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Modulation of gut microbiota and their metabolites by functional mulberry juice non-thermally pasteurized using microfiltration

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ABSTRACT

Background: Mulberry fruit is a source of phenolic compounds and has biological properties. This study aimed to assess the effects of isomaltooligosaccharide (IMO) added to mulberry juice on prebiotic activity and gut fermentation properties.

Objectives: The study aimed to produce mulberry juice supplemented with IMO, which might generate short chain fatty acids (SCFA), and to examine the effects of a prebiotic activity after fecal fermentation.

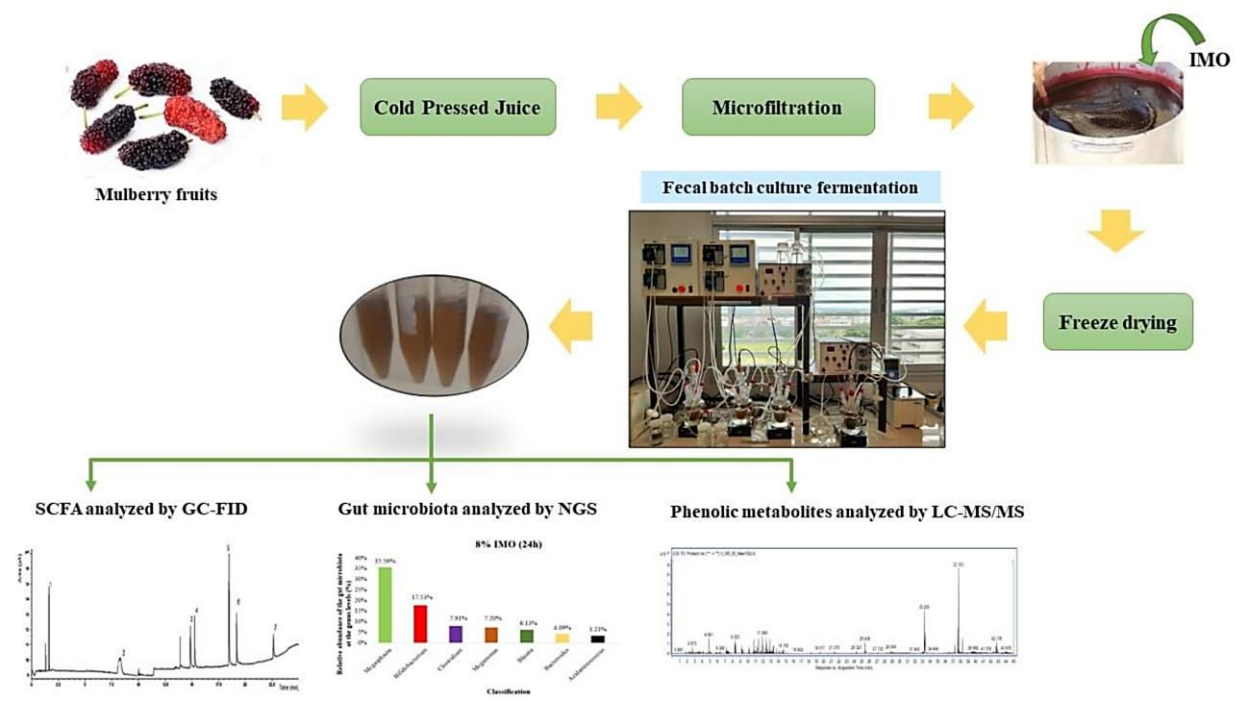
Materials and methods: Functional mulberry juices were prepared with three different levels of isomaltooligosaccharide (IMO), namely 0% (MBI0), 2% (MBI2), and 8% (MBI8). The study tested mulberry juices supplemented with IMO, which generated short-chain fatty acids (SCFA), phenolic metabolites, and favored beneficial gut bacteria, and examined the prebiotic activity after fecal fermentation by the colonic microbiota. A crossflow hollow fiber microfiltration system with a 0.22 µm pore size was employed to deliver permeates considered non-thermally pasteurized juices.

Results: The results show that short chain fatty acids (SCFA) included large propionic acid and butyric acid concentrations at 48h. The percent of bifidobacteria significantly increased to 5.03% and 17.53% in 24 hours fermentation of MBI2 and MBI8, respectively. After fecal batch culture fermentation, some anthocyanin metabolites such as 3-(2-hydroxyphenyl)

propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, and aminocaproic acid, were detected. Therefore, IMO can serve as a potential prebiotic ingredient added in mulberry juice for promoting the growth of beneficial gut microbiota.

Conclusion: The results show that IMO favored beneficial microorganisms in the gut and contributed to biologically active compounds such as metabolites of polyphenols and anthocyanins in the gut.

Keywords: Mulberry, isomaltooligosaccharide, prebiotic, gut microbiota, metabolites



INTRODUCTION

Mulberry (*Morus* spp.) belongs to the family Moraceae. It is grown worldwide in Europe, Africa, America, and Asia [1]. *Morus*, *Ficus*, and *Artocarpus* are genera studied extensively for their health benefits [2]. Zhu et al. [3] point out that every plant in the Moraceae family contains flavonoids. In Thailand, white and black mulberry is widely cultivated since they are easy to grow and produce high yields. Mulberry fruit is rich in nutritive and bioactive compounds, such as anthocyanins, rutin, quercetin, and vitamins, with concentrations dependent on the variety, cultivars, and maturity stage of the mulberry fruit**.** It has been reported that mulberry contains bioactive compounds, including anthocyanins, phenolic acids, and flavonoids, with anti-thrombotic, antioxidant, antimicrobial, and anti-inflammation properties [4]. Cyanidin-3-glucoside and cyanidin-3-rutinoside are the major anthocyanins [5]. Bioactive compounds are non-essential substances in diet or beverages but can control metabolic processes to have a net positive effect on health. They can be compounds current in food or by addition, fortification, or

supplementation. These bioactive compounds are phytochemicals commonly originating in plants, such as flavonoids, phenolic acids, alkaloids, saponins, polysaccharides, and others. Bioactive compounds from plants are classified under secondary metabolites, 'phytochemicals.' Bioactive compounds from animals may be observed in their entirety or in the form of hydrolysates, a mixture of peptides and amino acids. Bioactive compounds have been observed to help various biological activities that may be measured as beneficial to those by lowering the risk of or suffering from the disease [6]. The Functional Food Center (FFC) has distinct functional food (FF) as "natural or processed foods that contain biologically active compounds; which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and manage their symptoms". The FFC has advised a 16-step course for the induction of foods into the category of functional food consisting of: (1) Establishing a goal of the functional food product, (2) Determining relevant bioactive compounds, (3) Establishing the appropriate dosage of bioactive compounds, (4) Determining the specific pathway and mechanism of action, (5) Establishing relevant biomarker, (6) Chooses an appropriate food vehicle for bioactive compounds, (7) Provides preclinical studies on efficacy and safety, (8) Provides clinical trials for dosage, efficacy, and safety, (9) Creates a unique label that informs the consumers of the most effective way to consume the product, (10) Publications are submitted to peer-reviewed journals, preferably in open access, (11) Educates the general public, (12) Sends information to credible governmental agencies, such as the FDA, for approval, (13) Official establishment of the accredited functional food product, (14) Release the functional food product to the market. (15) Provides epidemiological studies. and (16) Provides after market research [7].

Similar to other juices, mulberry juice is extracted from the fruit. Itis usually processed into juice or concentrated juice to extend its shelf-life for commercial purposes. Generally, thermal pasteurization, sterilization, and concentration are used in juice processing. Recently, non-thermal pasteurization and concentration by membrane technology have been shown to be promising technology for fruit juice processing [8-10]. The major advantages of using this technology over conventional thermal processing include lesser degradation of heat-sensitive bioactive components and sensory properties and reduced energy consumption. The demand for functional drinks derived from juices is increasing by supplementing bioactive ingredients for increased health benefits. Prebiotics are a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota [11]. Isomaltooligosaccharides (IMO) are a type of prebiotics prepared from starch by microbial enzymatic transformation [12]. Structurally, isomaltooligosaccharides contain glucose oligomers with α-D-(1,6)-links, including isomaltose, panose, and isomaltotriose isomaltotetraose, isomaltopentaose, and higher oligosaccharides [13]. IMO can modulate the composition and metabolic activity of the gut microbiota, can be used as a sweetener, and improves the health of the host organism [13]. In patients, IMO is associated with a significant reduction in triglyceride and cholesterol levels [14]. Previous studies have discovered that the beneficial microorganisms *Bifidobacterium* and

Lactobacillus increase in fecal matter when IMO is supplemented [15].

In this study, functional mulberry juice supplemented with IMO was formulated and non-thermally pasteurized by microfiltration. The effects of such products on gut microbiota modulation and production of short-chain fatty acids (SCFAs) and metabolites of phenolic compounds were evaluated. A colonic system was experimentally simulated using pH-controlled batch culture fermentation with human fecal slurry to evaluate beneficial effects on bacteria in the colon. The gut microbiota was determined by next-generation sequencing (NGS) to assess the proportions in the gut microbiota community. Gas chromatography-flame ionization detection (GC-FID) was used to determine the amount of SCFA, and phenolic metabolites were analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Materials: All chemicals and reagents used for fecal batch culture fermentation were analytical grade purchased from Sigma-Aldrich. The isomaltooligosaccharide (IMO) at 63.84% concentration was obtained from the previous study. Mulberry fruits were supplied by Ban Suan Kaset Nai Fun (Na Mom District, Songkhla, Thailand). The mulberry fruits were washed with water and extracted using a fruit juice extractor. The extract was then filtered through a filter cloth to remove solids. The mulberry juice was obtained with a total soluble solid of 8°Brix. Functional mulberry juice was formulated by the addition of isomaltooligosaccharide (IMO) at specific concentrations (0, 2, and 8%). Hollow fiber microfiltration with membrane pore size 0.22 µm was employed to produce non-thermally pasteurized mulberry functional juice. Subsequently, the functional mulberry juice added IMO was further freeze-dried to obtain the powders.

Collection and preparation of fecal slurry: Samples of fresh feces were collected from four healthy donors to prepare the fecal slurry used as inoculum in the simulated colon system (batch culture). The inclusion and exclusion criteria for fecal donors were set. The donors had not consumed products containing prebiotics or probiotics nor received antibiotic treatment for at least 3 months before donating the feces and had no digestive system disease. The donors were healthy subjects between the ages of 25 and 45 years. The ethical clearance and consent were performed before collecting the feces. The donors collected the fresh feces in the morning. Fresh feces of the donors were put into an anaerobic chamber, combined, and put in a stomacher's bag lined with filler. The 0.1 M phosphate-buffered saline (PBS; pH 7.0) was used to dilute the fresh feces at a ratio of 1:10 (w/v) or 10% concentration. After that, the stomacher homogenized the fecal slurry for 2 minutes before it was inoculated into the sterile vessels.

Fecal batch culture fermentation: The medium was prepared by mixing 0.8 g peptone water, 0.045 g NaCl, 0.9 g yeast extract, 0.018 g KH_2PO_4, 0.018 g K_2HPO_4, 0.045 g $CaCl_2{\cdot}6H_2O$, 0.045 g $MgSO_4{\cdot}7H_2O$, 0.9 g $NaHCO_3$, 0.225 g bile salts, 0.225 g L-cysteine·HCl, 0.0225 g hemin, 0.9 mL Tween 80, 4.5 µL vitamin K, and 0.025% resazurin. The basal medium was sterilized at 121ºC for 15 minutes. The sterile vessel was filled with 90 mL of sterile basal medium and 1.0 g freeze-dried mulberry added IMO

sample in a sterile basal medium, then continuously stirred using a magnetic bar in anaerobic conditions by flushing with nitrogen gas throughout fecal fermentation. The pH in the vessel was controlled at 6.8±0.1 by a pH controller, and the temperature was controlled at 37ºC by circulating warm water into the double jacket of the vessel using a circulating water bath. These conditions were mimicked conditions in the human colon. The fecal slurry (10 mL) was filled into 90 mL of sterile basal media to obtain a final concentration of 1% (v/v). Fecal fermentation was performed for 48 h, and samples were taken at 0, 6, 12, 24, and 48 h of fermentation. Samples were analyzed of gut microbiota by 16S rRNA gene sequencing (next generation sequencing, NGS). Short-chain fatty acids (SCFAs) were analyzed by gas chromatography with a flame ionization detector (GC-FID). Moreover, phenolic metabolites were analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS). All samples were kept at -20ºC before analyses.

Short-chain fatty acids analysis by GC-FID: Samples (1125 µL) were centrifuged at 13,000 x *g* at 4ºC for 5 minutes. The supernatant was filtered through a nylon membrane filter (0.22 µm). Aliquots of the filtered supernatant were mixed with acetone in a 1:1 ratio. Acetic, propionic, and butyric acid were used for the standard curves of each SCFA. GC-FID analyzed short-chain fatty acid production. The GC column was HP-INNOWax of 30 m x 0.32 mm size with 0.25 µm thick film. The initial oven temperature was set to 60°C and was increased to 100°C in 2 minutes. Then the temperature was increased to 250°C in 5 minutes (at 15°C/minute). Helium (99.99%), hydrogen, and oxygen were used as carrier gases in the system for GC-FID. The total run time was 25.67 minutes. The total volume injected was 2 µL. Standard acetic, propionic, and butyric acid curves were used to determine the concentrations of SCFAs in the samples by comparing the peak areas (mAU*s) for samples at respective retention times.

Phylogenetic analysis: The bacterial DNA was compared to the NCBI database for 16S rRNA gene sequence. Then, the 16S rRNA gene sequences were aligned using ClustalW [16]. The maximum likelihood tree (Kimura two-parameter model, 100 replications) was constructed using MEGA software version 11 [17].

Determination of gut microbiota by next-generation sequencing (NGS): Samples were taken, and their DNA amount was measured to obtain an initial DNA of about 5 ng/µL for PCR. 1µM primer F (5 µL), 1µM primer R (5 µL), 5 ng/*u*l a DNA template (2.5 µL), and 2x KAPA HiFi HotStart Ready Mix (12.5 µL) were included. The tube was put in the Gene Amp following the user instructions of Gene Amp PCR 9700. The first step was an initial denaturation at 95°C for 3 minutes. Then the following was repeated for 25 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and a final 72°C for 5 minutes. Then, the sample was held at 4°C. The PCR product was tested by electrophoresis using Gel Documentation for DNA testing, according to the manufacturer's instructions. The amplicon was approximately 550 bp. The 1st PCR clean-up used 20 AMPure XP Beads per sample, 80% freshly prepared ethanol, and 10 mM Tris pH8.5. The 2nd PCR step had an index attached. After reacting on ice with chemical addition, a 5 µL sample of extracted DNA was taken for

the second PCR (Index) and placing it into a 0.2 mL PCR tube to mix with Nextera XT Index Primer 1(N7xx) (5 µL), Nextera XT Index Primer 2 (S5xx) (5 µL), 2x KAPA HiFi HotStart Ready Mix (25 µL), and PCR grade water (10 µL). The operating procedures were followed using the Gene Amp PCR 9700 as follows. The first step was to perform an initial denaturation at 95°C for 3 minutes. Then the following was repeated for 8 cycles: 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and a final 72°C for 5 minutes. Then, the sample was held at 4°C. The 2nd PCR clean-up was done with AMPure XP Beads one more time. After that, the Library Quantification and QC process included an electrophoresis test of PCR product using 2 µL samples with Gel Documentation. For DNA testing, amplicon with approximately 630 bp was used in calculations, and the DNA concentration was diluted to 4 nM for next generation-sequencing.

In library normalization and pooling, the conversion of the DNA concentration unit to nM was calculated, and then DNA was diluted to 4 nM.

$$\frac{\textbf{(Concentration in ng/µl)}}{\textbf{(660 mol x average library size)}} \textbf{X} \quad 10^6$$

For library denaturation and MiSeq sample loading, PhiX was prepared to a concentration of 4 nM, and denatured PhiX was diluted to 20 pM, then further to 4 pM (the same concentration as denatured DNA library), and DNA Library was combined with the PhiX control.

RESULTS AND DISCUSSION

Short-chain fatty acids (SCFA) production: Short-chain fatty acids (SCFA) were found in the simulated colon from bacterial fermentation. Microorganisms produce mainly acetic acid, propionic acid, and butyric acid in the colon. Gas chromatography with a flame ionization detector (GC-FID) was used to analyze SCFAs in the samples. It was found that SCFA in mulberry juices (0% IMO, 2% IMO, and 8% IMO) at 0 and 24 hours, as shown in Table 1. In this study, the SCFA produced by intestinal microorganisms was primarily propionic acid and butyric acid. According to [18], butyric acid and propionic acid are the main SCFA components generated during fermentation. However, the acetic acid concentration in that study is lower than in this current study.

Statistical analysis: The effects of sample type on fecal batch culture fermentation were determined by one-way analysis of variance (ANOVA) using SPSS version 20.0 software with $p < 0.05$ required for statistical significance in Duncan's test. The results are shown as mean ± SD.

MBI2 had a high acetate concentration, and MBI8 also had a high acetate concentration at 24 and 48 hours, while the control sample with 0% IMO (as well as MBI2 and MBI8 fermented for 0, 6, or 12 hours) had no significant acetate concentration ($p > 0.05$).

Propionate had an increasing trend of concentration during fermentation. The increase was significant ($p < 0.05$) at 24 hours for all samples (MBI0 4.00 mM, MBI2 5.06 mM, and MBI8 7.03 mM). Wu et al. [18] found propionic acid significantly higher than acetic acid and butyric acid. The control showed a significant increase ($p < 0.05$) at 24 hours of fecal fermentation (4.00 mM). So, the results indicate that propionic acid was generated by the bacterial strains in the colon from the substrates in samples [19].

On the other hand, MBI2 and MBI8 showed the highest concentrations of butyric acid, namely 9.55 ± 1.01 mM and 10.79 ± 0.33, respectively, at 48 hours, while the control had no increase. Butyric acid has anti-inflammatory activity, reducing colonic inflammation and the occurrence of colon cancer [20].

Table 1. Short chain fatty acid (SCFA) profiles in fecal batch culture fermentation of mulberry juice.

Sample	SCFA concentration (mM)			
	Acetic acid	Propionic acid	Butyric acid	Total SCFA
Control (0% IMO)				
0 hr	1.35 ± 0.02^{a}	0.78 ± 0.13^{c}	1.19 ± 0.01^{b}	3.31 ± 1.06^{c}
6 hr	1.45 ± 0.07^{a}	2.13 ± 1.25^{b}	2.21 ± 0.11^{b}	5.79 ± 1.42^{b}
12 hr	1.45 ± 0.08^{a}	2.34 ± 0.09^{b}	2.37 ± 2.44^{b}	6.18 ± 0.25^{b}
24 hr	2.01 ± 0.01^{a}	4.00 ± 0.07^{a}	3.44 ± 3.36^{b}	9.45 ± 0.15^{a}
48 hr	2.14 ± 0.07^{a}	4.98 ± 0.28^{a}	4.68 ± 5.45^{a}	11.81 ± 1.14^{a}
MBI2 (2% IMO)				
0 hr	1.56 ± 0.14^{c}	1.04 ± 0.13^{c}	2.63 ± 0.25^{c}	5.23 ± 0.53^{c}
6 hr	1.46 ± 0.06^{c}	1.49 ± 0.07^{c}	1.84 ± 0.05^{c}	4.79 ± 0.18^{c}
12 hr	4.01 ± 0.12^{b}	2.45 ± 0.12^{c}	2.39 ± 0.17^{c}	8.85 ± 0.41^{c}
24 hr	5.87 ± 0.11^{b}	5.06 ± 0.12^{b}	4.24 ± 3.50^{b}	15.16 ± 3.73^{b}
48 hr	9.7 ± 4.96^{a}	11.66 ± 1.69^{a}	9.55 ± 1.01^{a}	30.91 ± 7.66^{a}
MBI8 (8% IMO)				
0 hr	3.39 ± 0.11^{bc}	7.45 ± 3.44^{b}	2.93 ± 0.14^{c}	13.77 ± 3.69^{b}
6 hr	1.59 ± 0.02^{c}	1.24 ± 0.07^{c}	2.32 ± 0.04^{c}	5.15 ± 0.12^{c}
12 hr	1.83 ± 0.04^{c}	0.84 ± 0.08^{c}	2.61 ± 0.12^{c}	5.28 ± 0.24^{c}
24 hr	6.01 ± 0.11^{b}	7.03 ± 0.19^{b}	5.86 ± 0.17^{b}	18.89 ± 0.47^{b}
48 hr	8.08 ± 0.16^{a}	13.68 ± 0.50^{a}	10.79 ± 0.33^{a}	32.56 ± 0.98^{a}

Values are given as mean ± SD of duplicate fermentation; Different superscripts within a column (in the rows for the same sample) indicate significant differences ($p < 0.05$).

Table 2a. Identification of phenolic compounds in fecal fermentation of the control initially (0% IMO at 0 hour) as analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.185	Citric acid	192.0272	192.027	$C_6H_8O_7$	-1.01	96.76	1745060
2	3.099	L-Phenylalanine	165.0791	165.079	$C_9H_{11}NO_2$	-0.88	92.77	648981
3	3.813	3,4-Dihydroxybenzoic acid	154.0264	154.0266	$C_7H_6O_4$	1.05	97.53	125963
4	4.151	Hydroxyphenyllactic acid	182.0577	182.0579	$C_9H_{10}O_4$	1.02	95.14	89641
5	4.79	3,4-Dihydroxybenzaldehyde	138.0313	138.0317	$C_7H_6O_3$	3.08	89.46	104315
6	8.449	3-(2-Hydroxyphenyl) propionic acid	166.0628	166.063	$C_9H_{10}O_3$	1.34	86.62	152197
7	8.737	D-(+)-3-Phenyllactic acid	166.0631	166.063	$C_9H_{10}O_3$	-0.68	89.47	366439
8	8.749	trans-Cinnamic acid	148.0522	148.0524	$C_9H_8O_2$	1.5	92.8	49403
9	14.838	Quercetin	302.0423	302.0427	$C_{15}H_{10}O_7$	1.31	94.21	45720
10	23.608	7-Ketodeoxycholic acid	406.2714	406.2719	$C_{24}H_{38}O_5$	1.24	49.19	229449
11	27.391	Cholic acid	408.2872	408.2876	$C_{24}H_{40}O_5$	0.8	96.41	452817
12	37.326	Allolithocholic acid	376.2976	376.2977	$C_{24}H_{40}O_3$	0.25	49.07	546142

Table 2b. Identification of phenolic compounds produced by fecal fermentation of the control (0% IMO) at 24 hours as analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.761	3-(3,4-Dihydroxyphenyl) lactate	198.0527	198.0528	$C_9H_{10}O_5$	0.43	86.87	91635
2	3.375	3,4-Dihydroxybenzoic acid	154.0266	154.0266	$C_7H_6O_4$	-0.24	94.9	66030
3	3.989	Dihydroxyphenylacetic acid	168.0423	168.0423	$C_8H_8O_4$	-0.04	97.66	43434
4	4.853	3,4-Dihydroxybenzaldehyde	138.0315	138.0317	$C_7H_6O_3$	1.25	86.76	235655
5	5.141	Hydroxyphenyllactic acid	182.0578	182.0579	$C_9H_{10}O_4$	0.42	98.84	218504
6	5.292	Pyrocatechol	110.0367	110.0368	$C_6H_6O_2$	0.39	87.67	115429
7	6.169	4-Hydroxybenzaldehyde	122.0367	122.0368	$C_7H_6O_2$	1.03	83.52	39795
8	7.321	2-Hydroxyphenylacetic acid	152.0472	152.0473	$C_8H_8O_3$	0.93	87.43	28886
9	7.547	Isoleucyl-phenylalanine	278.1629	278.163	$C_{15}H_{22}N_2O_3$	0.45	98.48	44601
10	7.722	Chenodeoxycholic acid sulfate	472.2516	472.2495	$C_{24}H_{40}O_7S$	-4.52	83.26	64931
11	8.048	Isocaproic acid	116.0834	116.0837	$C_6H_{12}O_2$	3.18	86.94	19926
12	21.729	Cholic acid	408.2875	408.2876	$C_{24}H_{40}O_5$	0.13	99.56	151425
13	37.339	Allolithocholic acid	376.2976	376.2977	$C_{24}H_{40}O_3$	0.28	98.41	409526

Identification of phenolic metabolites from fecal fermentation analyzed by liquid –chromatography-mass/mass spectrometry (LC-MS/MS): The liquid chromatography-mass/mass spectrometry (LC-MS/MS) technique was used to analyze the phenolic compounds in mulberry juice in the fecal batch culture fermentations. The results are presented in Table 2. The experiment had gut microbiota interact with the substrate (IMO) and assessed the changes in phenolic compounds. Phenolic compounds in mulberry juice were detected at 254 nm, 280 nm, and 310nm, as they are usually detectable at these wavelengths [21]. The results showed that the added prebiotic likely contributed higher concentrations of some phenolics than in the control. Tables 3c and 3d show that the most abundant phenolic compounds were 3-(2-hydroxyphenyl) propionic acid, 3,4-dihydroxybenzaldehyde, L-phenylalanine, aminocaproic acid, 3,4-dihydroxybenzaldehyde, and cholic acid. Bao et al. [22] studied phenolic components in digested mulberry cultivars. During *in vitro* fermentation, the anthocyanins increased and then decreased with time, and some anthocyanin metabolites (such as 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, etc.) were produced by bifidobacteria and lactobacilli. Cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside were the major phenolic compounds in mulberry analyzed by LC-MS/MS after *in vitro* fermentation. Colonic bacteria included *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Eubacterium hallii,* and *Clostridium barlettii*. Isomaltooligosaccharide (IMO) in mulberry juice promoted gut microflora. Owolabi et al. [5] studied *in vitro* fermentation of purple rice and detected microbial populations. Some phenolic compounds, including 3-(4-hydroxyphenyl) propionic acid, phenyl propionic acid, and pyrocatechol, were affected by the dihydroxylation of C3G.

Table 2c. Identification of phenolic compounds produced by fecal fermentation of 2% IMO for 24 hours as analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.692	3-(3,4-Dihydroxyphenyl) lactate	198.0526	198.0528	$C_9H_{10}O_5$	1.29	85.56	49768
2	3.043	L-Phenylalanine	165.0787	165.079	$C_9H_{11}NO_2$	1.42	87.43	85107
3	4.083	Dihydroxyphenylacetic acid	168.0421	168.0423	$C_8H_8O_4$	0.84	98.67	47527
4	4.797	3,4-Dihydroxybenzaldehyde	138.0315	138.0317	$C_7H_6O_3$	1.44	87.22	181050
5	5.123	Hydroxyphenyllactic acid	182.0578	182.0579	$C_9H_{10}O_4$	0.79	99.21	69396
6	5.298	Pyrocatechol	110.0366	110.0368	$C_6H_6O_2$	1.82	87.37	39297
7	8.054	Isocaproic acid	116.0834	116.0837	$C_6H_{12}O_2$	2.77	86.03	21834
8	21.785	Cholic acid	408.2872	408.2876	$C_{24}H_{40}O_5$	0.93	98.76	32731
9	27.297	Ursodeoxycholic acid	392.2922	392.2927	$C_{24}H_{40}O_4$	1.15	97.89	25957

Table 2d. Identification of phenolic compounds produced by fecal fermentation of 8% IMO for 24 hours as analyzed by liquid chromatography-mass/mass spectrometry (LC-MS/MS).

S/N	Retention Time (Min)	Compound Name	m/z (Calculated)	m/z (Observed)	Molecular Formula	Diff (ppm)	Score (%)	Abundance
1	2.915	Pyrocatechol	109.0296	110.0368	$C_6H_6O_2$	-0.9	87.83	71933
2	2.94	3,4-Dihydroxybenzoic acid	153.0193	154.0266	$C_7H_6O_4$	-0.4	98.91	46386
3	3.065	L-Phenylalanine	165.0791	165.079	$C_9H_{11}NO_2$	-0.86	99.33	166048
4	4.757	3,4-Dihydroxybenzaldehyde	138.0316	138.0317	$C_7H_6O_3$	0.73	99.29	129761
5	5.045	Hydroxyphenyllactic acid	182.058	182.0579	$C_9H_{10}O_4$	-0.47	99.58	75475
6	5.521	Parahydroxyphenylacetic acid	152.048	152.0473	$C_8H_8O_3$	-4.21	89	20190
7	5.797	Aminocaproic acid	131.0947	131.0946	$C_6H_{13}NO_2$	-0.55	87.43	165237
8	7.701	Chenodeoxycholic acid sulfate	472.2516	472.2495	$C_{24}H_{40}O_7S$	-4.57	82.61	93019
9	8.051	Isocaproic acid	116.0836	116.0837	$C_6H_{12}O_2$	0.74	99.38	14485
10	8.402	3-(2-Hydroxyphenyl) propionic acid	166.0634	166.063	$C_9H_{10}O_3$	-2.69	85.77	1049504
11	8.603	D-(+)-3-Phenyllactic acid	166.0631	166.063	$C_9H_{10}O_3$	-0.5	89.21	39014
12	8.628	Lithocholic acid sulfate	456.2566	456.2546	$C_{24}H_{40}O_6S$	-4.52	84.21	41956
13	21.281	1-(3,4-Dihydroxyphenyl)-5-hydroxy-3-decanone	280.1679	280.1675	$C_{16}H_{24}O_4$	-1.72	83.39	41433
14	21.732	Cholic acid	408.2874	408.2876	$C_{24}H_{40}O_5$	0.33	99.55	124285

Gut microbiota population: A phylogenetic tree was constructed using MEGA11 with its maximum-likelihood method to classify the gut microbiota. There were three main bacterial groups in the fecal batch culture fermentation (Figure 1). This study used next-generation sequencing (NGS) to analyze the microbiome, finding beneficial bacteria for health such as *Bifidobacterium scardovii, Bifidobacterium stercoris, Megasphaera elsdenii, Bacteroides vulgatus,* and *Lachnospira pectinoschiza*. The population of *Bifidobacterium,* including *Bifidobacterium scardovii* and *Bifidobacterium stercoris* was significantly ($p < 0.05$) increased in IMO 2% and 8% cases when compared to 0% IMO after 24 hours of fermentation (Figure 2B-2D). *Megasphaera elsdenii* are beneficial bacteria in the intestine that release ammonia from oligopeptides [23]. The bacteria groups *Sporobacter termitidis*, *Clostridium leptum*, *Oscillospira guillermondii*, and *Lachnospira pectinoschiza* are anti-inflammatory and help against bowel disease (IBD) [24]. *Pseudobutyrivibrio xylanivorans* and *P. ruminis* are butyrate-producing bacteria that are beneficial in the human colon [25].

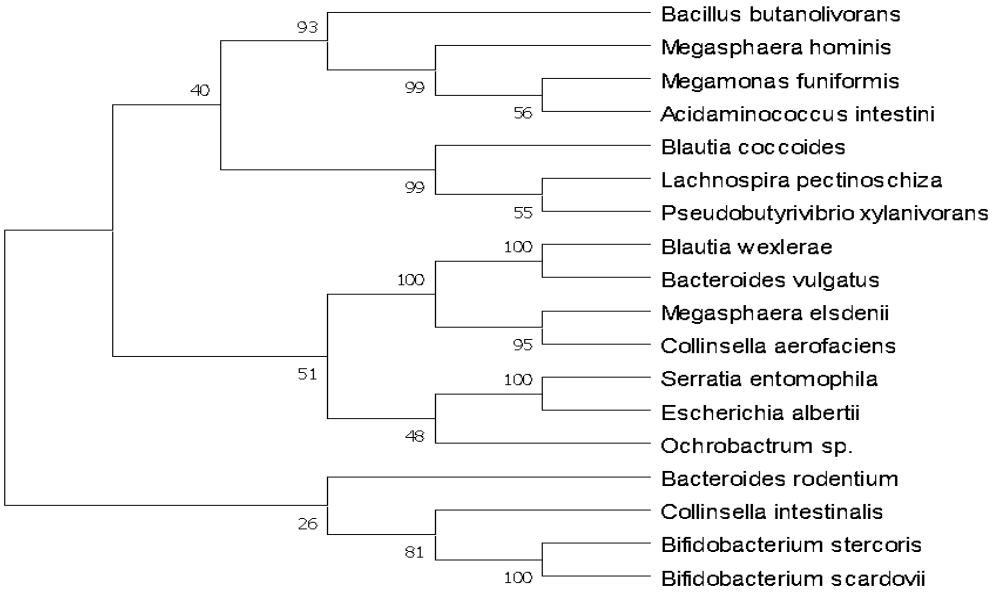
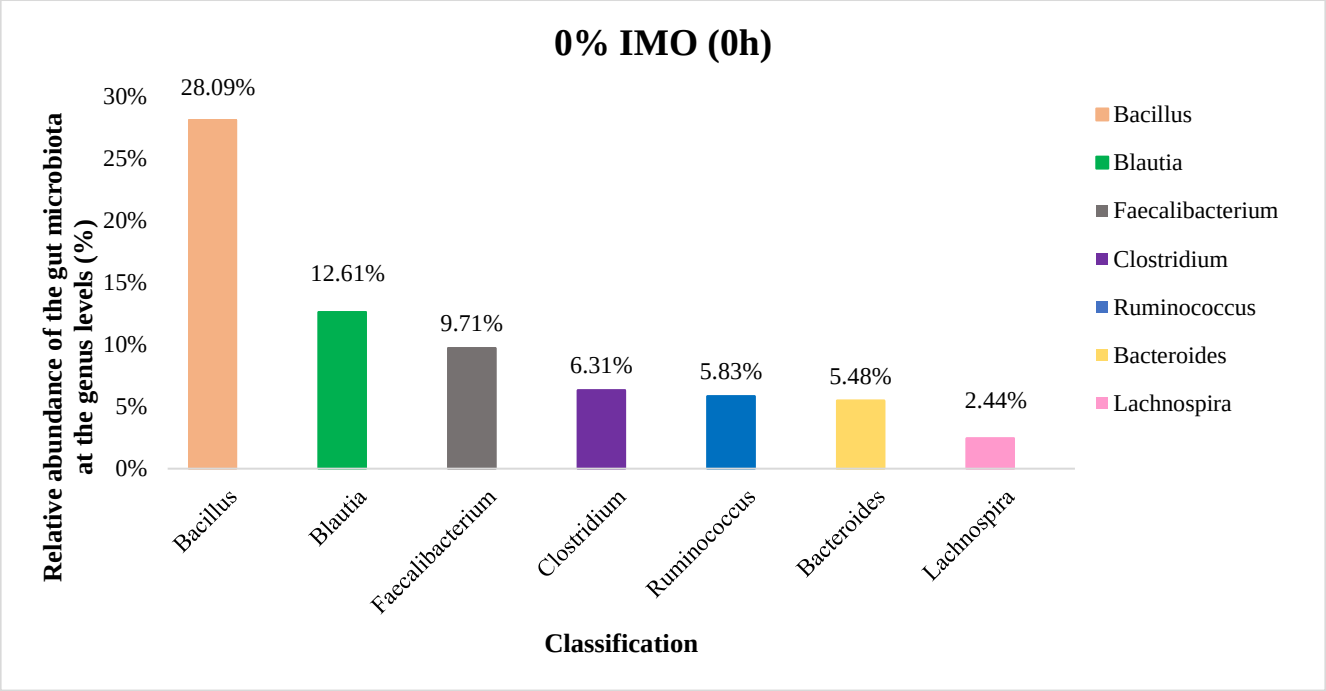


Figure 1. Phylogenetic analysis of the 16s rRNA sequence. The maximum likelihood tree (100 bootstraps) was constructed using16s rRNA sequences collected from the NCBI database

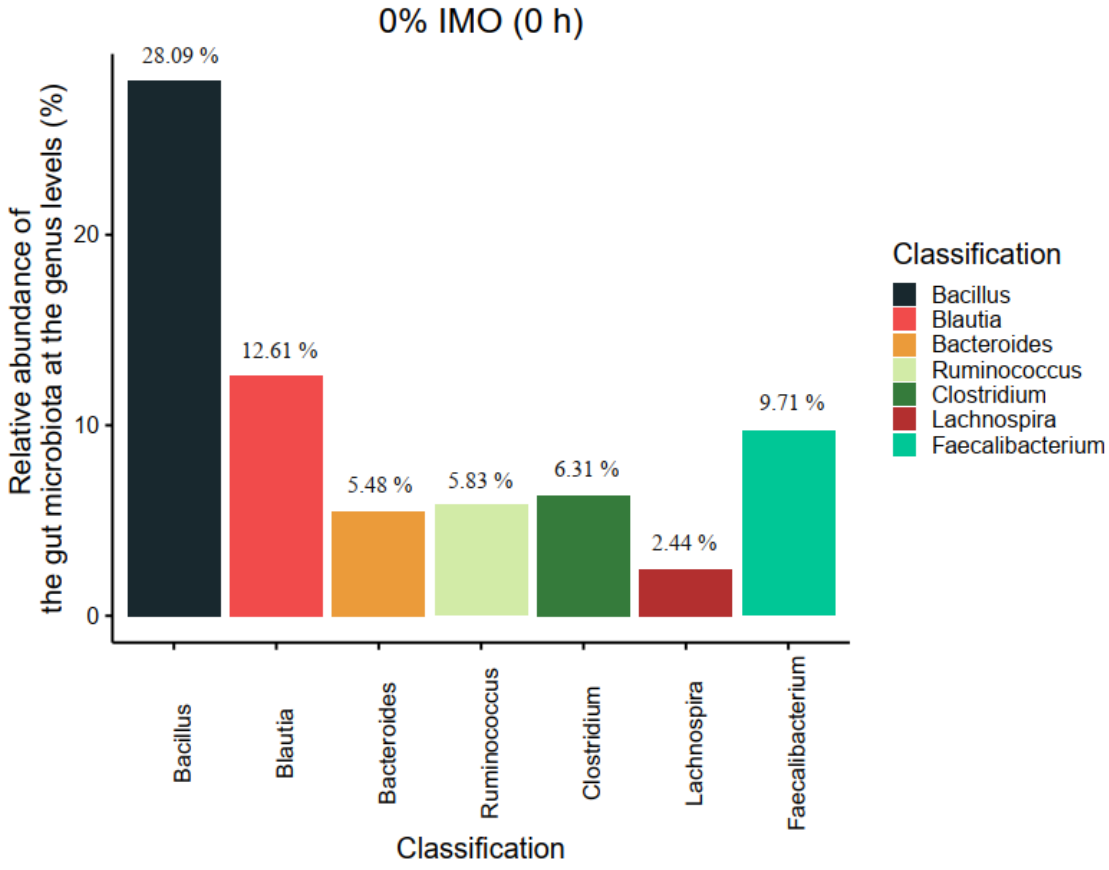
The four samples tested show more significant increases in bifidobacteria than in strains of non-beneficial bacteria. Figure 2 shows the populations of bifidobacteria. The percent of bifidobacteria significantly ($p < 0.05$) increased, as indicated in Figures 2C (5.03%) and 2D (17.53%) in 24 hours of fermentation when comparedwith Figures 2A and 2B. Therefore, the IMO stimulated the growth of 502ifidobacterial in the experimentally simulated colon. Zhang et al. [26] showed the effects of dietary IMO levels on the gut microbiota. IMO is prebiotic and supports the growth of bacteria in the colon, such as bifidobacteria. Plongbunjong et al. [27] produced isomaltooligosaccharides (IMOs) from Sangyod rice starch. In *in vitro* fermentation, the population of bifidobacteria and lactobacilli increased, which are beneficial bacteria in the body. This current experiment showed a significant increase in bifidobacteria ($p < 0.05$). On the other hand, Bacteroides, which are pathogenic bacteria, decreased significantly ($p < 0.05$) when IMO was

added to mulberry juice. The species classification detected *Megasphaera hominis* after 24 hours of fermentation (Figure 2B-2D), and *Megasphaera* species is more common in women, especially in postmenopausal cases with decreased lactobacilli.

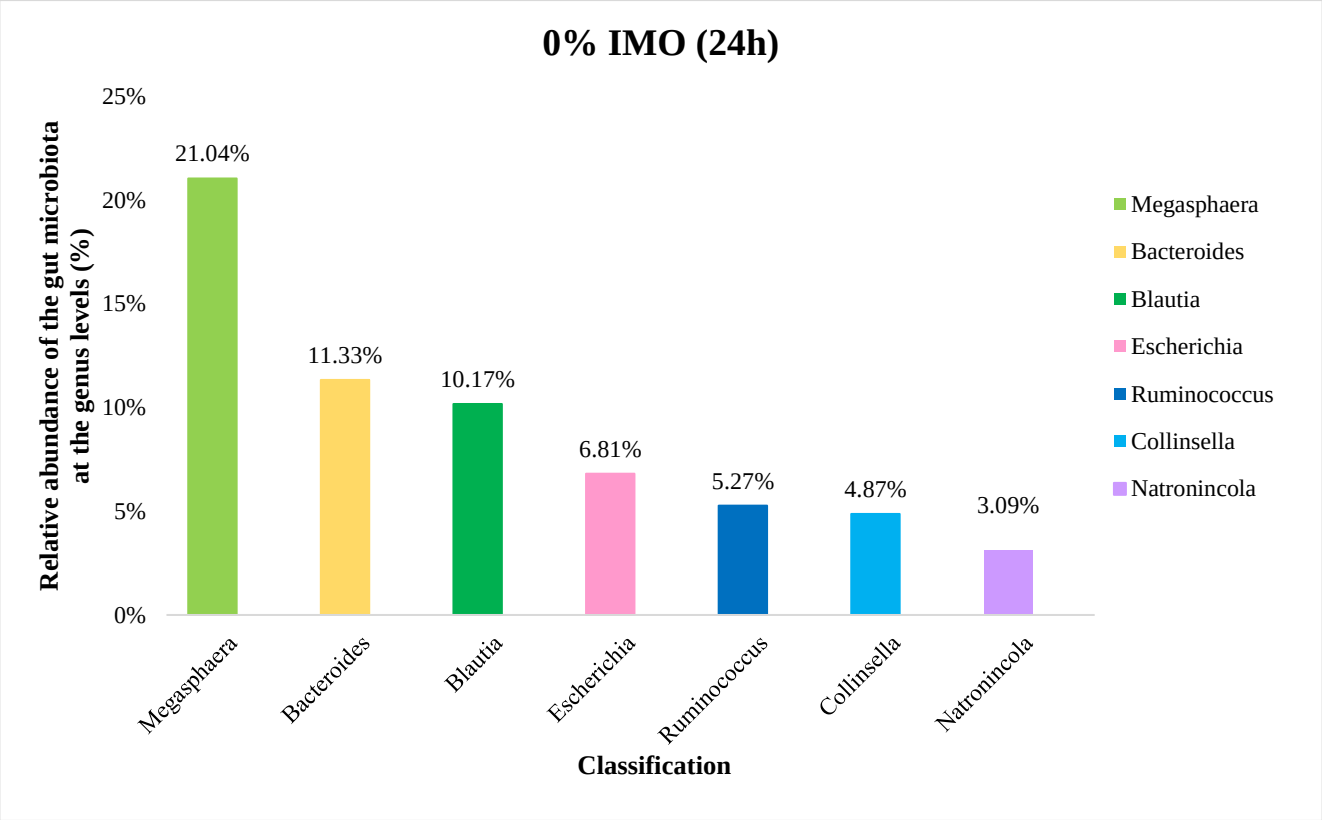
A)



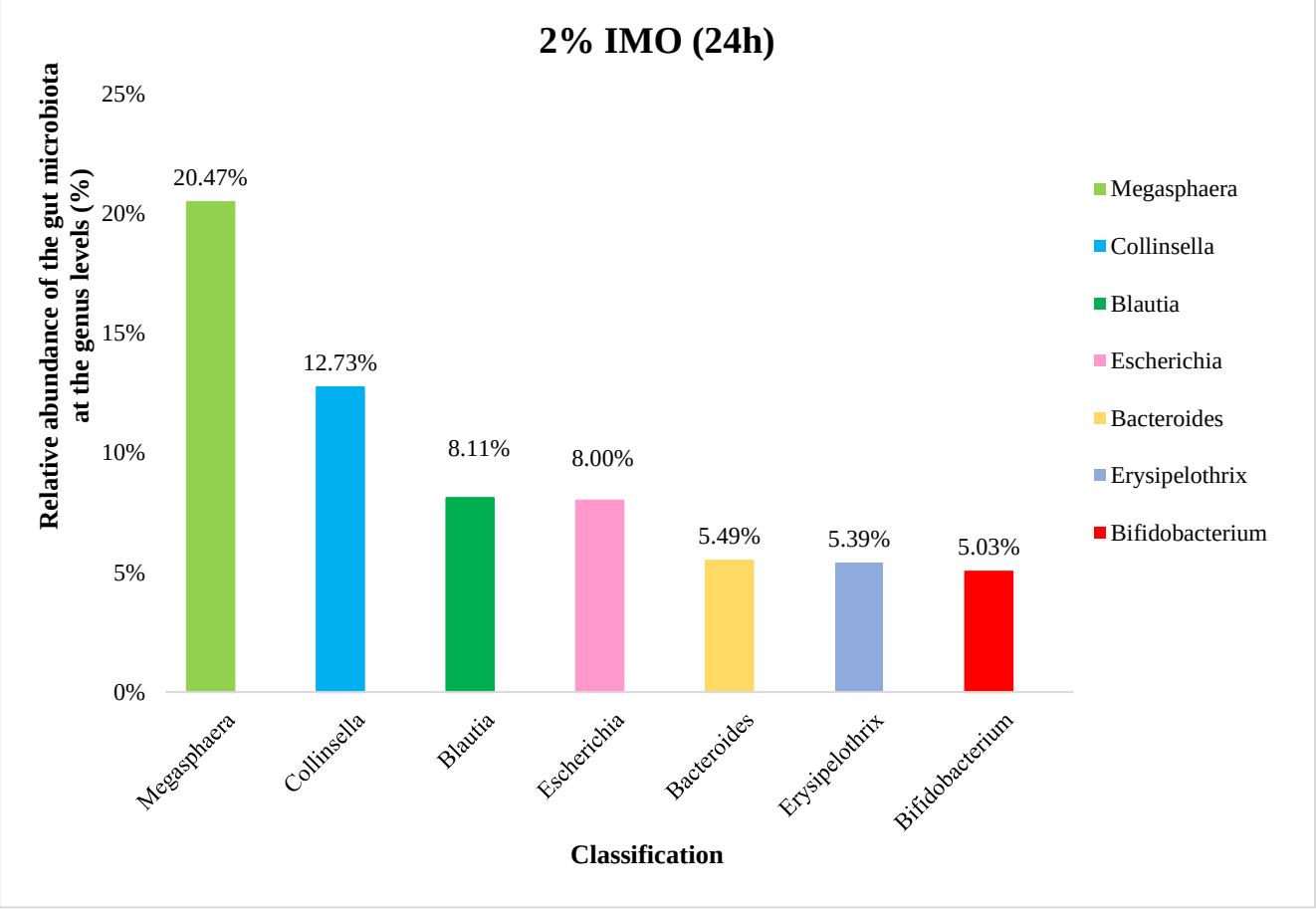
B)



C)


0% IMO (24h)
Relative abundance of the gut microbiota at the genus levels (%)
25%
20%
15%
10%
5%
0%
21.04%
11.33%
10.17%
6.81%
5.27%
4.87%
3.09%
Megasphaera
Bacteroides
Blautia
Escherichia
Ruminococcus
Collinsella
Natronincola
Classification
Megasphaera
Bacteroides
Blautia
Escherichia
Ruminococcus
Collinsella
Natronincola

D)


2% IMO (24h)
Relative abundance of the gut microbiota at the genus levels (%)
25%
20%
15%
10%
5%
0%
20.47%
12.73%
8.11%
8.00%
5.49%
5.39%
5.03%
Megasphaera
Collinsella
Blautia
Escherichia
Bacteroides
Erysipelothrix
Bifidobacterium
Classification
Megasphaera
Collinsella
Blautia
Escherichia
Bacteroides
Erysipelothrix
Bifidobacterium

E)

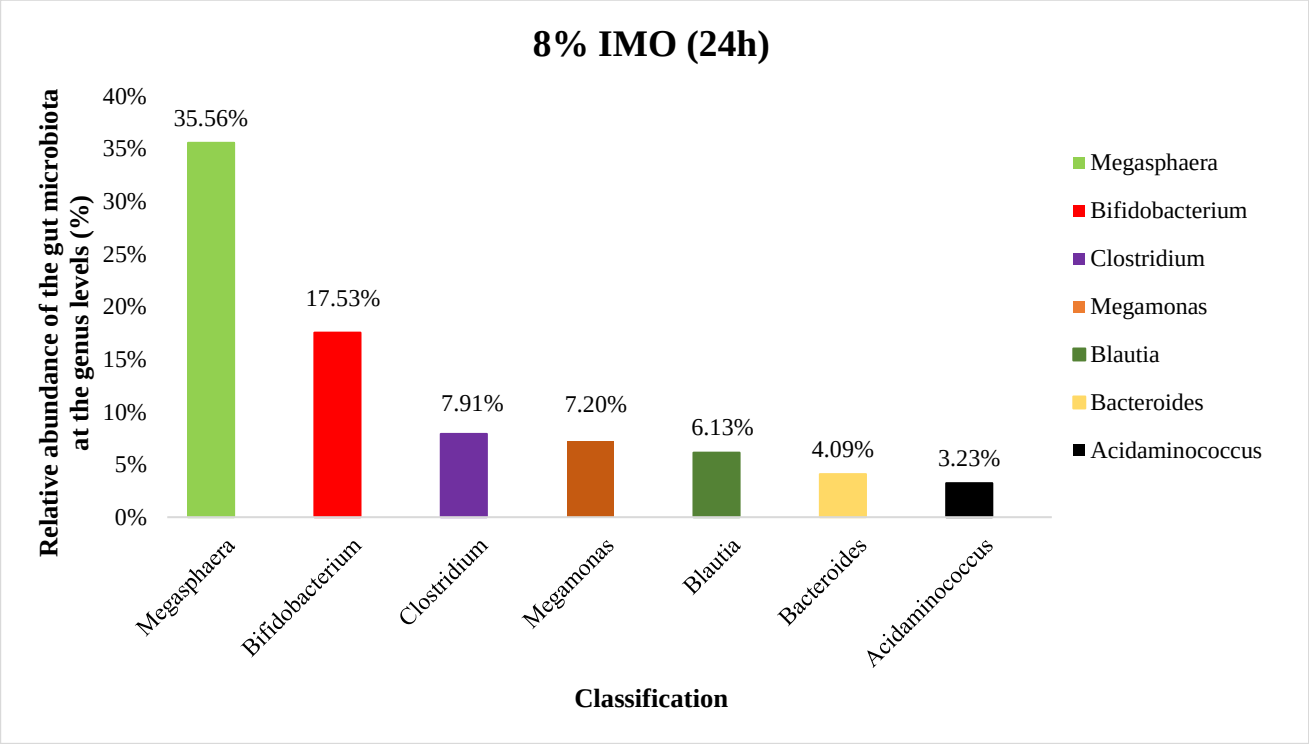


Figure 2. Percentages of mean relative abundances of the bacteria at the genus levels in (A) 0% IMO (0 hour); (B) 0% IMO (24 hours); (C) 2% IMO (24 hours); and (D) 8% IMO (24 hours).

CONCLUSION

This study has shown that adding isomaltooligosaccharide (IMO) to mulberry juice favored the growth of beneficial microorganisms in the large intestine, especially bifidobacteria. Experimentally *in vitro* gut fermentation of IMO produced a large amount of propionic acid. As the percentage of IMO increased, *Bifidobacterium* population and SCFA concentration increased. Therefore, supplementing IMO promoted bacteria beneficial to gut health.

Abbreviations: C3G: Cyanidin-3-glucoside, GC-FID: Gas chromatography-flame ionization detection, IBD: Inflammatory bowel disease, IMO: Isomaltooligosaccharide, LC-MS/MS: Liquid chromatography-mass/mass spectrometry, MBI: Mulberry juice added isomaltooligosaccharide, NCBI: The National Center for Biotechnology Information, NGS: Next generation sequencing, PCR: Polymerase chain reaction, SCFA: Short chain fatty acid, UHPLC: Ultra high liquid chromatography.

Author's contributions: T.K. performed experiments, analyzed data, and drafted the manuscript. W.Y. edited the manuscript and supervision, acquired the research grant. Z.L. edited the manuscript and comments. S.W. supervised, design experiment in gut fermentation, revised and edited the manuscript.

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