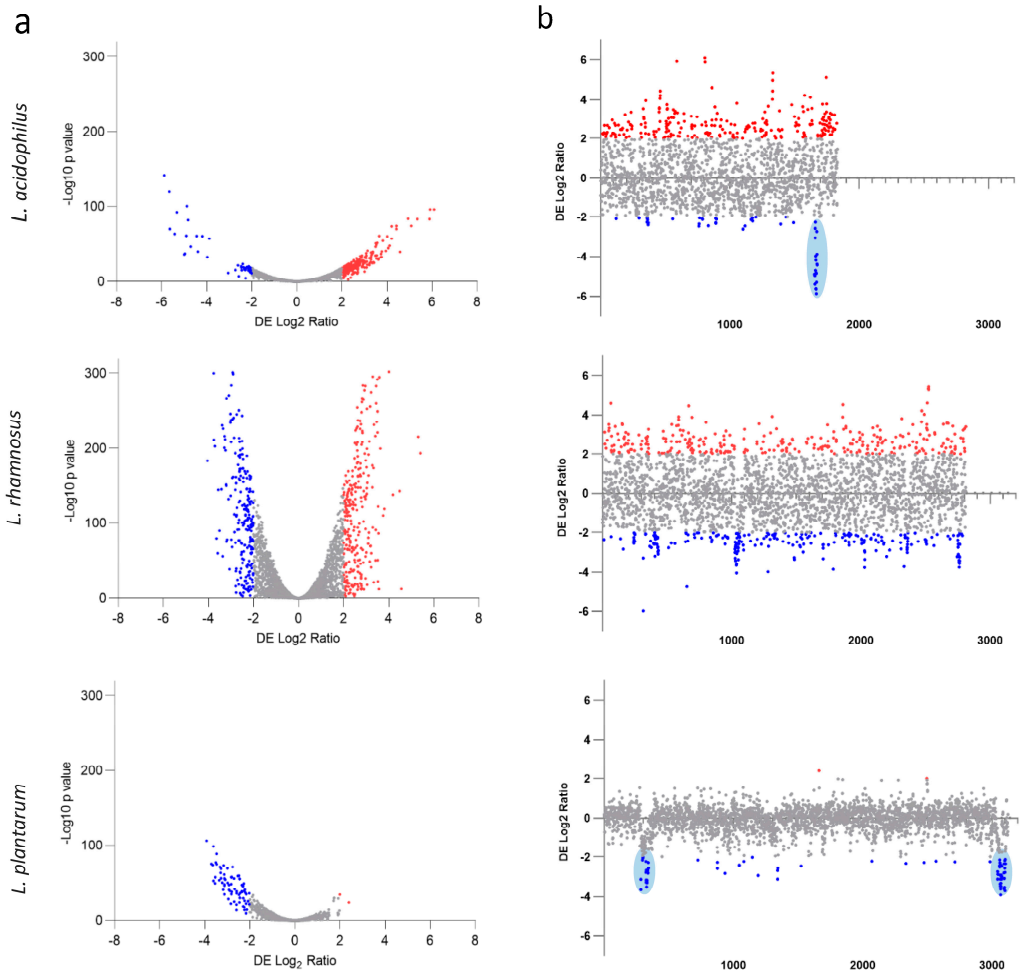


Figure 3. Whole-transcriptome response of probiotic strains to POM extract. Volcano plots comparing Log2 fold change to −Log 10 statistical significance (**a**). Differential gene expression across the chromosome for each of the three strains (**b**). Colors indicate genes with a Log2 fold change ≥ 2 in red and ≤ -2 in blue with a p value ≤ 0.05. Blue ovals indicate regions discussed in the text.

Subsequently, we used the differentially expressed gene sets to determine the clusters of orthologous groups (COG) grouping for each strain (Figure 4). The COG distribution

was different amongst the three strains. For *L. acidophilus* (Figure 4a) and *L. rhamnosus* (Figure 4b) strains, the function unknown [S], amino acid metabolism and transport [E], carbohydrate metabolism and transport [G] and inorganic ion transport and metabolism [P] COGs contained a larger representation of genes, whereas, as expected given the low number of differentially expressed genes for *L. plantarum*, there was less representation across the COGs (Figure 4c,d).

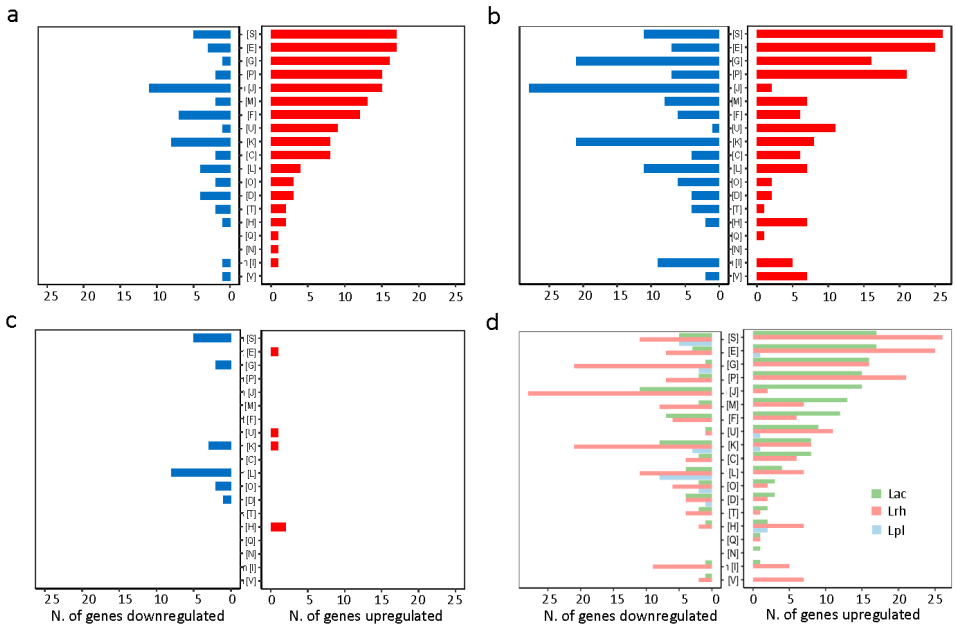


Figure 4. Determination of the clusters of orthologous groups (COG) grouping. COG grouping was assigned to significant genes (Log2 fold change ≥ 2 in red and ≤ -2 in blue with a p value ≤ 0.05) using the EggNOG database for each strain, (**a**) *L. acidophilus*, Lac, (**b**) *L. rhamnosus*, Lrh, and (**c**) *L. plantarum*, Lpl, and (**d**) an overview of data from the three strains. The COG categories are function unknown [S], amino acid metabolism and transport [E], carbohydrate metabolism and transport [G], inorganic ion transport and metabolism [P], translation [J], cell wall/membrane/envelope biogenesis [M], nucleotide metabolism and transport [F], intracellular trafficking and secretion [U], transcription [K], energy production and conversion [C], replication and repair [L], post-translational modification [O], cell cycle control and mitosis [D], signal transduction mechanisms [T], coenzyme metabolism [H], secondary metabolites biosynthesis [Q], cell motility [N], lipid metabolism [I] and defense mechanism [V].

3.4. Individual Differential Gene Expression after Growth in POM Extract

We next looked at individual genes of interest that were differentially expressed in each strain after exposure to POM extract. In the case of *L. acidophilus*, differential gene expression ranged from Log2 ± six-fold, with numerous hypothetical proteins amongst the genes with the highest differential gene expression (Table S2). Genes with higher expression in SDM with POM extract compared to the control included a di-tripeptide transport protein (*dtpT*, Log2 ratio 2.99) and genes encoding for multidrug transporter proteins (Log2 ratio 3.74 and 3.36), permeases (Log2 ratio 3.49 and 3.14) and a glycosidase (Log2 ratio 4.12) (Table S2). Interestingly, with the exception of one permease (lba0753), none of these genes were reported as differentially expressed in a previous study from our group, in which the ability of *L. acidophilus* NCFM to metabolize the dietary plant glucosides amygdalin, esculin and salicin was studied [33]. In the case of lba0753, it was found to be upregulated (Log2 ratio 2.3) when grown on esculin compared to lactose. Similarly, for *L. rhamnosus*, differential gene expression ranged from Log2 ± six-fold, with numerous hypothetical proteins amongst the genes with the highest differential gene expression (Table S2). The most upregulated genes included three adjacent genes, ecdB (Log2 ratio 5.41), a putative UbiX-like flavin prenyltransferase gene; *bsdC* (Log2 ratio 5.32), a phenolic

acid decarboxylase subunit C gene; and a hypothetical protein (Log2 ratio 5.27). Similar to *L. acidophilus*, a *dtpT* gene was upregulated (Log2 ratio 5.32). Numerous transporter proteins, including those annotated as riboflavin transporters, were also amongst the most upregulated genes, in addition to permeases (Table S2). In the case of *L. plantarum*, only two genes were upregulated, and the expression levels were lower than in the other two strains, with the gene with the highest expression encoding for a transcriptional regulator (*padR*, Log2 ratio 2.39). Other induced genes included, similar to *L. acidophilus* and *L. rhamnosus*, a *dtpT* protein-encoding gene (Log2 ratio 1.97) and a permease gene (Log2 ratio 1.94). Riboflavin synthase (Log2 ratio 1.92) was also one of the seven upregulated genes. As reported above, large proportions of the downregulated genes were part of two separate prophages in the genome of *L. plantarum* (Table S2 and Figure 3D).

3.5. Target Metabolite Analysis

While lactobacilli are generally considered to lack the required enzymes to biotransform the main constituents of POM, we measured select biologically relevant metabolites to determine their concentrations after 24 h of growth of the three probiotic strains. Using UPLC-MS/MS, we determined the concentrations of punicalagin, ellagic acid and gallic acid (Figure 5), while urolithins A, C and D were undetected (data not shown). Of the three metabolites, punicalagin was detected in the highest amount (Figure 5a). However, we noted a significant decrease in detection in the control sample after 24 h at 37 °C. This could be due to chemical instability and the degradation of the extract under these conditions. Remarkably, when all three probiotic strains were present, the detected levels of punicalagin were more similar to those in the SDM control medium with P400 (control) at T0, indicating that the presence of the probiotic strains in the medium prevented punicalagin degradation via an undetermined method (Figure 5A). The amount of ellagic acid detected significantly increased at T24 in the control medium and samples with *L. acidophilus* and *L. rhamnosus* but not *L. plantarum* (Figure 5B). In the case of the control T24 *L. acidophilus* samples, this increase could be due to the degradation of punicalagin, whereas the reason for the increased detection in the *L. rhamnosus* sample would need further investigation. The detected amount of gallic acid was not significantly different for *L. rhamnosus* but was significantly reduced for the other two probiotic strains and in the control at T24 compared to T0 (Figure 5C). These data point to the limitations of this experiment, as these results could be further investigated to better understand the interaction of the POM extract, its constituents and their chemical–physical properties, such as solubility and stability in relation to time, temperature, pH and the presence of probiotic strains.

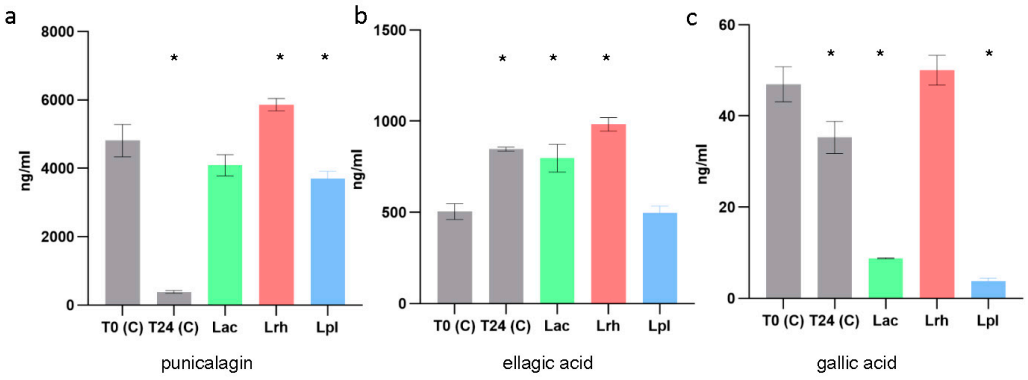


Figure 5. Targeted metabolite analysis for probiotic strains grown with POM extract 400 µg/mL. The ng/mL amount for each metabolite is shown on the y-axis, and the condition is on the x-axis. Punicalagin (**a**), ellagic acid (**b**) and gallic acid (**c**). Gray bars show the control conditions without bacteria. T0 (C): SDM control medium with P400 at 0 h; T24 (C): SDM control medium with P400 at 24 h; Lac: *L. acidophilus*; Lrh: *L. rhamnosus*; and Lpl: *L. plantarum*. Error bars show standard deviation of the mean, and * indicates a p value ≤ 0.05 when compared to the control T0.

4. Discussion

Functional foods are the subject of increased research interest in order to determine the diverse attributes of foods beyond basic nutrition, such as their effects on and biotransformation by constituents of the GIT [34,35]. Pomegranate is one such functional food consumed in various forms (fruit, juice or extract), which has documented health benefits and encompasses a host of beneficial compounds that are further metabolized into bioactive metabolites in the GIT [11,36]. While the benefits of POM have been postulated for centuries, the beneficial constituents have only recently been identified and studied using scientific methods, including clinical trials [11]. In this study, we set out to further understand the relationship and activities of the functional food POM with lactobacilli and, more specifically, commercially available probiotic bacteria. We focused on commercially available strains from three different species, as these strains are well studied, currently sold as probiotic strains for human consumption and ready for use in formulations with functional foods.

In total, 48 strains of lactobacilli were tested across 14 species, and depending on the strain or species, growth in POM extract had varied effects on OD values (Figure S1). These preliminary results also indicated that, for some strains, P800 was inhibitory and/or slowed the growth rate; therefore, we used a concentration of P400 for subsequent experiments. Our results show that the three probiotic strains displayed different responses to POM. For *L. acidophilus*, POM had a positive effect, as cell growth results showed that growth in POM increased cell numbers at eight hours, and this was further enhanced if the cells had been preadapted by transferring them in POM prior to growth assays. These prior transfers in POM extract may have affected bacterial growth by priming gene expression, such as in the induction of a stress response. There is also the possibility that the addition of POM extract provided minor additional nutritional components. While the increase in cell number was statistically significant in these growth conditions for *L. acidophilus,* we note that the overall increase was relatively minor when compared to the total viable count. Flow cytometry analysis also showed a lower amount of cell death when *L. acidophilus* was grown with POM, indicating a protective effect on the cells yet to be established. Given the results we observed with *L. acidophilus* in particular and the lack of previously published studies with similar observations for this species, it would be interesting for future studies to further establish any potential mutual benefit. *L. plantarum* and *L. rhamnosus* grew to the same extent as the controls in the presence of POM, and the preadaptation did not result in enhanced growth; this was confirmed by enumerating cell counts. Previous work also determined that the growth of probiotic species was relatively unaffected by the inclusion of POMx in the growth medium [18]. A subsequent study determined that POMx significantly enhanced lactobacilli numbers in the batch culture fermentation of fecal slurry [19]. A third study determined an increase in the cell counts of total lactobacilli with P400; however, the individual species' details are not provided [20].

We next grew all three strains in the presence of POM extract and determined the whole-transcriptome response during the logarithmic phase. Strikingly, we determined three different global responses amongst the three strains. Exposure to POM extract had the most significant and highest level of whole-transcriptome response for *L. rhamnosus*, followed by *L. acidophilus*, where the upregulation of the transcriptome was more impacted. The least responsive transcriptome to POM extract was *L. plantarum*'s, with less than three percent of the genome impacted and few upregulated genes. Both *L. plantarum* and *L. rhamnosus* have significantly larger genomes (~3.2 and 3 Mb, respectively) than *L. acidophilus* (~2 Mb) and are therefore presumed to be less fastidious. In particular, since many *L. plantarum* strains are derived from plants and fermented foods [37], it is plausible that this species has evolved with plant products and thus has a distinct transcriptional response. Genes encoding for numerous types of transporters, such as phosphotransferase (PTS) system, multidrug, di-tripeptide transporters and members of the major facilitator superfamily (MFS) transporter families, were upregulated. In addition, genes encoding for glucosidases, permeases, hydrolases and hypothetical proteins were upregulated. While all

three strains showed a markedly different global transcriptional response, there was some commonality; in particular, homologs of certain genes were upregulated, for example, *dtpT*, a di-tripeptide transporter, strongly indicating their involvement in the exposure and/or growth of these strains in the presence of POM extract. Further, in silico analysis of these genes of interest and the construction of strains devoid of these genes could be performed to determine the role of these genes in the phenotypes described in this study. For example, future studies should determine whether the expression of these genes contributes to the lower number of dead cells determined by flow cytometry for *L. acidophilus* when grown in P400. Likewise, it should be assessed whether induction of prophages from the *L. plantarum* genome occurred and contributed to cell death in the total cell population.

We also detected specific metabolites derived from POM after growth across the three strains. We included these metabolites, as punicalagin is the precursor to the more bioavailable ellagic acid, and all three have shown beneficial attributes in the gut [11]. For example, punicalagin, ellagic acid and gallic acid demonstrated anti-inflammatory effects [38,39] and pathogen inhibition [40–42]. The lack of detection of urolithins was not unexpected, as this correlates with previous reports, including studies with lactobacilli [20,43], and other members of the gut microbiota have been shown to possess the genes required for the conversion to urolithin [44]. While none of the urolithins were detected, we were able to measure the concentrations of punicalagin, ellagic acid and gallic acid. We did note a lack of degradation in the case of punicalagin when compared to the control, indicating a possible protective effect by the probiotic strains. This protective effect could result in the greater bioavailability of punicalagin to other members of the microbiome that are known to metabolize punicalagin to urolithins [44]. However, these preliminary data reveal that additional studies are needed to determine the interaction of the POM extract and its constituents with probiotic strains. Future considerations should include their chemical–physical properties, such as solubility and stability in relation to time and temperature.

5. Conclusions

Overall, this study investigates the interplay between a beneficial dietary compound and commercially relevant probiotic bacteria, shows that POM extract triggers species-specific responses by probiotic strains and substantiates the rising interest in using POM as a prebiotic compound. The data presented here point to the need for continued investigation to further discover the constituents of POM that result in the phenotypes we have observed. Especially intriguing is how a functional food such as POM can discern and act to inhibit pathogenic members of the GIT while leaving beneficial bacteria such as *Lactobacillaceae* species unscathed or even enhanced in number. We newly report on the transcriptome response of the three strains, emphasizing the results we have determined throughout: POM extract triggers species-specific responses by probiotic strains. Also compelling is the lack of punicalagin degradation in the media containing the probiotic strains. Overall, this study suggests an under-appreciated evolutionary complexity between functional foods and/or their metabolites and the bacteria of the GIT, and opens new avenues to include POM in probiotic formulations and expand our understanding of how select dietary compounds impact the physiology of beneficial bacteria and ultimately the health of the host.

6. Patents

S.O.F. and R.B. are inventors on patent 17/442,776: Probiotic bacteria capable of adaptive response to pomegranate extract and methods of production and use thereof.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms11020404/s1. Figure S1: Schematic of growth conditions; Figure S2: OD values of strains grown with POM extract; Table S1: Details of strains used in Figure S2; Table S2: List of differentially expressed genes from whole-transcriptome analysis.

Author Contributions: Conceptualization, S.O. and R.B.; methodology, S.O. and N.C.; writing—original draft preparation, S.O. and N.C.; writing—review and editing, S.O. and R.B.; funding acquisition, R.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The RNA-seq data were deposited in the NCBI Short Read Archive (SRA) database under the BioProject ID PRJNA907268. The SRA accessions number are SAMN31967636–SAMN31967647.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Granato, D.; Barba, F.J.; Kovačević, D.B.; Lorenzo, J.M.; Cruz, A.G.; Putnik, P. Functional Foods: Product Development, Technological Trends, Efficacy Testing, and Safety. *Annu. Rev. Food Sci. Technol.* **2020**, *11*, 93–118. [CrossRef] [PubMed]
2. Available online: https://www.precedenceresearch.com/functional-food-market (accessed on 30 June 2022).
3. Gill, S.R.; Pop, M.; Deboy, R.T.; Eckburg, P.B.; Turnbaugh, P.J.; Samuel, B.S.; Gordon, J.I.; Relman, D.A.; Fraser-Liggett, C.M.; Nelson, K.E. Metagenomic analysis of the human distal gut microbiome. *Science* **2006**, *312*, 1355–1359. [CrossRef] [PubMed]
4. Gomaa, E.Z. Human gut microbiota/microbiome in health and diseases: A review. *Antonie Van Leeuwenhoek* **2020**, *113*, 2019–2040. [CrossRef] [PubMed]
5. Zmora, N.; Soffer, E.; Elinav, E. Transforming medicine with the microbiome. *Sci. Transl. Med.* **2019**, *11*, eaaw1815. [CrossRef] [PubMed]
6. Ozcan, E.; Rozycki, M.R.; Sela, D.A. Cranberry Proanthocyanidins and Dietary Oligosaccharides Synergistically Modulate Lactobacillus plantarum Physiology. *Microorganisms* **2021**, *9*, 656. [CrossRef] [PubMed]
7. National Center for Complementary and Integrative Health. NCCIH Strategic Plan FY 2021–2025: Mapping the Pathway to Research on Whole Person Health. Available online: https://www.nccih.nih.gov/about/nccih-strategic-plan-2021-2025 (accessed on 30 June 2022).
8. Mackler, A.M.; Heber, D.; Cooper, E.L. Pomegranate: Its health and biomedical potential. *Evid. Based Complement. Alternat. Med.* **2013**, *2013*, 903457. [CrossRef]
9. Binda, S.; Hill, C.; Johansen, E.; Obis, D.; Pot, B.; Sanders, M.E.; Tremblay, A.; Ouwehand, A.C. Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. *Front. Microbiol.* **2020**, *11*, 1662. [CrossRef] [PubMed]
10. Henning, S.M.; Yang, J.; Lee, R.P.; Huang, J.; Hsu, M.; Thames, G.; Gilbuena, I.; Long, J.; Xu, Y.; Park, E.H.; et al. Pomegranate Juice and Extract Consumption Increases the Resistance to UVB-induced Erythema and Changes the Skin Microbiome in Healthy Women: A Randomized Controlled Trial. *Sci. Rep.* **2019**, *9*, 14528. [CrossRef]
11. Johanningsmeier, S.D.; Harris, G.K. Pomegranate as a functional food and nutraceutical source. *Annu. Rev. Food Sci. Technol.* **2011**, *2*, 181–201. [CrossRef]
12. Pirzadeh, M.; Caporaso, N.; Rauf, A.; Shariati, M.A.; Yessimbekov, Z.; Khan, M.U.; Imran, M.; Mubarak, M.S. Pomegranate as a source of bioactive constituents: A review on their characterization, properties and applications. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 982–999. [CrossRef]
13. Li, P.; Du, R.; Chen, Z.; Wang, Y.; Zhan, P.; Liu, X.; Kang, D.; Chen, Z.; Zhao, X.; Wang, L.; et al. Punicalagin is a neuraminidase inhibitor of influenza viruses. *J. Med. Virol.* **2020**, *93*, 3465–3472. [CrossRef]
14. Lin, L.T.; Chen, T.Y.; Lin, S.C.; Chung, C.Y.; Lin, T.C.; Wang, G.H.; Anderson, R.; Lin, C.C.; Richardson, C.D. Broad-spectrum antiviral activity of chebulagic acid and punicalagin against viruses that use glycosaminoglycans for entry. *BMC Microbiol.* **2013**, *13*, 187. [CrossRef] [PubMed]
15. Li, G.; Xu, Y.; Pan, L.; Xia, X. Punicalagin Damages the Membrane of Salmonella Typhimurium. *J. Food Prot.* **2020**, *83*, 2102–2106. [CrossRef] [PubMed]
16. Kujawska, M.; Jodynis-Liebert, J. Potential of the ellagic acid-derived gut microbiota metabolite—Urolithin A in gastrointestinal protection. *World J. Gastroenterol.* **2020**, *26*, 3170–3181. [CrossRef] [PubMed]
17. Lee, G.; Park, J.S.; Lee, E.J.; Ahn, J.H.; Kim, H.S. Anti-inflammatory and antioxidant mechanisms of urolithin B in activated microglia. *Phytomedicine* **2019**, *55*, 50–57. [CrossRef] [PubMed]
18. Bialonska, D.; Kasimsetty, S.G.; Schrader, K.K.; Ferreira, D. The effect of pomegranate (Punica granatum L.) byproducts and ellagitannins on the growth of human gut bacteria. *J. Agric. Food Chem.* **2009**, *57*, 8344–8349. [CrossRef]

19. Bialonska, D.; Ramnani, P.; Kasimsetty, S.G.; Muntha, K.R.; Gibson, G.R.; Ferreira, D. The influence of pomegranate by-product and punicalagins on selected groups of human intestinal microbiota. *Int. J. Food Microbiol.* **2010**, *140*, 175–182. [CrossRef] [PubMed]
20. Li, Z.; Summanen, P.H.; Komoriya, T.; Henning, S.M.; Lee, R.P.; Carlson, E.; Heber, D.; Finegold, S.M. Pomegranate ellagitannins stimulate growth of gut bacteria in vitro: Implications for prebiotic and metabolic effects. *Anaerobe* **2015**, *34*, 164–168. [CrossRef]
21. Sukumar, M.R.; Konig, B. Pomegranate extract specifically inhibits Clostridium difficile growth and toxin production without disturbing the beneficial bacteria in vitro. *Infect. Drug Resist.* **2018**, *11*, 2357–2362. [CrossRef]
22. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S.; et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef]
23. De Filippis, F.; Pasolli, E.; Ercolini, D. The food-gut axis: Lactic acid bacteria and their link to food, the gut microbiome and human health. *FEMS Microbiol. Rev.* **2020**, *44*, 454–489. [CrossRef]
24. Li, Z.; Henning, S.M.; Lee, R.P.; Lu, Q.Y.; Summanen, P.H.; Thames, G.; Corbett, K.; Downes, J.; Tseng, C.H.; Finegold, S.M.; et al. Pomegranate extract induces ellagitannin metabolite formation and changes stool microbiota in healthy volunteers. *Food Funct.* **2015**, *6*, 2487–2495. [CrossRef]
25. Heber, D.; Seeram, N.P.; Wyatt, H.; Henning, S.M.; Zhang, Y.; Ogden, L.G.; Dreher, M.; Hill, J.O. Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size. *J. Agric. Food Chem.* **2007**, *55*, 10050–10054. [CrossRef] [PubMed]
26. Chamberlain, M.; O'Flaherty, S.; Cobian, N.; Barrangou, R. Metabolomic Analysis of Lactobacillus acidophilus, L. gasseri, L. crispatus, and Lacticaseibacillus rhamnosus Strains in the Presence of Pomegranate Extract. *Front. Microbiol.* **2022**, *13*, 863228. [CrossRef]
27. Henning, S.M.; Zhang, Y.; Rontoyanni, V.G.; Huang, J.; Lee, R.P.; Trang, A.; Nuernberger, G.; Heber, D. Variability in the antioxidant activity of dietary supplements from pomegranate, milk thistle, green tea, grape seed, goji, and acai: Effects of in vitro digestion. *J. Agric. Food Chem.* **2014**, *62*, 4313–4321. [CrossRef]
28. Kimmel, S.A.; Roberts, R.F. Development of a growth medium suitable for exopolysaccharide production by Lactobacillus delbrueckii ssp. bulgaricus RR. *Int. J. Food Microbiol.* **1998**, *40*, 87–92. [CrossRef]
29. Klotz, C.; Goh, Y.J.; O'Flaherty, S.; Johnson, B.; Barrangou, R. Deletion of S-Layer Associated Ig-Like Domain Protein Disrupts the Lactobacillus acidophilus Cell Surface. *Front. Microbiol.* **2020**, *11*, 345. [CrossRef] [PubMed]
30. O'Flaherty, S.; Klaenhammer, T.R. Multivalent Chromosomal Expression of the Clostridium botulinum Serotype A Neurotoxin Heavy-Chain Antigen and the Bacillus anthracis Protective Antigen in Lactobacillus acidophilus. *Appl. Environ. Microbiol.* **2016**, *82*, 6091–6101. [CrossRef] [PubMed]
31. Kearse, M.; Moir, R.; Wilson, A.; Stones-Havas, S.; Cheung, M.; Sturrock, S.; Buxton, S.; Cooper, A.; Markowitz, S.; Duran, C.; et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **2012**, *28*, 1647–1649. [CrossRef]
32. Adams, K.J.; Pratt, B.; Bose, N.; Dubois, L.G.; St John-Williams, L.; Perrott, K.M.; Ky, K.; Kapahi, P.; Sharma, V.; MacCoss, M.J.; et al. Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics. *J. Proteome Res.* **2020**, *19*, 1447–1458. [CrossRef] [PubMed]
33. Theilmann, M.C.; Goh, Y.J.; Nielsen, K.F.; Klaenhammer, T.R.; Barrangou, R.; Abou Hachem, M. Lactobacillus acidophilus Metabolizes Dietary Plant Glucosides and Externalizes Their Bioactive Phytochemicals. *mBio* **2017**, *8*, e01421-17. [CrossRef]
34. Durazzo, A.; Nazhand, A.; Lucarini, M.; Atanasov, A.G.; Souto, E.B.; Novellino, E.; Capasso, R.; Santini, A. An Updated Overview on Nanonutraceuticals: Focus on Nanoprebiotics and Nanoprobiotics. *Int. J. Mol. Sci.* **2020**, *21*, 2285. [CrossRef] [PubMed]
35. Nazhand, A.; Souto, E.B.; Lucarini, M.; Souto, S.B.; Durazzo, A.; Santini, A. Ready to Use Therapeutical Beverages: Focus on Functional Beverages Containing Probiotics, Prebiotics and Synbiotics. *Beverages* **2020**, *6*, 26. [CrossRef]
36. Kandylis, P.; Kokkinomagoulos, E. Food Applications and Potential Health Benefits of Pomegranate and its Derivatives. *Foods* **2020**, *9*, 122. [CrossRef]
37. Duar, R.M.; Lin, X.B.; Zheng, J.; Martino, M.E.; Grenier, T.; Perez-Munoz, M.E.; Leulier, F.; Ganzle, M.; Walter, J. Lifestyles in transition: Evolution and natural history of the genus Lactobacillus. *FEMS Microbiol. Rev.* **2017**, *41*, S27–S48. [CrossRef]
38. BenSaad, L.A.; Kim, K.H.; Quah, C.C.; Kim, W.R.; Shahimi, M. Anti-inflammatory potential of ellagic acid, gallic acid and punicalagin A&B isolated from Punica granatum. *BMC Complement. Altern. Med.* **2017**, *17*, 47. [CrossRef]
39. Nguyen-Ngo, C.; Salomon, C.; Lai, A.; Willcox, J.C.; Lappas, M. Anti-inflammatory effects of gallic acid in human gestational tissues in vitro. *Reproduction* **2020**, *160*, 561–578. [CrossRef]
40. Macedo, N.S.; Barbosa, C.; Bezerra, A.H.; Silveira, Z.S.; da Silva, L.; Coutinho, H.D.M.; Dashti, S.; Kim, B.; da Cunha, F.A.B.; da Silva, M.V. Evaluation of ellagic acid and gallic acid as efflux pump inhibitors in strains of Staphylococcus aureus. *Biol. Open* **2022**, *11*, bio059434. [CrossRef] [PubMed]
41. Song, W.; Wang, L.; Jin, M.; Guo, X.; Wang, X.; Guan, J.; Zhao, Y. Punicalagin, an Inhibitor of Sortase A, Is a Promising Therapeutic Drug to Combat Methicillin-Resistant Staphylococcus aureus Infections. *Antimicrob. Agents Chemother.* **2022**, *66*, e0022422. [CrossRef]
42. Tian, Q.; Wei, S.; Su, H.; Zheng, S.; Xu, S.; Liu, M.; Bo, R.; Li, J. Bactericidal activity of gallic acid against multi-drug resistance Escherichia coli. *Microb. Pathog.* **2022**, *173*, 105824. [CrossRef]

43. Caballero, V.; Estevez, M.; Tomas-Barberan, F.A.; Morcuende, D.; Martin, I.; Delgado, J. Biodegradation of Punicalagin into Ellagic Acid by Selected Probiotic Bacteria: A Study of the Underlying Mechanisms by MS-Based Proteomics. *J. Agric. Food Chem.* **2022**, *70*, 16273–16285. [CrossRef] [PubMed]
44. Garcia-Villalba, R.; Beltran, D.; Espin, J.C.; Selma, M.V.; Tomas-Barberan, F.A. Time course production of urolithins from ellagic acid by human gut microbiota. *J. Agric. Food Chem.* **2013**, *61*, 8797–8806. [CrossRef] [PubMed]