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Influence of Human Age on the Prebiotic Effect of Pectin-Derived Oligosaccharides Obtained from Apple Pomace

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Abstract: The aim of this study was to evaluate the prebiotic effect of pectin-derived oligosaccharides (POS) obtained from apple pomace on the growth and metabolism of microbiota from the human gastrointestinal tract as a function of the age of the host. The counts of major bacterial groups *Bifidobacterium* sp., lactobacilli, *Clostridium* sp., *Bacteroides* sp., *Enterococcus* sp. and Enterobacteria were assessed during long-term *in vitro* fermentation of mixed cultures in a prebiotic medium. Comparative assessment of bacterial diversity in the human fecal microbiota was performed relative to the age of the host, from childhood to old age, through younger years and middle age. The age group of the host was found to be an important factor that determined the prebiotic effect of POS, which was related to both the qualitative and quantitative composition of fecal microbiota and its metabolism. In contrast to the microbiota of elderly subjects, the child-derived intestinal microbiota underwent significantly different alterations in terms of the proportion and composition of lactic acid bacteria, leading to a more favorable pattern of short-chain fatty acids (SCFA) and lactic acid levels.

Keywords: pectin-derived oligosaccharides (POS); prebiotics; apple pomace; human age

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1. Introduction

The digestive tract is a unique habitat for a rich microbiota which performs important functions: metabolic, trophic and immunological. The human intestinal microbiota is one of the most diverse ecosystems. The number of microbial cells in the human gut was estimated to be comparable in magnitude to the number of human cells [1]. The exact composition of the intestinal microbiota is specific to the individual. The presence of certain species can predispose the host to the development of numerous diseases, including inflammatory bowel disease, cancer, allergies or obesity [2–4].

Factors that influence the number and diversity of gut microbiota include age, diet, antibiotic therapies, peristalsis and the production of metabolites by bacteria. The development of gut microbiota starts at birth. It has been proven that following natural births, the microorganisms in children reflect the vaginal microbiota of the mother, with the presence of bacteria such as lactobacilli and *Prevotella* sp. In contrast, in children born via caesarean section, the predominant bacteria include the *Staphylococcus*, *Corynobacterium* and *Propionibacterium* genera, which mainly colonize the surface of the mother's skin [5,6]. The milk formula will also determine the gut microbiota of babies. In the case of breastfeeding, lactobacilli and bacteria of the genus *Bifidobacterium* have been shown to be dominant, whereas if artificial milk is used the genus *Bacteroides* and species belonging to various

clostridial clusters are found in majority [7]. The gut microbiota remains unstable until the child is 2–3 years old, and also shows reduced diversity. The composition of the microbiota of a 2–3 years old child resembles that found in adulthood [8], as an increase in the proportion of *Bacteroides* sp. and decreasing numbers of *Bifidobacterium* sp. and lactobacilli are observed. The composition of human gut microbiota is relatively stable throughout adulthood. However, in the elderly there is a decrease in the number of *Bacteroides* sp. with a simultaneous increase in the number of *Enterococcus* sp. and *Escherichia coli* [9,10], there are no essential differences between young adults and elderly groups under 75–80 years of age. However, centenarians were found to have low microbiota diversity with a significantly different composition, including higher levels of Proteobacteria.

Diet and biogeography are not the only factors that influence the gut microbiota. Age-related changes in gut physiology, such as gastric motility disorders, achlorhydria, and ageing of mucosal immune and motor systems, as well as degenerative changes in the enteric nervous system, can have a significant impact on the gut microbiome pattern [11,12]. According to the recent literature, probiotic and prebiotic supplements can increase the numbers of beneficial intestinal microbiota, providing protection against intestinal problems and contributing to the overall improvement of health. Despite the significant increase in publications in the subject of human intestinal microbiota diversity, few studies have linked the impact of prebiotics on gut microbiota relative to the age of the host. Among them, the role of gut microbiota, dietary fiber, and prebiotics in early life functional gastrointestinal disorders was reviewed by Wegh et al. [13]. On the other hand, it was also evidenced, both *in vivo* and *in vitro*, that prebiotic galactooligosaccharides (GOS) can exert positive effects upon bifidobacteria in the gut microbiota of elderly persons, enhancing the intestinal microbiota during ageing [14,15].

Recently, there has been great interest in the possibility of producing novel forms of prebiotic carbohydrates using various renewable sources. Apple pomace is a by-product generated during the processing of apple juice and can be considered a functional ingredient in food and nutraceutical applications. Several authors have suggested pectin-derived oligosaccharides (POS) as new-generation prebiotics [16–19]. A prebiotic is defined as: 'a substrate that is selectively utilized by host microorganisms conferring a health benefit'. Health effects of prebiotics are evolving but currently include benefits such as improvements in bowel function, immune response, glucose and lipid metabolism, bone health, and regulation of appetite and satiety [20].

To our knowledge, there are no data available on the influence of POS on the diversity of gut microbiota relative to human age. Therefore, the aim of this study was to evaluate the prebiotic effect of POS obtained from apple pomace on the growth and metabolism of microbiota from the human gastrointestinal tract as a function of the age of the host, from childhood to teenaged years, middle age and old age.

2. Materials and Methods

2.1. Materials

The research material was a prebiotic preparation of apple pomace obtained on an industrial scale according to the enzymatic method developed by our team for the Agros Nova company (Łowicz, Poland).

The biological material consisted of 3 samples of human feces obtained from healthy volunteers: one child aged 7 (male), two adults aged 24 and 67 (male) and one sub-elderly individual aged 69 (male); they were non-smoking, without a history of gastrointestinal tract disorders, on a basal diet, who had not consumed prebiotic or probiotic supplements, nor received antibiotic treatment within 6 months prior to the study [21]. All volunteers were on a typical Western-type diet, without prevalence of some group of food (meats or vegetables); they were not vegetarians or vegans. No invasive or inconvenient methods were used to collect fecal samples from healthy volunteers (fecal samples were acquired naturally). Therefore, the institution awarding the grant funding (National Centre of Research and Development, NCBR, Warszawa, Poland) as well as our research unit (Institute

of Fermentation Technology and Microbiology, TUL) did not require the approval of the Ethics Committee to conduct research using human feces inoculum in *in vitro* tests [21]. All donors were volunteers, who were informed about the planned analytical procedures and agreed to publish the obtained data.

Feces were collected into sterile containers, kept in oxygen-limited conditions (in the presence of 5% (*v*/*v*) CO_2 in a Heal Force® HF 212 UV incubator) and used within a maximum of 1 h after collection.

2.2. Methods

2.2.1. High-Performance Anion-Exchange Chromatography

Chromatographic analysis was performed according to the method developed in our previous study [21], using an ion chromatograph DIONEX ICS-3000 (Dionex, Sunnyvale, CA, USA) with a PED electrochemical detector, equipped with a Dionex Carbo Pac PA200 (3 × 250 mm) analytical column and Dionex Carbo Pac PA200 guard column (3 × 50 mm). Gradient separation at 0.8 mL/min flow rate was used with the following eluents: eluent 1: 200 mM NaOH (solution 50% *v*/*v* in water NaOH, Sigma-Aldrich®, Europe) in 550 mM NaOAc (Sigma-Aldrich®, St. Louis, MO, USA); eluent 2: 250 mM NaOH (solution 50% *v*/*v* in water NaOH, Sigma-Aldrich®, St. Louis, MO, USA); and eluent 3: distilled water (18 MΩ). The oligomer standards were obtained according to the procedure described in [22].

2.2.2. *In Vitro* Fecal Fermentation and Qualitative and Quantitative Assessment of the Microbiome

To obtain a starting suspension, 1 g of human feces was mixed with 20 mL of sterile 0.85% saline solution. One milliliter of inoculum was introduced into 100 mL of sterile POS preparation (pH = 7) and incubated under oxygen-limited conditions (in the presence of 5% (*v*/*v*) CO_2 in the Heal Force® HF 212 UV incubator) at 37 °C for 14 days.

2.2.3. Koch's Plating Method

The numbers of selected fecal microorganisms were determined by plating using selective microbial media: lactobacilli-MRS (BTL), *Bifidobacterium* sp.-Bifidobacterium medium (MERCK), *Bacteroides* sp.-Schaedler Agar-KV (MERCK), *Clostridium* sp.-Tryptose Sulfite Cycloserine Agar (MERCK), *Enterococcus* sp.-Bile Aesculin Agar (MERCK), and Enterobacteriaceae-Mac Conkey Agar (MERCK).

2.2.4. Fluorescence In Situ Hybridization (FISH)

Selected bacteria groups in the human feces were measured on the basis of the method described by Gullon et al. [23], using synthetic oligonucleotide probes labeled with fluorescent dye (Table 1). One milliliter of feces inoculum (prepared according to the method described in Section 2.2.2) was incubated with 3 mL of 4% paraformaldehyde at 4 °C for 24 h and then centrifuged. The biological material was then rinsed with PBS buffer three times. The resulting sediment was suspended in 0.5 mL of 50% (*v*/*v*) ethanol. After vortexing, 50 µL of the mixture was collected in small PCR tubes. Then, 20 µL 0.01% (*m*/*v*) lysozyme solution in TRIS-EDTA (composed of 0.211 g TRIS, 1.611 g EDTA, 100 mL of redistilled water) was added and the samples were incubated at 37 °C for 30 min. The samples were centrifuged, the supernatant was discarded and the sediment was rinsed with 100 µL of PBS. The bacterial material was then mixed with 50 µL hybridization buffer (composed of 0.242 g TRIS, 12.56 g NaCl, 0.01 g SDS, 20 mL of formamide, 100 mL of redistilled water) and 10 µL of oligonucleotide probe. The time and temperature of the hybridization process are shown in Table 1, depending on the oligonucleotide probe used.

Table 1. Characteristics of oligonucleotide probes used in the FISH method.

Probe Code	Target Microorganisms	Fluorescent Dye	Temperature [°C]	Time [h]
Bac 303	*Bacteroides/Prevotella* sp.	5′ Cy3	56	3
Bif 164	*Bifidobacterium* sp.	5′ Cy3	58	18
E. coli	*Eschericha coli*	5′ Cy3	51.7	18
Enter 1432	*Enterobacteriaceae*	5′ Cy3	43	18
Erec 484	*Clostridium coccoides*	5′ Cy3	57	16
Lab 158	*lactobacilli/Enterococcus* sp.	5′ Fluo	45	24

In the next stage, the samples were centrifuged, the supernatant was poured off and 150 µL of washing buffer (composed of 0.242 g TRIS, 12.56 g NaCl, 100 mL of redistilled water) was added. After 30 min of incubation at a temperature suitable for the oligonucleotide probe used, the samples were centrifuged. After removing the supernatant, 100 µL of PBS was introduced into the tube, which was vortexed and centrifuged again. The pellet was suspended in 50 µL of PBS.

From each sample, 7 µL was applied to a glass slide. Microscopic observations were made using a fluorescence microscope (Nikon Eclipse Ci) at 400× magnification. The numbers of microorganism cells were counted in 15 fields of view.

2.2.5. Short-Chain Fatty Acid (SCFA) Analysis

A Thermo Scientific liquid chromatograph equipped with RI Plus and PDA detectors (Thermo Scientific, Waltham, MA, USA) and an Aminex HPX 87H (300 × 7.8 mm id; Bio-Rad, Hercules, CA, USA) column were used. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min. The analysis was carried out for 40 min at 60 °C. Identification of individual metabolites generated during *in vitro* fecal fermentations was performed on the basis of a comparison of the peak retention times with those for formic acid, propionic acid, acetic acid and butyric acid standards (Sigma) [21].

2.2.6. Lactic Acid Isomers Analysis

To determine L(+) and D(−) forms of lactic acid in cultivars of human gastrointestinal microorganisms in the apple pomace hydrolyzate, Megazyme enzymatic tests were used (D-Lactic Acid (D-Lactate) Rapid Assay Kit, L-Lactic Acid (L-Lactate) Assay Kit).

2.2.7. Statistical Analysis

Analysis of variance (one-way ANOVA) and Tukey's Honestly Significant Differences (HSD) test ($p < 0.05$) were conducted to determine the differences between the analyzed samples with respect to microbiome quantity and metabolites. SPSS for Windows (version 17.0, SPSS Institute Inc., Chicago, IL, USA) was used for all statistical analyses. For each age group, three biological replicates were performed.

3. Results

3.1. Quantitative and Qualitative Composition of POS

Figure 1 shows the quantitative and qualitative composition of the apple pomace hydrolysate. Oligosaccharides with different degrees of polymerization in the range of DP 2–17 were observed. The total amount of POS in the hydrolysate was equal to 5.2 g/100 g of dry weight.

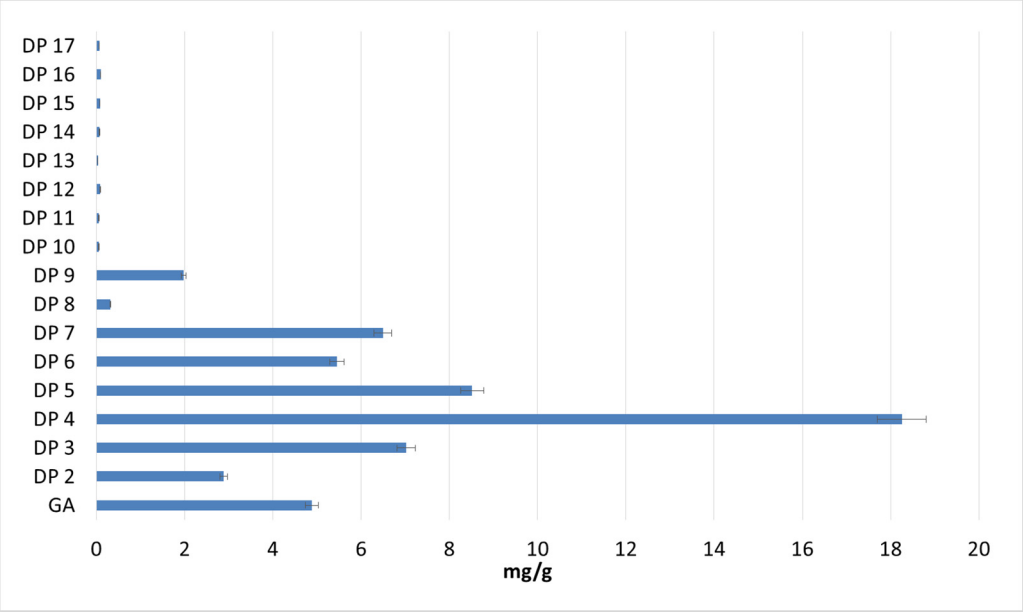

Figure 1. Concentration (mg/g) of glucuronic acid (GA) and pectic oligosaccharides with different degrees of polymerization (DP 2–DP 17) in a prebiotic preparation of apple pomace. The data represent means from one experiment repeated three times. Error bars denote the SD.

3.2. Growth Dynamics of Microorganisms Colonizing the Human Digestive Tract during In Vitro Fermentations in Apple Pomace Hydrolysate

Due to the limitations of different methods, in this study the standard Koch's plating method and FISH as a comparative method were applied independently to quantify the changes in human fecal microbiome in the presence of apple pomace hydrolysate. An important limitation of the use of Koch's plating method is the fact that within the fecal microbiome, up to 80% of microorganisms are constituted by unculturable species, which do not grow *in vitro* with the application of the standard plating method [24]. FISH does not require cultivation of the organisms. However, the detection limit is quite low—10^6 cells/mL. If the concentration of bacteria is below that number, the microorganisms will not be detected in the microscope observation field. Furthermore, in contrast to the FISH method, which detects both living and dead cells simultaneously, in the Koch's plating method only the living and actively proliferating cells are quantified. This is a very important advantage, if determination of the long-term growth rate is a target.

In the first stage of this study, a comparative assessment was performed of bacterial diversity in the human fecal microbiota, relative to the age of host (Figures 2 and 3). Based on the results obtained using the Koch's plating method, it was found that in the samples of feces from a seven-year-old child, bacteria of the genus *Bacteroides* predominated (28.8%), followed by *Clostridium* (21.2%), *Bifidobacterium* (20.9%), Enterobacteriaceae (12.1%) and lactobacilli (16.1%). According to the FISH method, the majority of these samples was constituted by the lactobacilli/*Enterococcus* sp. (40.4%), followed by *Clostridium coccoides* (21.1%), *Bacteroides/Prevotella* sp. (14.7%), Enterobacteriaceae (13.4%), and *Bifidobacterium* sp. (10.9%). The literature emphasizes that the colonic microbiota of infants can be described as adult-like after the age of 2–3 years, although the populations of facultative anaerobes are often observed to be larger than those of healthy adults. Thus, our findings are in accordance with the literature, which indicates that *Bacteroides* sp., *Bifidobacterium* sp. and *Clostridium* sp. are predominant in adults (determination was assessed using plating method) [25].

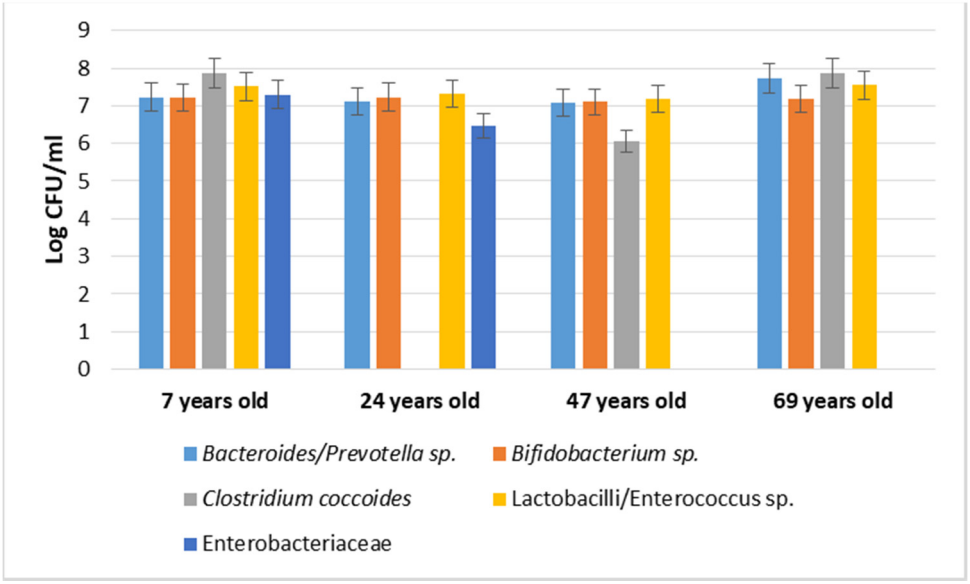

Figure 2. The content of bacteria populations in human feces in relation to host age, determined with the application of the fluorescence in situ hybridization (FISH) method. Data represent means from three repetitions of one experiment.

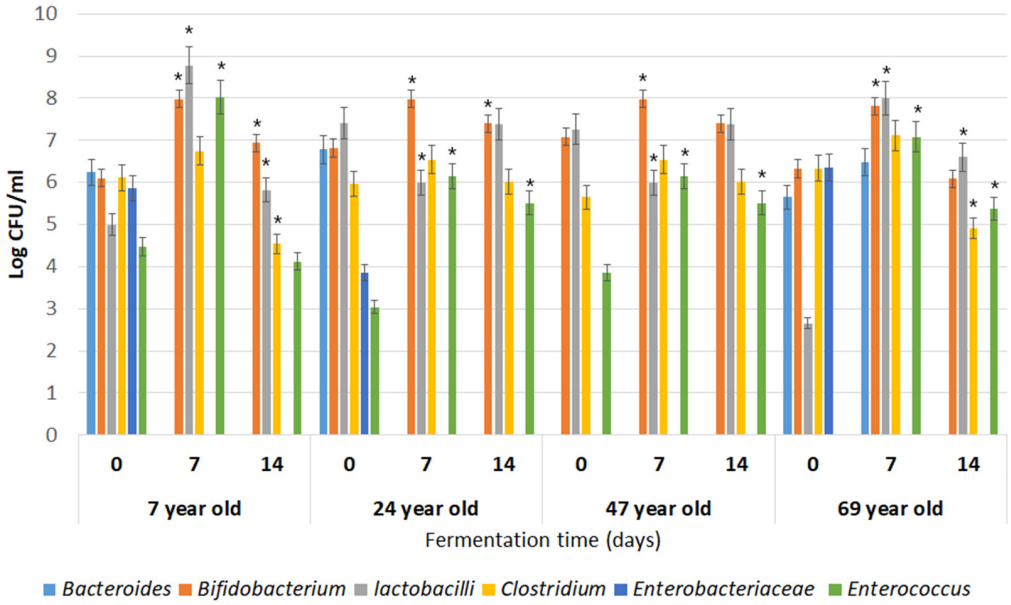

Figure 3. Bacteria concentration during *in vitro* fecal fermentations in AP hydrolysate (conducted over 14 days at 7-day intervals) in relation to the host's age, determined using Koch's plating method. The data represent the means from three repetitions of each experiment. Error bars denote the SD. * Results significantly differ from the unexposed control, ANOVA ($p < 0.05$).

However, the composition of fecal microbiota varied markedly between the tested child and adults. In the child feces, the population of the Enterobacteriaceae family was 104-fold and 144-fold higher when compared with that of the 24-year-old and 49-year-old, respectively (Figure 3). Similar results were obtained by Hopkins et al. [25]. No Enterobacteriaceae were detected in the feces from 24-year-old or 49-year-old using the FISH method. The microbiota of young adults and adults were generally characterized by high levels of lactic acid bacteria (LAB). In the samples from the young adult of 24 years, the most numerous were lactobacilli, which constituted over 65% of the total of microbiota analyzed. *Bacteroides* sp. (15.6%), *Bifidobacterium* sp. (16.9%) and *Clostridium* sp. (2.4%) were

also found in large amounts, whereas *Enterococcus* sp. and Enterobacteriaceae constituted only around 1% of the total microorganisms. The samples of feces from the person of 47 years were distinguished by the highest number of *Bifidobacterium* sp. (65%) and lactobacilli (32.5%). The amount of *Clostridium* sp. bacteria was found to be 2.2%, which was comparable with the amount found in the young adult samples. The predominance of lactic acid bacteria in the abovementioned samples was confirmed using the FISH method. According to the literature, once the climax microbiota has become established, the major bacterial groups in the feces of adults remain relatively constant over time. The differences in the patterns of microorganisms for individuals of between 24 and 47 years observed in our study could be explained partially by individual health state associated with the starting of aging processes.

According to Salazar et al. [10], the ageing process deeply affects the composition of the microbiota, reducing diversity and the proportion of beneficial bacteria while changing the dominant species. Viable counts of the predominant bacterial species isolated in our study from the feces of adults and elderly individuals showed some variation, such as higher levels of Enterobacteriaceae (32.5%) and *Clostridium* sp. (30.9%), and lower amounts of *Bacteroides* sp. (6.4%) in the latter group (Figure 3). However, in a study by Biagi et al. [11], carried out using molecular methods of microbial identification, no essential differences were found between the microbiotas of young adults and elderly people under 75–80 years of age. In contrast, a study by Claesson et al. (also using pyrosequencing 16S rRNA gene V4 region) [26] found that the gut populations of people over the age of 65 showed a substantial increase in the proportion of *Bacteroides* sp. and *Clostridium* sp. compared to younger individuals. The microbiotas of centenarians have been shown to be less diverse than those of adults, and to have decreased levels of *Bifidobacterium* sp., *Bacteroides* sp. and Enterobacteriaceae, with increased levels of *Clostridium* sp. [11].

In vitro fermentations were carried out to confirm the effect of POS oligosaccharides contained in the apple pomace hydrolysate on the growth and metabolism of mixed bacteria populations from the human feces, relative to the host age group (Figure 3). The growth efficiency of *Bifidobacterium* sp., *Bacteroides* sp., *Clostridium* sp., *Enterococcus* sp., lactobacilli and Enterobacteriaceae bacteria was determined over 14 days at 7-day intervals. On the basis of the conducted tests, it was found that the POS caused substantial growth in the amounts of bacteria of lactobacilli (for samples from the 7-, 47-, and 69-year-olds) and *Bifidobacterium* genus (for the 24-year-old), already by day seven of the fermentation process in all tested environments. The forward stage of fermentation revealed further dynamics in the microbiota pattern, towards the quantitative domination of LAB. In the final stage of the *in vitro* fermentations, *Bifidobacterium* sp. was highly represented only in the child microbiota (92.3%), while the adult and elderly subjects exhibited different ratios of lactobacilli and *Bifidobacterium* sp. in favor of lactobacilli. After the first week of cultivation, a decrease in the number of *Clostridium* bacteria was observed. Bacteria from the Enterobacteriaceae family were not able to grow in studied samples. In the elderly person, the obtained results were not in full accordance with Toward et al. [15], who observed that prebiotic galactooligosaccharides exert significant effects only upon bifidobacteria in the gut microbiota both *in vivo* and *in vitro*.

3.3. Metabolism of Microorganisms Colonizing the Human Digestive Tract during In Vitro Fermentations in Apple Pomace Hydrolysate

Table 2 and Figure 4 show the effects of microbiota diversity and dynamics on lactic acid and SCFA accumulation during *in vitro* fermentation, performed by fecal microorganisms differentiated by age group. The main product of the metabolism of fecal microbiota cultured in apple pomace hydrolyzate was lactic acid. Lactate production is connected with the metabolic activity of lactic acid bacteria and *Bifidobacterium*. The highest concentration of lactic acid was determined in the fecal samples of the child of 7 years (above 524 mg/100 mL). In the remaining samples, its content ranged between 210.6 mg/100 mL (for the 47-year-old) and 257.1 mg/100 mL (for the 69-year-old). It was also found that the ratio of the L (+) and D (−) isomers of lactic acid in the cultures of human fecal mi-

croorganisms varied, depending on the ages of the individuals and the fermentation time. In the samples from hosts aged 24 and 69, the L (+) form of lactic acid prevailed during the whole fermentation period. However, in the later stages of the *in vitro* process it was noticed that all of the samples were characterized by a higher ratio of the L (+) form of lactic acid. The L (+) isomer is metabolized much more quickly by the human body due to the presence of L-lactate dehydrogenase and does not affect the health of the host. In contrast, the D (−) lactic acid isomer remains for a longer time in the digestive tract and may accumulate temporarily, causing acidification (lactacidemia). The D (−) lactic acid form also has the important function of lowering the pH of the intestine, which inhibits the growth of pathogens [27].

Table 2. Lactic acid concentration and ratio of L (+) and D (−) isomers of lactic acid during *in vitro* fecal fermentations in AP hydrolysate (conducted over 14 days at 7-day intervals) in relation to host age.

Host Age	Fermentation Period [days]	Lactic Acid Concentration [mg/100 g]	L (+)/D (−)
7 years	7	524.55 ± 26.93 ^{a}	0.88
	14	534.72 ± 32.40 ^{a}	2.36
24 years	7	262.32 ± 15.72 ^{b}	1.12
	14	254.52 ± 14.11 ^{b}	1.03
47 years	7	225.93 ± 11.65 ^{bc}	0.85
	14	210.64 ± 9.02 ^{c}	1.15
69 years	7	213.89 ± 6.86 ^{c}	2.09
	14	257.14 ± 7.94 ^{b}	2.58

Data represent means from three repeats in one experiment. Different letters ($^{a, b, c}$) represent significance differences ($p < 0.05$).

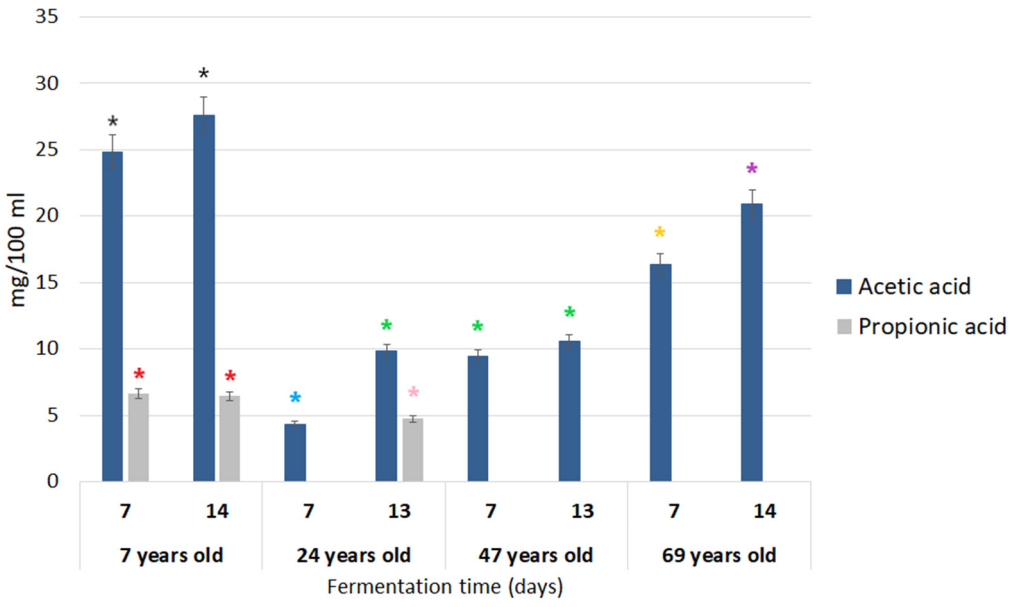

Figure 4. Short-chain fatty acid concentrations during *in vitro* fecal fermentations in AP hydrolysate (conducted over 14 days at 7-day intervals) in relation to host age. Data represent means from three repetitions of one experiment. Error bars denote the SD. Different colors used in the asterisks (*) represent significant difference for each fermentation period differentiated by age group, ANOVA ($p < 0.05$).

Human digestive enzymes are capable of degrading only starch and the disaccharides sucrose and lactose. The wide variety of complex polysaccharides become substrates for specialized enzymes (including glycoside hydrolases, glycosyltransferases, polysaccharide

lyases, and carbohydrate esterases) encoded by the gut microbiome. SCFAs are the main end products of non-digestible carbohydrate fermentation by fecal communities [28–30]. The highest levels of SCFAs are found in the proximal colon, where they are used locally by enterocytes or transported across the gut epithelium into the bloodstream [31,32]. Acetate, propionate, and butyrate are regarded as predominant SCFAs.

In this study, the short chain fatty acids identified in the cultures were: acetic acid at concentrations of 12.9–28.5 mg/100 mL and propionic acid at up to 6.8 mg/100 mL. However, no butyric acid was found, which is the main fermentation product of bacteria of the *Clostridium* genus. The highest concentration of SCFAs was found in the media fermented with microorganisms from the child feces (Figure 4), in which the molar ratio of acetate:propionate in the fermentation media was 78.9:21.1. Similar molar ratios of SCFA (60:20) detected in human large intestine were reported by Cummings et al. [31]. However, Gullon et al. [23] obtained the ratio 1:0.08 using an *in vitro* model.

4. Discussion

Discussing the limitations of our study, it is worth noting that microbiota profiles differ across sections of the gastrointestinal tract. Lactobacilli, *Veillonella*, and *Helicobacter* are more abundant in proximal gut, whereas *Bacilli*, Streptococcaceae, Actinomycinaeae, and Corynebacteriaceae are predominant in duodenum, jejunum, or ileum. The colon is dominated by the families Prevotellaceae, Bacteroidaceae, Rikenellaceae, Ruminococcaceae, and Lachnospiraceae. Moreover, there are also differences in the microbial patterns between the gut epithelium (colonized mainly by *Clostridium*, lactobacilli, *Enterococcus*) and gut lumen [33–35]. Despite the fact that there may be significant differences in microbial composition between intestine mucosa and feces, fecal specimens are frequently used in many studies as substitutions of intestine/colon microbiota. According to Zmora et al. [36], even the microbiota composition in the lower digestive tract, which is closest to feces, is significantly different from that of feces. Nevertheless, fecal samples are naturally collected, whereas endoscopic procedures, surgery, or intestinal aspiration used to acquire tissue samples and luminal contents are very invasive and not suitable for healthy people. They also provide cross-contamination and disturbance to normal intestinal physiology [37].

The gut microbiome is a key element for maintaining human health. The proper development of its composition from the moment of birth plays a crucial role in ensuring optimal functioning of the human body. Prebiotics can alter the composition and metabolism of intestinal microbiota from the first days of human life. Human milk oligosaccharides (HMOs) are known to enhance the development of a bifidogenic microbiome in infants [38,39]. To imitate HMOs, a mixture of short-chain GOS and long-chain FOS has been developed [40]. According to Hoeflinger et al. [41], many bifidobacteria and bacteroide species are able to metabolize HMOs, whereas Enterobacteriaceae are able to consume non-HMO prebiotics, such as GOS and maltodextrin. In healthy adults, the microbiota is generally stable, but heavily affected by ageing.

In our study, *in vitro* fermentations were carried out to confirm the effect of POS oligosaccharides contained in apple pomace hydrolysate on the growth and metabolism of mixed bacteria populations from human feces, relative to the host age group. *Bifidobacterium* sp. was highly stimulated by the POS only in the microbiota of a 7-year-old child, while the adult and elderly subjects exhibited different ratios of lactobacilli and *Bifidobacterium* sp. in favor of lactobacilli. These results are in accordance with several previous studies. The growth of beneficial lactic acid bacteria and *Bifidobacterium* stimulated by apple pomace oligosaccharides, determined *in vitro* using fecal inocula from adult donors, has also been noted by Gullon et al. [23]. The joint populations of bifidobacteria and lactobacilli increased from 19% to 29%, 34%, and 32% in *in vitro* cultures with pectic oligosaccharides obtained from lemon peel, sugar beet pulp, and FOS, respectively [20]. The dynamics of the microbial population were assessed by FISH. Moon et al. [42] noted the bifidogenic effect of linear arabino-oligosaccharides (LAOS) *in vitro* with fecal inocula from adults, which they determined using molecular methods of microbial identification. Fecal bifidobacteria

have also been found to increase with GOS treatment in an in vivo study of adolescent girls aged 10–13 years [43].

The gut microbiota and its metabolites play a key role in maintaining health in all age groups, especially among the elderly. Clinical trials have demonstrated that prebiotics possess the potential to reverse age-related decline in bifidobacteria and modulate associated health parameters. For instance, Vulevic et al. [44] assessed the effect of a GOS mixture on gut microbiota, markers of immune function, and metabolites, in 40 elderly (aged 65–80 years) volunteers in a randomized double-blind placebo (maltodextrin)-controlled, cross-over study. Their study revealed that B-GOS consumption led to significant increases in *Bacteroides* sp. and bifidobacteria—the latter correlating with increased lactic acid in fecal waters. The bifidogenic effect of GOS *in vivo* was also confirmed by Walton et al. [45], on a population of men and women aged 50 years and above. Furthermore, GOS supplementation had a bifidogenic effect in all *in vitro* system vessels.

With age, the composition of the microbiota deteriorates, affecting the processes it carries out. The gut microbiota of elderly subjects contains fewer beneficial microorganisms and shows an increase in facultative anaerobes. These changes limit SCFA production. A shift from a predominantly saccharolytic metabolism toward a predominantly putrefactive metabolism is also observed with aging [46]. Age-dependent alterations, such as changes in eating habits and lifestyle, as well as changes in the intestinal microbiota ecosystem (reduced species diversity of the microbiota, imbalance, constant variations, a reduction in the number of beneficial lactobacilli and bifidobacteria, increased amounts of *Clostridium* and *Eubacterium* genera), may be involved in the transformation and activation of promutagenic compounds [26,47,48]. This can contribute to many diseases in older people, such as diabetes, cardiovascular disease, colorectal cancer, inflammatory bowel disease, and frailty [11,48–50]. It has been shown that prebiotics can have a protective effect with respect to microbial bioactivation of potentially carcinogenic substances, such as heterocyclic aromatic amines [48]. One possible explanation for this mechanism is that inulin-type fructo-oligosaccharides alter bacterial composition and metabolism by decreasing the activity of bacterial β-glucuronidase. Prebiotics alter the fermentation pattern of the environment towards the saccharolytic and acidic, which may be the basis of their protective effect. Generally, lower proteolytic activity of the residual microbiota is related to health benefits. The presence of prebiotics increases the production of SCFA and lactic acid in the GIT (gastrointestinal tract). The end products of prebiotic fermentation by lactobacilli are SCFA. Their presence in the colon is desirable, due to their pro-health effects, especially as anti-carcinogenic agents [51].

To sum up, to diminish variability among studies, researchers should be aware of the effect that age can cause, since significant differences between children, young adults, adults, and elderly people have been reported. The differences in fecal batches at baseline within tested age groups should be taken into consideration as well, since the gut microbiota pattern also changes with diet, the prevalence of some groups of food, prebiotic, or probiotic supplements, antibiotic treatment and when patients have a history of gastrointestinal tract disorders.

5. Conclusions

This study set out to evaluate the prebiotic effect of pectin-derived oligosaccharides (POS) obtained from apple pomace on the growth and metabolism of microbiota from the human gastrointestinal tract as a function of the age of the host, from childhood to old age, through teenaged years and middle age. Several previous studies have focused on the determination of the composition of the human gut microbiota depending on the age of the host. Recent research has also shown that prebiotic oligosaccharides result in increased numbers of beneficial intestinal microbiota, providing many valuable health benefits. Our results suggest that the age group of the host may be an important factor that determines the effect of prebiotics, in terms of the qualitative and quantitative composition of fecal microbiota and its metabolism. Even though the obtained results are promising, they

can be only considered an approximation due to the limited number of subjects included and parameters evaluated. Therefore, *in vivo* tests on humans of different ages should be performed to confirm these findings.

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References

1. Sender, R.; Fuchs, S.; Milo, R. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* **2016**, *14*, e1002533. [CrossRef] [PubMed]
2. McGarr, S.E.; Ridlon, J.M.; Hylemon, P.B. Diet, anaerobic bacterial metabolism and colon cancer: A review of the literature. *J. Clin. Gastroenterol.* **2005**, *39*, 98–109. [PubMed]
3. Ley, R.E.; Turnbaugh, P.J.; Klein, S.; Gordon, J.I. Microbial ecology: Human gut microbes associated with obesity. *Nature* **2006**, *444*, 1022–1023. [CrossRef]
4. Frank, D.N.; St Amand, A.L.; Feldman, R.A.; Boedeker, E.C.; Harpaz, N.; Pace, N.R. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13780–13785. [CrossRef]
5. Biasucci, G.; Benenati, B.; Morelli, L.; Bessi, E.; Boehm, G. Cesarean delivery may affect the early biodiversity of intestinal bacteria. *J. Nutr.* **2008**, *138*, 1796–1800. [CrossRef]
6. Dominguez-Bello, M.G.; Costello, E.K.; Contreras, M.; Magris, M.; Hidalgo, G.; Fierer, N.; Knight, R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11971–11975. [CrossRef]
7. Bui, T.P.N.; Troise, A.D.; Nijsse, B.; Roviellof, G.N.; Foglianog, V.; de Vos, W.M. Intestinimonas-like bacteria are important butyrate producers that utilize Nε-fructosyllysine and lysine in formula-fed infants and adults. *J. Funct. Foods* **2020**, *70*, 103974. [CrossRef]
8. Bezirtzoglou, E. The intestinal microflora during the first weeks of life. *Anaerobe* **1997**, *3*, 173–177. [CrossRef]
9. Yatsunenko, T.; Rey, F.E.; Manary, M.J. Human gut microbiome viewed across age and geography. *Nature* **2012**, *486*, 222–227. [CrossRef]
10. Salazar, N.; Arboleya, S.; Valdes, L. The human intestinal microbiome at extreme ages of life. Dietary intervention as a way to counteract alterations. *Front. Genet.* **2014**, *5*, 1–9. [CrossRef]
11. Biagi, E.; Nylund, L.; Candela, M.; Ostan, R.; Bucci, L.; Pini, E.; Nikkı, J.; Monti, D.; Satokari, R.; Franceschi, C.; et al. Through ageing, and beyond: Gut microbiota and inflammatory status in seniors and centenarians. *PLoS ONE* **2015**, *5*, e10667.
12. Konturek, P.C.; Haziri, D.; Brzozowski, T.; Hess, T.; Heyman, S.; Kwiecien, S.; Konturek, S.J.; Koziel, J. Emerging role of fecalmicrobiota therapy in the treatment of gastrointestinal andextra-gastrointestinal diseases. *J. Physiol. Pharmacol.* **2015**, *66*, 483–491. [PubMed]
13. Wegh, C.A.M.; Schoterman, M.H.C.; Vaughan, E.E.; Belzer, C.; Benning, M.A. The effect of fiber and prebiotics on children's gastrointestinal disorders and microbiome. *Expert Rev. Gastroenterol. Hepatol.* **2017**, *11*, 1031–1045. [CrossRef]
14. Scheida, M.M.A.; Franco Moreno, Y.M.; Maróstica, M.R., Jr.; Pastorea, G.M. Effect of prebiotics on the health of the elderly. *Food Res. Int.* **2013**, *53*, 426–432. [CrossRef]
15. Toward, R.; Montandon, S.; Walton, G.; Gibson, G.R. Effect of prebiotics on the human gut microbiota of elderly persons. *Gut Microbes* **2012**, *3*, 57–60. [CrossRef]
16. Gullón, B.; Garrote, G.; Alonso, J.L.; Parajo, J.C. Production of L-lactic acid and oligomeric compounds of apple pomace by simultaneous saccharification and fermentation: A response surface methodology assessment. *J. Agric. Food Chem.* **2007**, *55*, 5580–5587. [CrossRef]
17. Hotchkiss, A.T., Jr.; Manderson, K.; Tuohy, K.M.; Widmer, W.W.; Nunez, A.; Gibson, G.R.; Rastall, R.A. Bioactive properties of pectic oligosaccharides from sugar beet and Valencia oranges. In Proceedings of the 233rd ACS National Meeting, Chicago, IL, USA, 25–29 March 2007.

18. Rabetafika, H.N.; Bchir, B.; Blecker, C.H.; Richel, A. Fractionation of apple by-products as source of new ingredients: Current situation and perspectives. *Trends Food Sci. Technol.* **2014**, *40*, 99–114. [CrossRef]
19. Gómez, B.; Gullón, B.; Yáñez, R.; Schols, H.; Alonso, J.L. Prebiotic potential of pectins and pectic oligosaccharides derived from lemon peel wastes and sugar beet pulp: A comparative evaluation. *J. Funct. Foods* **2016**, *20*, 108–121. [CrossRef]
20. Gibson, G.R.; Hutkins, R.; Sanders, M.E.; Prescott, S.L.; Reimer, R.A.; Salminen, S.J.; Scott, K.; Stanton, C.; Swanson, K.S.; Cani, P.D.; et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 491–502. [CrossRef]
21. Wilkowska, A.; Nowak, A.; Antczak-Chrobot, A.; Motyl, I.; Czyżowska, A.; Paliwoda, A. Structurally different pectic oligosaccharides produced from apple pomace and their biological activity in vitro. *Foods* **2019**, *8*, 365. [CrossRef]
22. Bonnin, E.; Clavurier, K.; Daniel, S.; Kauppinen, S.; Mikkelsen, J.D.M.; Thibault, J.-F. Pectin acetylesterases from Aspergillus are able to deacetylate homogalacturonan as well as rhamnogalacturonan. *Carbohydr. Polym.* **2008**, *74*, 411–418. [CrossRef]
23. Gullon, B.; Gullon, P.; Sanz, Y.; Alonso, J.L.; Parajo, J.C. Prebiotic potential of a refined product containing pectic oligosaccharides. *Food Sci. Tech.* **2011**, *44*, 1687–1696. [CrossRef]
24. Eckburg, P.B.; Bik, E.M.; Bernstein, C.N. Diversity of the human intestinal microbial flora. *Science* **2005**, *308*, 1635–1638. [CrossRef] [PubMed]
25. Hopkins, M.J.; Sharp, R.; Macfarlane, G.T. Variation in human intestinal microbiota with age. *Digest Liver Dis.* **2002**, *34*, 12–28. [CrossRef]
26. Claesson, M.J.; Cusack, S.; O'Sullivan, O.; Greene-Diniz, R.; de Weerd, H.; Flannery, E.; Marchesi, J.R.; Falush, D.; Dinan, T.; Fitzgerald, G.; et al. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4586–4591. [CrossRef]
27. Mack, D.R. D (–)-lactic acid-producing probiotics, D (–)-lactic acidosis and infants. *Can. J. Gastroenterol.* **2004**, *18*, 671–675. [CrossRef]
28. Cantarel, B.L.; Lombard, V.; Henrissat, B.; Hu, Y.; Walker, S.; Laine, R.; Varki, A.; Sharon, N.; Varki, A.; Hooper, L. Complex carbohydrate utilization by the healthy human microbiome. *PLoS ONE* **2012**, *7*, e28742. [CrossRef]
29. El Kaoutari, A.; Armougom, F.; Gordon, J.I.; Raoult, D.; Henrissat, B. The abundance and variety of carbohydrateactive enzymes in the human gut microbiota. *Nat. Rev. Microbiol.* **2013**, *11*, 497–504. [CrossRef]
30. Holscher, H.D. Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut Microbes* **2017**, *8*, 172–184. [CrossRef]
31. Cummings, J.H.; Pomare, E.W.; Branch, H.W.J.; Naylor, E.; Macfarlane, G.T. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **1987**, *28*, 1221–1227. [CrossRef]
32. De Vadder, F.; Kovatcheva-Datchary, P.; Goncalves, D.; Vinera, J.; Zitoun, C.; Duchampt, A.; Backhed, F.; Mithieux, G. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* **2014**, *156*, 84–96. [CrossRef]
33. Sekirov, I.; Russell, S.L.; Antunes, L.C.; Finlay, B.B. Gut microbiota in health and disease. *Physiol. Rev.* **2010**, *90*, 859–904. [CrossRef] [PubMed]
34. Dieterich, W.; Schuppan, D.; Schink, M.; Schwappacher, R.; Wirtz, S.; Agaimy, A.; Neurath, M.F.; Zopf, Y. Influence of low FODMAP and gluten-free diets on disease activity and intestinal microbi;ota in patients with non-celiac gluten sensitivity. *Clin. Nutr.* **2018**, *38*, 697–707. [CrossRef]
35. Dieterich, W.; Schink, M.; Zopf, Y. Microbiota in the Gastrointestinal Tract. *Med. Sci.* **2018**, *6*, 116. [CrossRef]
36. Zmora, N.; Zilberman-Schapira, G.; Suez, J.; Mor, U.; Dori-Bachash, M.; Bashiardes, S. Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell* **2018**, *174*, 1388–1405. [CrossRef] [PubMed]
37. Tang, Q.; Jin, G.; Wang, G.; Liu, T.; Liu, X.; Wang, B.; Cao, H. Current Sampling Methods for Gut Microbiota: A Call for More Precise Devices. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 151. [CrossRef] [PubMed]
38. Bunesova, V.; Lacroix, C.; Schwab, C. Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense. *BMC Microbiol.* **2016**, *16*, 248. [CrossRef] [PubMed]
39. Asakuma, S.; Hatakeyama, E.; Urashima, T.; Yoshida, E.; Katayama, T.; Yamamoto, K.; Kumagai, H.; Ashida, H.; Hirose, J.; Kitaoka, M. Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. *J. Biol. Chem.* **2011**, *286*, 34583–34592. [CrossRef]
40. Vandenplas, Y.; Berger, B.; Carnielli, V.P.; Ksiazyk, J.; Lagstrom, H.; Sanchez Luna, M.; Migacheva, N.; Mosselmans, J.-M.; Picaud, J.-C.; Possner, M.; et al. Human Milk Oligosaccharides: 2 -Fucosyllactose (2 -FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. *Nutrients* **2018**, *10*, 1161. [CrossRef]
41. Hoeflinger, J.L.; Davis, S.R.; Chow, J.; Miller, M.J. In vitro impact of human milk oligosaccharides on Enterobacteriaceae growth. *J. Agric. Food Chem.* **2015**, *63*, 3295–3302. [CrossRef] [PubMed]
42. Moon, J.S.; Shin, S.Y.; Choi, Y.S.; Joo, W.; Cho, S.K.; Li, L.; Kang, J.-H.; Kim, T.-J.; Han, N.S. In vitro digestion and fermentation properties of linear sugar beet arabinan and its oligosaccharides. *Carbohydr. Polym.* **2015**, *131*, 50–56. [CrossRef] [PubMed]
43. Whisner, C.M.; Martin, B.R.; Schoterman, M.H.C.; Nakatsu, C.H.; McCabe, L.D.; McCabe, G.P.; Wastney, M.E.; van den Heuvel, E.G.H.M.; Weaver, C.M. Galacto-oligosaccharides increase calcium absorption and gut bifidobacteria in young girls: A double-blind cross-over trial. *Br. J. Nutr.* **2013**, *110*, 1292–1303. [CrossRef] [PubMed]

44. Vulevic, J.; Juric, A.; Walton, G.E.; Claus, S.P.; Tzortzis, G.; Toward, R.E.; Gibson, G.R. Influence of galacto-oligosaccharide mixture (B-GOS) on gut microbiota, immune parameters and metabonomics in elderly persons. *Br. J. Nutr.* **2015**, *114*, 586–595. [CrossRef] [PubMed]
45. Walton, G.E.; van den Heuvel, E.G.H.M.; Kosters, M.H.W.; Rastall, R.A.; Tuohy, K.M.; Gibson, G.R. A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *Br. J. Nutr.* **2012**, *107*, 1466–1475. [CrossRef]
46. Woodmansey, E.J.; McMurdo, M.E.T.; Macfarlane, G.T.; Macfarlane, S. Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Appl. Environ. Microbiol.* **2004**, *70*, 6113–6122. [CrossRef]
47. Rondanelli, M.; Giacosa, A.; Faliva, M.A.; Perna, S.; Allieri, F.; Castellazzi, A. Review on microbiota and effectiveness of probiotics use in older. *World J. Clin. Cases* **2015**, *3*, 156–162. [CrossRef]
48. Nowak, A.; Czyżowska, A.; Huben, K.; Sójka, M.; Kuberski, S.; Otlewska, A.; Śliżewska, K. Prebiotics and age, but not probiotics affect transformation of 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ) by faecal microbiota–An in vitro study. *Anaerobe* **2016**, *39*, 124–135. [CrossRef]
49. Duncan, S.H.; Flint, H.J. Probiotics and prebiotics and health in ageing populations. *Maturitas* **2013**, *75*, 44–50. [CrossRef]
50. Saffrey, M.J. Aging of the mammalian gastrointestinal tract: A complex organ system. *Age* **2014**, *36*, 1019–1032. [CrossRef]
51. Patel, R.; DuPont, H.L. New approaches for bacteriotheraphy: Prebiotics, new-generation probiotics, and synbiotics. *Clinic. Inf. Dis.* **2015**, *60*, 108–121. [CrossRef]