Dietary Isomalto/Malto-Polysaccharides Increase Fecal Bulk and Microbial Fermentation in Mice

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Scope: The prevalence of metabolic-syndrome-related disease has strongly increased. Nutritional intervention strategies appear attractive, particularly with novel prebiotics. Isomalto/malto-polysaccharides (IMMPs) represent promising novel prebiotics that promote proliferation of beneficial bacteria in vitro. The present study investigates for the first time the in vivo effects of IMMP in mice.

Methods and results: C57BL/6 wild-type mice received control or IMMP-containing (10%, w/w) diets for 3 weeks. IMMP leads to significantly more fecal bulk (+26%, $p < 0.05$), higher plasma non-esterified fatty acids (colorimetric assay, +10%, $p < 0.05$), and lower fecal dihydrocholesterol excretion (mass spectrometry, −50%, $p < 0.05$). Plasma and hepatic lipid levels (colorimetric assays following lipid extraction) are not influenced by dietary IMMP, as are other parameters of sterol metabolism, including bile acids (gas chromatography/mass spectrometry). IMMP is mainly fermented in the cecum and large intestine (high-performance anion exchange chromatography). Next-generation sequencing demonstrates higher relative abundance of *Bacteroides* and butyrate producers (*Lachnospiraceae, Roseburia Odoribacter*) in the IMMP group.

Conclusion: The combined results demonstrate that IMMP administration to mice increases fecal bulk and induces potentially beneficial changes in the intestinal microbiota. Further studies are required in disease models to substantiate potential health benefits.

1. Introduction

Increased consumption of "Western-style" diets and a sedentary lifestyle are considered major contributing factors to the increasing prevalence of the metabolic syndrome, comprising obesity, insulin resistance, type 2 diabetes, hypertension, non-alcoholic fatty liver disease, and cardiovascular disease.[1] Epidemiological studies have indicated a relationship between low dietary fiber intake and risk of developing metabolic syndrome.[2,3] Whether the epidemiologically beneficial effects of fiber intake are a result of lower glycemic index, reduced energy content or the fact that dietary fibers stimulate fermentation by gastrointestinal (GI) microbiota is currently not clear. Nevertheless, the American Heart Association recommends a daily dietary fiber intake of 25–38 g d^{-1} (14 g per 1000 kcal d^{-1}).[4,5] Accumulating evidence on the potential benefits of dietary fibers has generated an increasing interest in foods that are low in glycemic index, slowly degraded, or completely escape digestion.

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Isomalto/malto-polysaccharides (IMMP) are a novel class of dietary fibers with a prebiotic potential.[6,7] IMMP contain a high proportion of α-(1→6) glycosidic linkages. Earlier studies showed that IMMP can stimulate proliferation and activity of *Bifidobacterium* and *Lactobacillus* during in vitro fermentation using adult human fecal inoculum as a microbial source.[7,8] In vitro studies showed that this modulatory effect on microbial communities was accompanied by the accumulation of succinate and short-chain fatty acids (SCFA), in particular acetate and propionate in the media.[8] These studies, as well as research on similar substrates, such as dextran and isomalto-oligosaccharides (IMOs), suggest that IMMP may have beneficial effects on metabolism and health.[9] However, currently no in vivo data are available supporting this assumption. Therefore, we designed the first in vivo study to investigate the effects of a particular IMMP in a mouse model. First, we aim to provide a thorough characterization of the impact of IMMP on the composition of the microbiota in different parts of the intestine. In addition, we chose to investigate effects of IMMP on (chole)sterol metabolism, which has high relevance with respect to cardiometabolic disease and integrates metabolic pathways of host and microbiome. Cholesterol is an important biomarker of cardiovascular disease.[10] The cholesterol molecule has a steroid nucleus that cannot be degraded by mammalian enzymes, hepatocytes can only metabolize cholesterol into primary bile acids.[11] Intestinal bacteria on the other hand can synthesize coprostanol and di-hydro-cholesterol out of cholesterol, poorly absorbable metabolites that promote cholesterol excretion from the body and thereby result in a depletion of the cholesterol pool.[12] Further, the microbiota can via bacterial bile salt hydrolase deconjugate primary bile acids and then further dehydroxylate, oxidize, and epimerize these into secondary bile acids.[11] Secondary bile acids are more efficiently excreted, thereby also favoring a depletion of the cholesterol pool as cholesterol is the parent molecule. In addition, bile acids play an important regulatory role in the control of glucose and lipid metabolism via signaling through, e.g., the nuclear receptor farnesoid X receptor (FXR) or the G-protein-coupled receptor TGR5.[11] Characterizing (chole)sterol metabolism therefore can reveal (patho)physiologically important read-outs that integrate diet, the metabolic activity of the microbiota and mammalian metabolic pathways.

2. Materials and Methods

2.1. Animal Experiments

C57BL/6OlaHsd female mice were obtained from Harlan (Horst, the Netherlands). All mice were 9 weeks old at the start of the dietary intervention. Animals were individually housed in a light- and temperature-controlled facility (12 h light–dark cycle, 21 °C). All animal experiments were approved by the Committee of Animal Experimentation at the University of Groningen (#6905AC) and performed in accordance with the Dutch National Law on Animal Experimentation as well as international guidelines on animal experimentation. IMMP-94 contained 94% α-(1→6) linkages (Avebe, Veendam, the Netherlands), was made by enzymatic conversion of potato starch using the GTFB 4,6-α-glucanotransferase enzyme purified from *E.coli* and pullulanase

(Promozyme D2, Novozymes, Bagsvaerd, Denmark) as described previously[7] and kindly supplied by Dr. Hans Leemhuis (Avebe). Animals were fed ad libitum with either control ($n = 6$) or IMMP (10% w/w, $n = 6$) supplemented diet for 21 days. Control baseline diet (Safe Diets, Augy, France) contained 60.94% corn starch, 0.06% cholesterol, 20% caseinate, 0.3% L-cystine, 7% carbohydrate mix (sucrose:maltodextrin, 50:50), 7% soya bean oil, 0.2% choline bitartrate, 3.5% mineral mixture, and 1% vitamin mixture (w/w). A modified diet containing 10% IMMP was obtained by replacing an equal amount of corn starch (50.94% corn starch, 0.06% cholesterol, 20% caseinate, 0.3% L-cystine, 7% carbohydrate mix, 7% soya bean oil, 0.2% choline bitartrate, 3.5% mineral mixture, and 1% vitamin mixture).

Fecal samples were freshly collected from the animals at time points 0, 24, 48 h, 3, 7, 14, and 21 days and stored at −80 °C until further analysis. Animals were weighed weekly. Food intake and fecal output were recorded on days 7, 14, and 21. At day 21, the gallbladder was cannulated under anesthesia (hypnorm 1 mg kg^{-1} body weight; diazepam 10 mg kg^{-1} body weight). Bile was collected continuously for 20 min and the rate of secretion was determined gravimetrically.[13] After termination, the GI tract was excised and the entire contents of stomach, small intestine, cecum, and large intestine were individually collected and immediately stored at −80 °C for later analysis.

2.2. Analysis of Bile, Plasma, and Liver

At the time of termination, a large blood sample was collected by heart puncture. Plasma was isolated and aliquots were stored at −80 °C until further analysis. Liver was excised at termination and homogenized. Extraction of lipids was performed from the homogenates using the Bligh and Dyer procedure and redissolved in water containing 2% Triton X-100.[13] Liver as well as plasma total cholesterol and triglycerides were measured using commercially available reagents (Roche, Diagnostic, Basel, Switzerland). Biliary bile acid and cholesterol concentrations were analyzed as described earlier.[14]

2.3. Fecal Sterol and Bile Acid Measurements

Feces were collected over a period of 24 h. Fecal samples were dried, weighed, and ground. A total of 50 mg of feces were used to extract neutral sterols and bile acids that were measured using gas–liquid chromatography as published.[15] Plasma bile acids were methylated with a mixture of acetyl chloride and trimethylsilylated with pyridine, N, O-Bis (trimethylsilyl) trifluoroacetamide, and trimethylchlorosilane. Plasma bile acids were then determined using liquid chromatography–mass spectrometry.[15]

2.4. DNA Extraction from Intestinal Content and Microbiota Analysis Using Next Generation Sequencing

Approximately 0.1 g of intestinal content sample were used for DNA extraction. Total bacterial DNA was extracted

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according to a previously described protocol with minor modifications.[8] Intestinal content samples were homogenized in 350 µL STAR buffer, with cooling at room temperature and the bead-beating step was repeated using 200 µL of fresh STAR buffer. The V4 region of 16S ribosomal RNA (rRNA) genes was amplified using uniquely barcoded primers 515F-n (5'-GTGCCAGCMGCCGCGGTAA-) and 806R-n (5'-GGACTACHVGGGTWTCTAAT) (200×10^{-9} м each).[16] Purified PCR products were pooled into libraries and sent for adapter ligation and HiSeq sequencing (GATC-Biotech, Konstanz, Germany). Data processing and analysis was carried out using NG-Tax.[16] In brief, libraries were filtered to contain only read pairs with perfectly matching barcodes that were subsequently used to separate reads by sample. Operational taxonomic units (OTUs) were assigned using an open reference approach and the SILVA_111_SSU 16S rRNA gene reference database (https://www.arb-silva.de/).[17]

2.5. Analysis of Fecal Short-Chain Fatty Acids

Feces were kept frozen before being processed for SCFA analysis. Approximately 50 mg of feces were mixed with 0.35 mL of 50 mmol L^{-1} sulfuric acid and 0.025 mL of 4 mg mL^{-1} 2-ethylbutyric acid. The mixture was homogeneously suspended by vortex mixing in the presence of glass beads (rinsed with Millipore water beforehand) in an Eppendorf tube. Subsequently the samples were centrifuged for 20 min at $18\,600 \times g$ and 4 °C, and the supernatant was analyzed by the high performance liquid chromatography–refractive index (HPLC–RI) method published previously.[18] The dry matter content of mouse feces was estimated by comparing the weight differences before and after freeze drying the feces.

2.6. Analysis of Oligosaccharide Profiles of Murine Digesta by HPAEC-PAD

The digesta samples from stomach, small intestine, cecum, and large intestine of six mice each from the control and the IMMP diet groups were freeze dried and then mixed in Millipore water at a concentration of 2.5 mg mL^{-1}. After mixing thoroughly by vortex, the tube containing the suspension was put in boiling water for 5 min and then centrifuged at $18\,600 \times g$ for 20 min. The supernatant was taken and analyzed by high performance anion exchange chromatography–pulsed amperometric detection (HPAEC–PAD). Ten microliters of sample was injected into a Dionex ICS 5000 system (Dionex) with a CarboPac PA-1 column (250 mm × 2 mm inner diameter (ID)) and a CarboPac PA guard column (25 mm × 2 mm ID). The temperature of the column was set at 20 °C. The flow rate of the two mobile phases (A) 0.1 м NaOH and (B) 1 м NaOAc in 0.1 м NaOH was set to 0.3 mL min^{-1}. The gradient elution was applied as follows: 0–40 min, 0–40% B; 40–40.1 min, 40–100% B; 40.1–45 min, 100% B; 45–45.1 min, 100–0% B; 45.1–60 min, 0% B. PAD (Dionex ISC-5000 ED) was used to monitor elution. HPAEC data were processed using ChromeleonTM 7.1 software (Dionex). Glucose, isomaltose, and maltodextrin standards, as well as an IMMP di-

gest treated with pure dextranase from *C. Erraticum*, were prepared and included in the HPAEC analysis, to identify oligosaccharide peaks as detailed previously.[8]
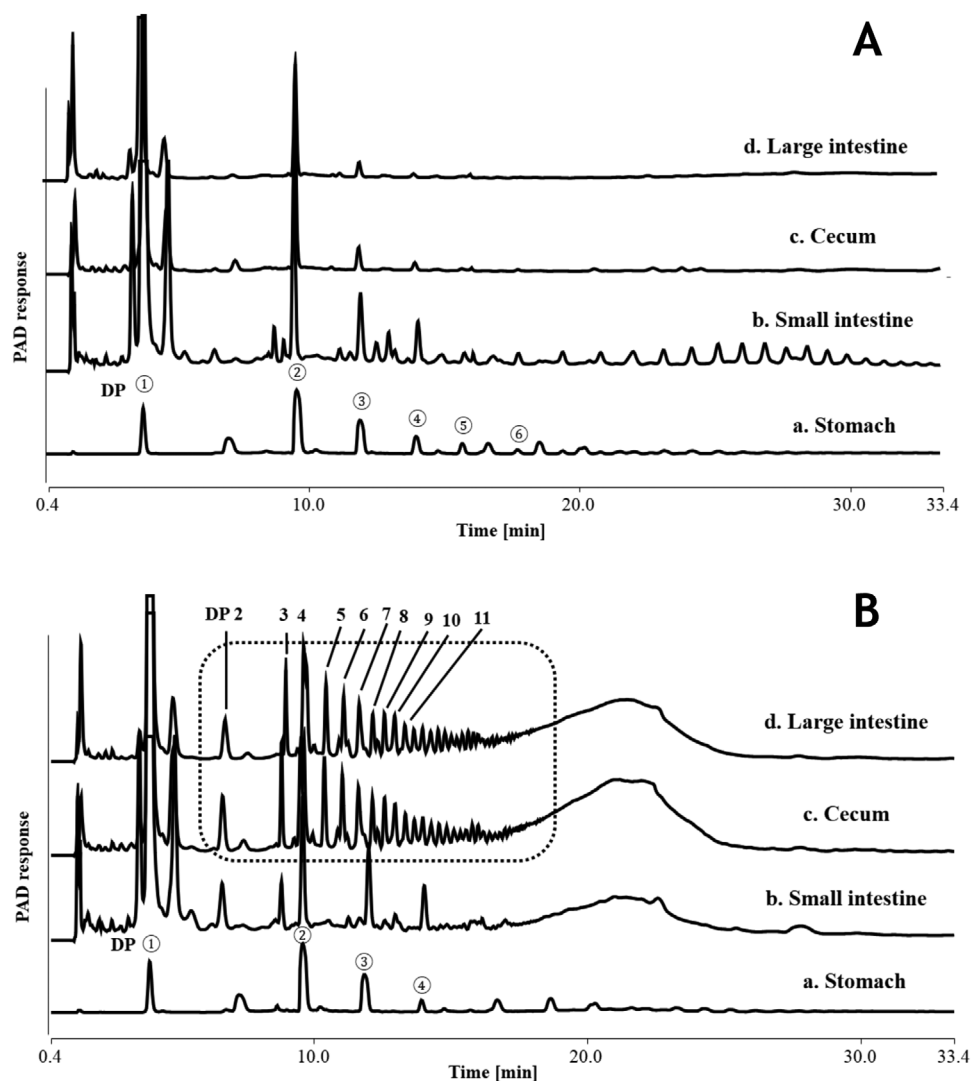
2.7. Statistics

Statistical analysis on metabolic parameters measured in plasma, liver, and feces was performed using GraphPad Prism software (San Diego, CA). All data are presented as mean ± SEM. Statistical differences between groups were assessed using the Mann–Whitney U-test. Statistical significance for all comparisons was assigned at $p < 0.05$. Microbial composition data were expressed as a relative abundance of each genus level taxon obtained with NG-Tax. A 5000 reads per sample rarefraction cut-off was used in alpha diversity indices (Shannon, Chao1, and PD Whole Tree). Calculations and group comparisons were done using nonparametric two-sample t-tests with Monte Carlo permutations in QIIME.[19] The association between microbiota composition and the dietary treatment group was investigated with RDA analysis in Canoco5, with significance assessed using a permutation test.[20] Beta diversity analysis, including weighted and unweighted unifrac distances estimates, and ANOSIM group comparisons were calculated in QIIME using rarefied data. Genus level taxa that differed significantly between different treatment groups were identified with Kruskal–Wallis analysis using QIIME.[19,21]

3. Results

3.1. IMMP Utilization along the GI Tract

The digesta from different parts of the GI tract of mice fed during a period up to 21 days with either control or IMMP containing diet were analyzed by HPAEC–PAD, in order to monitor the degradation of these fiber polymers from the diets and the formation of oligosaccharides. The figure depicts representative changes reproducibly observed in each individual animal investigated. α-1-4-linked maltodextrin peaks were present in the stomach and small intestine digesta of animals from both dietary treatment groups (**Figure 1**). These maltodextrin peaks were products of starch digestion by murine digestive enzymes. Differences were also noticed in the small intestine between the two groups of mice: the isomaltose peak (elution at ≈6 min) and a broad peak (17–24 min) were only present in the mice receiving IMMP supplemented diets. The broad peak corresponded to the unseparated IMMP polymer fraction, whereas the isomaltose peak indicated ongoing microbial fermentation at a very low level. When comparing the digesta of cecum and large intestine between the two groups, a series of separated α-1-6-linked isomalto-oligosaccharide peaks were clearly seen in the IMMP mice, whereas hardly any carbohydrate peak was detected in digesta of control mice. The observation that there were no substantial differences between cecum and large intestine suggests that the major IMMP degradation by bacterial dextranases occurs in the cecum. Looking to individual peak heights, a slight further degradation of the broad peak at 17–24 min and an almost equal

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Figure 1. High performance anion exchange chromatography (HPAEC) elution patterns of digesta from mice fed with A) control and B) IMMP supplemented diets. The digesta are taken from different parts of the gastrointestinal tract: a) stomach, b) small intestine, c) cecum, and d) large intestine. IMMP peaks (2–11) in a box and maltodextrin peaks (①–⑥) are annotated, with the number indicating the degree of polymerization (DP).
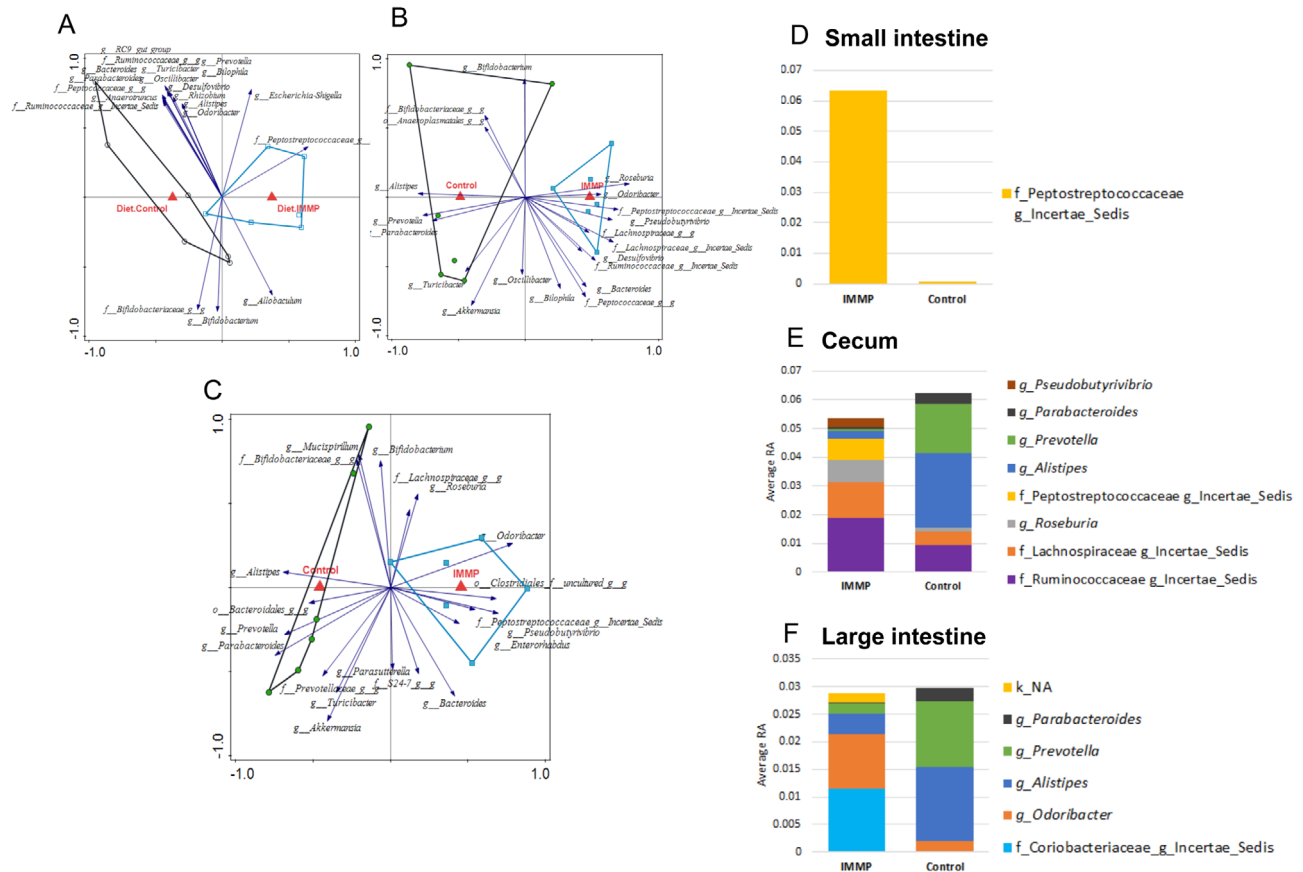
level of oligosaccharides present points to an enduring utilization of the IMMP structures. Please note that the glucose contents in these profiles are difficult to be quantitatively compared, since released glucose is rapidly utilized. Some remaining isomalto-oligosaccharides were still present in the fecal samples (result not shown). The release of the isomalto-oligosaccharides indicated the degradation of the polysaccharides during microbial fermentation of IMMP, and this fermentation took mainly place in the cecum and large intestine, although low level fermentation activity was also already detected in the small intestine.

3.2. Changes in GI Microbiota Composition

The total number of sequencing reads obtained for the 60 samples was 11 440 993 (min = 1643, max = 564 991, median =

173 066.5, mean = 190 683.2, SD = 130 379.2). Samples were rarefied at 5000 reads per sample depth prior to alpha diversity analyses. Alpha diversity estimates included Shannon diversity, Chao1, and PD Whole Tree; however, no significant differences were detected in any of the measures between control and treatment groups (Figure S1, Supporting Information).

The RDA analysis of fecal microbiota on day 2 showed that diet could explain 10.6% of the variation in the relative abundance of genus level taxa, however, the difference between the control and IMMP groups was not significant (FDR = 0.252, data not shown). At day 21, contents from different parts of the murine GI tract were used for detailed microbiota analyses. In small intestine digesta, diet explained 12.1% of the microbiota variation, but the difference was not statistically significant (FDR = 0.208, **Figure 2**A). In cecum, however, diet explained 20.3% of the variation and this effect was significant (FDR = 0.014, Figure 2B). Finally, diet significantly explained 16.8% of

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Figure 2. Constrained analysis (RDA) in the control and IMMP supplemented diet groups and their association with the genus level microbiota profiles in different parts of the gastrointestinal tract. The samples were collected from A) small intestine, B) cecum, and C) large intestine. Samples are labeled and enveloped based on their assignment to different treatment groups. Genus-level taxa that significantly differed in their relative abundance between control and IMMP supplemented diet groups in D) small intestine, E) cecum, and F) large intestine. When the taxonomic assignment could not be made at genus level, the lowest classifiable taxonomy assignment is used instead, and unidentified genus is indicated with "g_g."

the microbiota variation at genus level classification in large intestine samples (FDR = 0.04, Figure 2C).

In the small intestine, 22 genus level taxa were detected in the IMMP group, and additional 19 taxa were found in the control group. The 19 taxa had low relative abundance and together contributed to 1.7% of the total bacteria detected in the small intestine of the control animals. ANOSIM analysis showed no significant difference between treatment groups when weighted or unweighted unifrac distances were used ($p = 0.47$ and $p = 0.11$, respectively). Kruskal–Wallis analysis showed no statistically significant differences in the relative abundance of genus level taxa between the two treatment groups (FDR adjusted $p > 0.05$). When unadjusted p-values were used a just significant result was detected for Peptostreptococcaceae_*Incertae_Sedis* ($p = 0.049$), which was 114 times more abundant in the IMMP group compared to the control (Figure 2D). In addition, animals receiving IMMP diet had on average an 11 times lower relative abundance of genus *Enterococcus*, 19 times lower *Akkermansia*, 24 times lower *Bacteroides*, and 50 times less *Turicibacter* compared to the control group (Table S1, Supporting Information). Overall, these four taxa accounted for a cumulative relative abundance of 15.7% in the control group, and only 0.7% in the IMMP group. There were no differences in the average relative abundance of *Bifi-*

dobacterium and *Lactobacillus* between the IMMP and the control group.

In cecum, 37 genus level groups were detected with four groups found only in the IMMP-fed animals and four other taxa detected only in the control group. ANOSIM analysis showed significant differences between treatment groups when weighted or unweighted unifrac distances were used ($p = 0.01$, for both). Kruskal–Wallis analysis showed no statistically significant differences between relative abundances of individual genus level taxa between the two treatment groups (FDR > 0.05, Figure 2E), but based on unadjusted p-values, we identified differences in the relative abundance of genera *Alistipes*, *Prevotella*, *Roseburia*, *Pseudobutyrivibrio*, *Parabacteroides*, and *Incertae Sedis* in families Peptostreptococcaceae, Ruminococcaceae, and Lachnospiraceae (each $p < 0.05$). Compared to the control group, the IMMP group had a fivefold higher average relative abundance for *Roseburia*, and a 26-fold lower relative abundance of *Prevotella*, 13-fold lower *Akkermansia*, and ninefold lower *Alistipes* and *Parabacteroides*. In addition, the IMMP treated animals had on average a twofold lower relative abundance of *Bifidobacterium*, and threefold higher relative abundance of *Lactobacillus* as compared to the control group, however, these differences were not statistically significant (Table S1, Supporting Information).

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In the large intestinal samples, 40 genus level taxa were detected, of which four were only found in the control animals and eight were only found in the IMMP group. ANOSIM analysis showed no significant difference between treatment groups when weighted unifrac distances were used ($p = 0.1$), however, the difference was significant when unweighted distances were compared ($p = 0.01$). Kruskal–Wallis analysis showed no statistically significant differences between the two treatment groups (FDR > 0.05, Figure 2F), however unadjusted p-values indicated differences in the relative abundance of *Odoribacter, Parabacteroides, Prevotella, Alistipes,* family Peptostreptococcaceae genus *Incertae Sedis*, and uncultured genus within the order Clostridiales. Compared to the control group, the IMMP animals showed a 11-fold lower relative abundance of *Parabacteroides* and *Turicibacter*, eightfold lower *Akkermansia*, a sevenfold lower level of unidentified genus within the order Bacteroidales, and a fivefold higher relative abundance of *Odoribacter*. The IMMP animals also had on average a two times lower relative abundance of *Bifidobacterium*, and a three times higher relative abundance of *Lactobacillus*, however, these differences were not statistically significant ($p = 0.87$ for both taxa).

3.3. SCFA Production in IMMP-Fed Mice

SCFA are among the major products of bacterial fermentation. Our analyses of SCFA and other acids in feces showed that average succinic and lactic acid production increased in both groups at day 2 and 3, and decreased thereafter, except on day 14 when an increase in lactic acid ($p < 0.05$) in feces from the IMMP group was noted (**Figure 3**A,B). Propionic acid concentrations decreased throughout the duration of the study, while the levels of butyric and acetic acid remained stable (Figure 3C–E). A significantly lower level of propionic acid was seen in IMMP fed mice on day 21 ($p < 0.05$) and a lower level of lactic acid on day 2 ($p < 0.05$). However, overall, no consistent differences in these parameters were observed, especially no substantial increase in fecal SCFA, lactic, and succinic acid levels in response to IMMP.

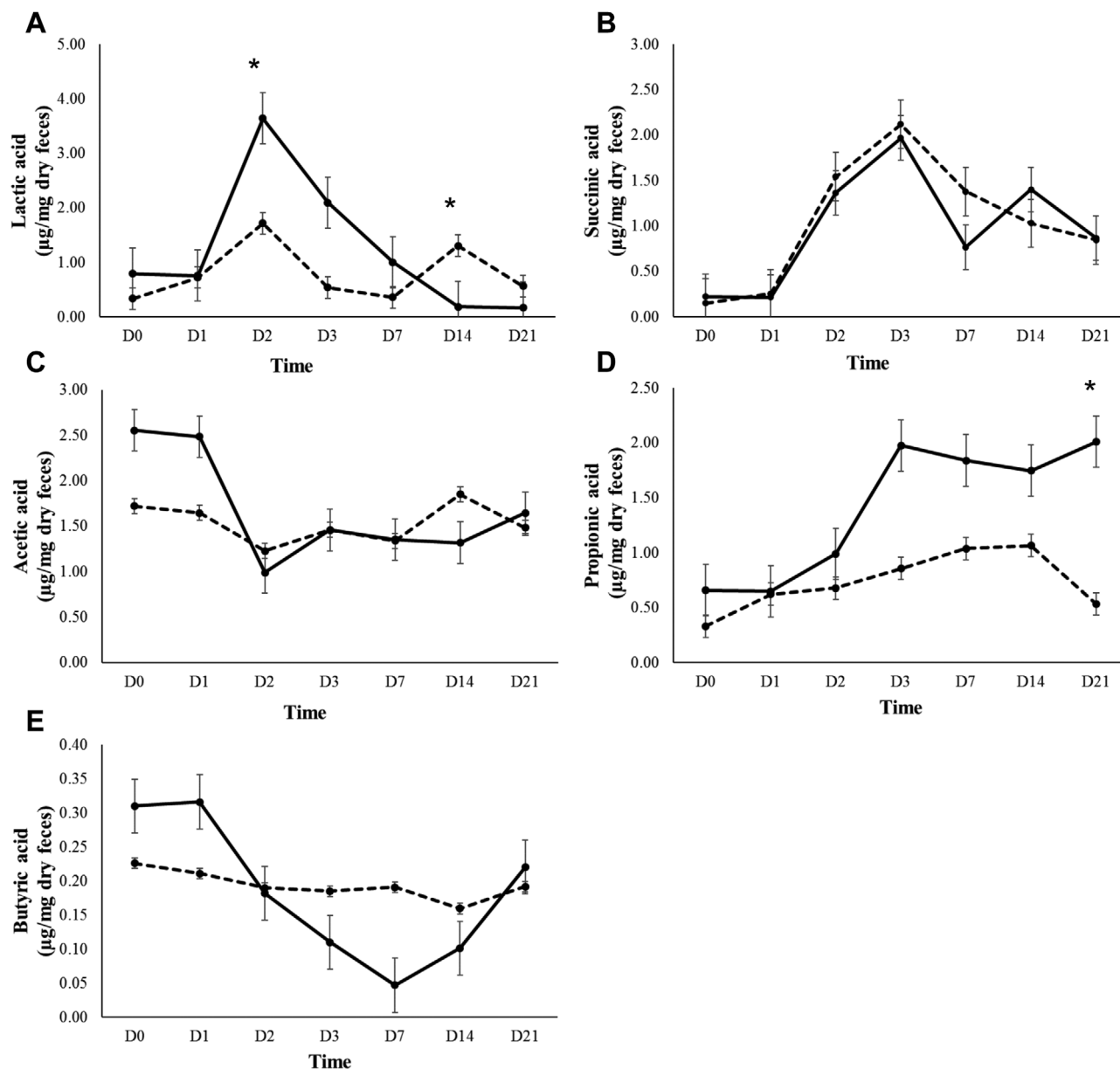### 3.4. Metabolic and Physiological Responses to IMMP

To study the potential physiological effects of the IMMP-derived SCFA, we investigated the impact of the different diets on lipid metabolism (**Table 1**). First, body weight and food intake in both groups remained stable throughout the dietary intervention period (Table 1). Plasma non-esterified fatty acids (NEFA) were higher (+8%, $p < 0.05$) in IMMP-fed mice compared with controls. Liver weight was unaffected and also liver cholesterol and triglyceride contents remained unchanged in either group. Also, the biliary excretion of bile acids and cholesterol did not change upon IMMP feeding. At the end of the dietary intervention, the IMMP-supplemented group had a significantly higher fecal bulk mass (+35%, $p < 0.05$) and lower levels of fecal dihydrocholesterol (−50%, $p < 0.05$), a product of bacterial metabolism. Fecal total neutral sterol and bile acid excretion remained unchanged. In terms of bile acid species in feces, control and IMMP-fed mice had a similar composition (**Figure 4**). Throughout the duration

of the experiment the fecal water content did not vary between the treatment groups, except on days 3 and 14, when it was significantly ($p < 0.05$) higher in the control group (d = 3: control = 14.0 ± 2.6%; IMMP = 10.5 ± 3.3% and d = 14: control = 20.9 ± 10.2%; IMMP = 10.2 ± 2.7%).

4. Discussion

This study is to the best of our knowledge the first to investigate in vivo properties and related physiological effects of IMMP supplementation in a murine model. Our data demonstrate that in mice fed IMMP-supplemented diet, the fermentation of IMMP occurred mostly in the cecum and large intestine and only at a very low level in the small intestine. This observation is in line with the changes observed in the microbiota composition in these regions of the GI tract. Beta diversity analysis with both weighted and unweighted unifrac distances, as well as genus level relative abundance based RDA analysis all showed that IMMP supplementation had a significant effect on microbiota composition in cecum and large intestine, but not in the ileum. In the large intestine, however, the significant effect was not detected when using weighted unifrac distances, suggesting that the significance was mostly due to changes in the composition of low abundance taxa.[22] In cecum, IMMP diet resulted in significantly higher levels of Lachnospiraceae *Incertae Sedis* ($p < 0.05$), and, although not statistically significant, increases in a highly abundant related genus within the Lachnospiraceae family (unidentified) and in *Bacteroides*. The increase in *Bacteroides* was also observed in the large intestine and this finding was in line with an earlier in vitro study showing that IMMP fermentation can be linked with an increase in both, the relative abundance and activity of *Bacteroides*.[8] An increased abundance of *Bacteroides* is largely regarded as a beneficial effect, based on data demonstrating a strong association between decreased *Bacteroides* and obesity as well as metabolic disease.[23] In the cecum, IMMP was further associated with a significant increase in *Roseburia*, and in both, cecum and large intestine, we detected a higher relative abundance of *Odoribacter*. *Lachnospiraceae*, and *Roseburia* are known to be saccharolytic groups associated with high fiber diets, while *Odoribacter* is largely asaccharolytic.[24,25] Lachnospiraceae, *Roseburia*, and *Odoribacter* are important producers of butyrate. Butyrate is a relevant metabolite produced by the gut microbiota that has been implicated in improving metabolic control, as well as having inhibiting effects on cancer cell growth, largely via inhibition of histone deacetylases (HDAC).[26,27] Further, butyrate upregulates the expression of endogenous host defense peptides in the gut and increases energy expenditure by activating brown adipose tissue.[28,29] Fecal analysis, though, did not reveal a significant increase in butyrate excretion in IMMP-fed mice, a finding likely attributable to the highly efficient uptake of this SCFA into colonocytes.[30,31]

In stomach and small intestine, we observed α-1-4-linked maltodextrins that were the main products of starch digestion by murine digestive enzymes. This was in line with previous in vitro studies in which small intestine extracts from rats were incubated with IMMP.[7] Given that IMMP represents a novel class of dietary fiber, it is essential to identify groups of bacteria that are promoted by dietary IMMP in vivo. Our study is the first

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Figure 3. Short-chain fatty acid profiles in murine feces from day 0 to day 21. In fecal samples, A) lactic acid, B) succinic acid, C) acetic acid, D) propionic acid, and E) butyric acid were measured. Solid line, control group; dashed line, IMMP group. $^{*}p < 0.05$.

to use next generation sequencing of PCR-amplified 16S rRNA genes to systematically and comprehensively explore the relative abundance of various groups of bacteria in different parts of the intestine of IMMP-fed mice. Previous in vitro studies with human fecal inoculum incubated with IMMP have shown increases in *Bifidobacterium* and *Lactobacillus*.[7,8] These strains are considered probiotic microorganisms as they confer health benefits on the host via generation of key metabolites such as SCFA. However, in the current study it appeared that while the relative abundance of *Lactobacillus* increased upon IMMP feeding, bifidobacteria were relatively reduced in their relative abundance, although it should be noted that none of these changes in the relative abundances were statistically significant. Interpreting these findings,

one needs to take into account that the murine gut ecosystem is different from that of the human GI tract. Therefore, further human studies seem warranted to corroborate these results.

Mammals lack digestive enzymes for degrading dietary fibers. In rodents, even though certain microbial communities are present in stomach and small intestine, large intestine and, importantly, cecum are the most active fermentation sites.[32] Thus, most dietary fibers pass through the upper gastrointestinal tract and are fermented in cecum and large intestine. Fermentation results in generation of multiple groups of metabolites such as intermediate acids as lactic and succinic acid and final metabolites as SCFA. SCFA can regulate several pathways related to lipid and glucose metabolism.[33–35] The present study followed the

Table 1. Animal characteristics, plasma, liver, and fecal parameters of lipid metabolism in mice fed control or IMMP diets. Values are shown as means per group ± SDs.

	Control Diet	IMMP Diet
Animal characteristics		
Body weight (d = 0)	19.48 ± 1.38	20.28 ± 0.66
Body weight at termination (d = 21)	19.93 ± 1.07	20.15 ± 1.00
Food intake [g d^{-1}]	3.18 ± 0.22	3.26 ± 0.21
Plasma		
Cholesterol [mmol L^{-1}]	2.32 ± 0.67	2.47 ± 0.17
Triglycerides [mmol L^{-1}]	0.31 ± 0.16	0.36 ± 0.14
NEFA$^{c)}$ [mmol L^{-1}]	1.01 ± 0.05	1.10 ± 0.03$^{a)}$
Total plasma bile acids, μmol·L^{-1}	3.05 ± 1.25	5.49 ± 3.23
Liver		
Liver weight (d = 21)		
-absolute [g]	0.80 ± 0.11	0.87 ± 0.07
-relative [% of body wt]	4.02 ± 0.47	4.32 ± 0.44
Triglycerides [nmol mg^{-1} liver]	21.31 ± 4.24	20.26 ± 7.23
Cholesterol [nmol mg^{-1} liver]	10.24 ± 3.31	9.30 ± 1.30
Bile flow [μL min^{-1} 100 g body wt^{-1}]	9.17 ± 2.21	9.34 ± 2.31
Biliary BA secretion [μmol d^{-1} 100 g body wt^{-1}]	34.06 ± 9.75	40.04 ± 5.46
Feces		
Feces (dry) [mg d^{-1} 1 g body wt^{-1}]	5.94 ± 0.49	8.05 ± 1.03$^{a)}$
Fecal Coprostanol [μmol d^{-1}]	0.95 ± 0.46	0.76 ± 0.58
Fecal Cholesterol [μmol d^{-1}]	1.55 ± 0.13	1.65 ± 0.34
Fecal DiH-Chol$^{b)}$ [μmol d^{-1}]	0.23 ± 0.03	0.18 ± 0.03$^{a)}$
Total fecal neutral sterols [μmol d^{-1}]	2.72 ± 1.69	2.60 ± 0.45
Total fecal bile acids [μmol d^{-1}]	2.67 ± 0.53	1.17 ± 1.40

$^{a)}$ $p < 0.05$; $^{b)}$ DiH-Chol, dihydrocholesterol; $^{c)}$ NEFA, nonesterified fatty acids.
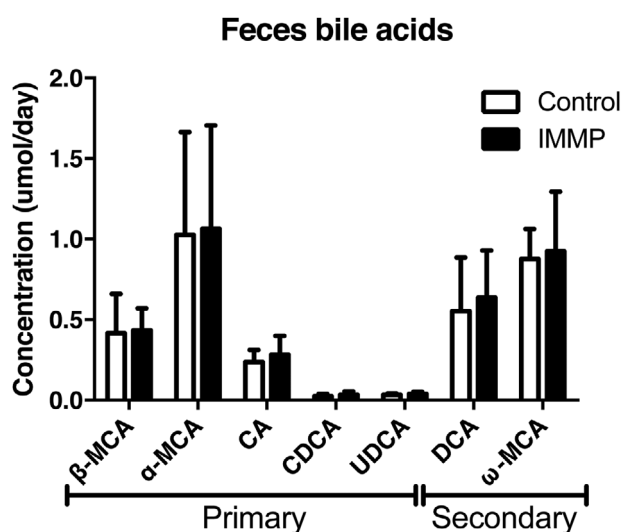
Figure 4. Bile acid composition in feces of control and IMMP-fed mice. At day 21, fecal samples were collected and processed for bile acid analysis as detailed in methods. Abbreviations: MCA, muricholic acid; CA, cholic acid; CDCA, chenodeoxy-cholic acid; DCA, deoxycholic acid; UDCA, ursodeoxy-cholic acid.

production of specific SCFA over several time periods. Subtle differences between both groups were observed for most SCFA and their precursors, such as lactic and succinic acids. At the end of the dietary intervention significantly lower propionic acid concentrations were detected in IMMP-fed animals. Propionic acid has been shown to attenuate lipid biosynthesis in the liver.[36,37] However, the unchanged hepatic lipid content observed in our study argues against a physiological significance of this result. On the other hand, lactic acid that is normally produced endogenously in high concentrations upon exercise in host muscles but only in low concentration in the intestine, is an intermediate of bacterial fermentation and can be used by some bacteria, together with acetic acid, to synthesize butyric acid. In adipose tissue, lactic acid has been shown to have a signaling function, being a natural ligand for GPR81 thereby inhibiting lipolysis.[38] At the end of the dietary intervention, higher lactic acid levels were detected in IMMP-fed mice compared to the control group. However, it is also important to point out that an increase in luminal SCFA does not always reflect uptake of SCFA by the host and the subsequent induction of metabolic effects.[39] Thus, it is possible that the uptake of SCFA is different in both groups that needs to be further investigated, ideally using animal models with the capacity to resemble human metabolic disease states.

Cholesterol homeostasis is maintained by a balance between cholesterol intake, cholesterol synthesis in the host, absorption of cholesterol in the intestine, and the removal of cholesterol via the feces. Cholesterol is also a key biomarker of cardiovascular disease. Gut bacteria can reduce cholesterol to its derivatives, such as dihydrocholesterol and coprostanol, which are not absorbed by the host, but largely excreted into the feces. In the present study a small but significant decrease in fecal dihydrocholesterol output was noted after feeding the IMMP diet. However, since dihydrocholesterol represents only a minor fraction of total fecal neutral sterol output, overall fecal neutral sterol excretion upon IMMP administration remained unaltered. The intestinal microbiota also plays an important role in modulating bile acids and thereby subsequently cholesterol turnover.[40] However, the present study shows that IMMP-fed mice have no substantial alterations in the respective composition of plasma, biliary, and fecal bile acids. Fecal bile acid profiles integrate endogenous synthesis and modifications by bacterial enzymes suggesting that based on our results no major effects either in the host or in microbial communities involved in bile acid metabolism are discernible.

A significant increase was seen in the total fecal output of IMMP fed mice, indicating improved fecal bulk in these animals. Bulk in the large intestine is associated with several beneficial effects such as stimulating defecation, diluting toxins, and distributing intracolonic pressure, while lower fecal weight is associated with constipation and colorectal cancer.[41–44] Since the total fecal neutral sterol and bile acid excretion remained comparable between the control and the IMMP fed groups, we believe that the changes in fecal bulk have no major impact on the (chole-)sterol balance. The overall weak metabolic response to IMMP supplementation might be explained by the fact that this dietary fiber is designed to function in the lower parts of the GI tract where it can be fermented by the microbiota. In mice, cecum and large intestine are the main fermentation sites, while the proximal intestine is metabolically more active.

In summary, IMMP supplementation increased fecal bulk and microbial fermentation in the intestine resulting in potentially beneficial alterations in microbiota composition without adversely impacting host metabolism. Subsequently, studies in disease models and humans are needed to investigate whether the intriguing changes observed here translate into actual health benefits.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

R.H.M., K.B., and F.G. contributed equally to this work. R.H.M. and K.B. were associated with study design, data acquisition, analysis and data interpretation, and drafting the article; F.G. was associated with data acquisition and analysis, and critical revision of the manuscript; H.A.S. and H.S. were associated with critical revision of the manuscript for important intellectual content. H.J.V. was associated with data interpretation and critical article revision for important intellectual content; U.J.F.T. was associated with study design, data interpretation, and drafting the article. All authors read and approved the final manuscript.

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