Food Research 3 (5) : 428 - 440 (October 2019)
Journal homepage: http://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Beneficial effectiveness of probiotic-low-fat ice cream containing *Krueo Ma Noy* (*Cissampelos pareira* L.) gum on colon microbiome under a dynamic gut model

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Article history:
Received: 10 February 2019
Received in revised form: 12 March 2019
Accepted: 14 March 2019
Available Online: 27 March 2019

Keywords:
Bifidobacterium lactis Bb-12,
Krueo Ma Noy,
Gut model,
Colon microbiome,
Low-fat ice cream

DOI:
https://doi.org/10.26656/fr.2017.3(5).068

Abstract

The combined effects of low-fat ice cream supplemented with *Bifidobacterium lactis* Bb-12 and *Krueo Ma Noy* gum (KMN) in modulating the gut microbiota of a dynamic gut model were evaluated. Experimental trials were held to assess overall microbial metabolites (i.e. short-chain fatty acids, lactic acid, ammonia and biogenic amines) and colon microbial populations of lactobacilli, bifidobacteria, clostridia, fecal coliforms and total anaerobes. Low-fat ice cream was either administered into the simulated system alone, with probiotic *B. lactis* Bb-12, KMN or both. Combined supplementation of low-fat ice cream with KMN and probiotic was shown to enhance the levels of beneficial microbial metabolites, *viz.* acetic, propionic, butyric and lactic acids, in proximal and distal colon vessels, while lowering the levels of ammonia and biogenic amines (i.e. cadaverine, putrescine, methylamine and tyramine) secretion as compared to pure low-fat ice cream. Observing from generated profiles of the colon microflora, the combined treatment yielded the greatest increase in populations of colon lactobacilli and bifidobacteria while suppressing the growth of fecal coliforms, clostridia and other harmful microbes. Such findings indicate the synbiotic potential and beneficial health effects of KMN and probiotic in low-fat ice cream in improving gut conditions.

1. Introduction

Functional foods, also known as foods exerting beneficial effects on specific organs or systems within the human body, are known for a variety of functions beyond energy and nutrient provision (Granato *et al*., 2010). In the present day, growing numbers of health-concerned consumers fueled the rapid expansion of functional foods in the market, especially probiotic dairy products. In terms of functional foods, dairy products are strongly linked to probiotics. Probiotic strains have become of prominent interest in food product development due to its various properties, such as bacteriocin production, acid and bile tolerance, epithelial cell adherence, gut survivability and colonization, and endurance to physiochemical conditions of food processing and storage, which contribute to numerous benefits on human health (Prado *et al.*, 2008). Such benefits are attributed by their antimicrobial, antimutagenic, anticarcinogenic and antihypertension properties. Regular ingestion has been shown to reduce symptoms of food allergy, stabilize host gut microflora and stimulate gut resistance to pathogens (Liong *et al*., 2009; Granato *et al*., 2010).

Dairy products, *viz.* probiotic yogurts, fermented beverages and ice cream, are regarded as the main vehicles of probiotic supplementation and subsequent ingestion. According to Shah (2007), at least 10^6 CFU/g of probiotic cells should be present in a dairy food to compensate subsequent population reduction during passage along the human gut. Probiotic supplemented ice cream has been reported to promote promising health benefits (Çaglar *et al*., 2008) and may potentially serve as vehicles and protective barriers for probiotics during gut transport (Cruz *et al*., 2009). The prevalence of non-communicable diseases, such as obesity and cardiovascular disease, has led to towards the innovation and development of low-fat ice cream formulations containing probiotic cultures, prebiotics and synbiotics (Akalin and Erisir, 2008; Chaikham and Rattanasena, 2017).

Krueo Ma Noy (*Cissampelos pareira* L.) is a woody, climbing medicinal plant of the *Menispermaceae* family indigenous to the tropical area of Asia, East Africa and South Africa. This plant grows abundantly in the

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eISSN: 2550-2166

Northern and Northeastern regions of Thailand. Due to its analgesic properties, the *Krueo Ma Noy* plant was commonly used by indigenous people to treat a number of ailments, including sores, asthma, dysentery, diuretic and traumatic pain (Vardhanaburi and Ikeda, 2006). Cold -water extraction of the leaves produced a dark green gel with cooling properties that can be used to treat fever. Gel formation occurred within a very short time after the extraction process, resulting from unique characteristics of *Krueo Ma Noy* pectin, a complex polysaccharide examined in the reports of Singthong *et al.* (2004, 2005). Pectin is commonly applied in food production and manufacturing processes as gelling and thickening agents (Barros *et al*., 2002). The water-soluble fiber is one of the major substrates in the colonic bacteria metabolism of carbohydrate hydrolysis to produce organic by-products, such as short-chain fatty acids (SCFA) and lactic acid (Slavin, 2013). Citrus pectin hydrolysate has been shown to exhibit prebiotic effects on tested non-fat milk probiotics, as demonstrated by growth enhancement, increased fermentation, and high levels of probiotic survivability (Ho *et al*., 2017).

Many researchers have incorporated dynamic models of the gastrointestinal system in the study of various probiotic functional foods, prebiotics and prebiotic candidates regarding their effects on colon bacteria viability, microbial composition and metabolite synthesis (Mäkivuokko *et al*., 2006; Mäkivuokko *et al*., 2007; Chaikham *et al*., 2012; Bianchi *et al*., 2014; Apichartsrangkoon *et al.*, 2015). Different *in vitro* gut models were used predominantly in nutrition studies involving compositional analysis of the colon microbial community. Normally, they are comprised of different segments of the human gastrointestinal tract, including stomach, small intestinal and colon compartments, with a stable microbial community resembling the human gut microbiota or conditions (Kontula *et al*., 2002; Possemiers *et al*., 2010; Sivieri *et al*., 2013). Probiotic administration has been shown to enhance the modulatory effects of low-fat ice cream on simulated microbial ecosystems of the gastrointestinal tract (Chaikham and Rattanasena, 2017). While the fermentation of different prebiotics and prebiotic candidates on probiotic foods have been subjected to the similar simulation systems, there are currently insufficient studies attributing to the potential prebiotic effects of *Krueo Ma Noy* gum (KMN). Therefore, the objective of this study was to examine the combined ability of KMN and *Bifidobacterium lactis* Bb-12 supplementation along with low-fat ice cream in adjusting microbial compositions of the simulated gut model. Colon microbial diversities were determined by monitoring the changes in populations of colon microorganisms, *viz.* lactobacilli, bifidobacteria, clostridia, fecal coliforms and total anaerobes, after feeding and fermentation with different treatments of low-fat ice cream. The ice cream samples were either administered pure, supplemented with *B. lactis* Bb-12, KMN or both. Metabolic products of microfloral fermentation, including SCFA (acetic, propionic, and butyric acids), lactic acid, ammonia and biogenic amines (cadaverine, putrescine, methylamine and tyramine) were also assessed within this study.

2. Materials and methods

2.1 Probiotic culture and chemicals

Lyophilized *B. lactis* Bb-12 was obtained from Chr. Hansen (Hørsholm, Denmark). Absolute ethanol, *L*-cysteine hydrochloride, sodium hydroxide (bacterial grade),hydrochloric acid (conc.), sodium hydroxide, *D* (+)-glucose, mucin from bovine submaxillary gland, phosphate buffer saline, boric acid, sodium hydroxide, bicarbonate, tryptose sulfite cycloserine (TSC) agar, *Clostridium perfringens* selective supplement, Tris (hydroxymethyl) aminomethane, sodium acetate, magnesium oxide, ammonia and acetic acid were purchased from Merck (Darmstadt, Germany). Sodium thioglycolate, sulfuric acid, xylan, arabinogalactan, *L*-cysteine, sodium thioglycolate, sodium hydrogen carbonate, pancreatin from porcine pancreas, ethylenediaminetetraacetic acid (EDTA), trichloroacetic acid (TCA), dansyl chloride and acetonitrile were supplied from Sigma-Aldrich (St. Louis, MO, USA). de Man, Rogosa and Sharpe (MRS) broth, MacConkey agar, yeast extract and peptone water were purchased from Hi-Media (Mumbai, India). Oxgall, brain heart infusion (BHI) agar and polyacrylamide gels were procured from Difco™ (BD, USA), Oxoid (Hampshire, United Kingdom) and Bio-Rad Laboratories Pty., Ltd. (Gladesville, New South Wales, Australia), respectively.

2.2 Extraction of Krueo Ma Noy gum

Krueo Ma Noy leaves were harvested from an orchard in Sakon Nakhon province, Thailand. The washed leaves were sun-dried for 15 hrs before blending. The blended leaves were extracted with distilled water at a ratio of 1:20 (w/v) at 75°C for 1 hr (Singthong *et al*., 2005). The extract was then filtered and precipitated with absolute ethanol at a ratio of 1:3 (v/v). Afterward, the precipitate was dried using a vacuum oven (XF050, France Etuves, Chelles, France) and powdered using a blender (National, Bangkok, Thailand).

2.3 Preparation of probiotic pellet

Lyophilized *B. lactis* Bb-12 was incubated in sterile MRS broth containing 0.05% (w/v) *L*-cysteine hydrochloride at 37°C for 20 hrs and then harvested by

centrifugation (Rotina 46R Centrifuge, Hettich®, Tuttlingen, Germany) with a rotary speed of 4,500 rpm at 4°C for 20 mins. Afterward, the precipitated cells were washed twice with 0.85% (w/v) sterile saline water and kept in a refrigerator at 4°C for 16 hrs (overnight). For activation, the pellet cells were warmed at 37°C for 1 hrs before supplementation into low-fat ice cream.

2.4 Production of low-fat ice cream

To produce low-fat ice cream, 76% (w/w) pasteurized skim milk plus 2% (w/w) milk butter were heated at 50°C for 5 mins before mixing with other solid ingredients, including 8% (w/w) skim milk powder, 12% (w/w) sucrose, 1% (w/w) corn starch and 1% (w/w) gelatin (240 Bloom) or 0.5% (w/w) gelatin plus 0.5% (w/w) KMN (Section 2.1).The mixture was blended for 2 mins, pasteurized at 85°C for 5 mins and then incubated in a refrigerator at 4°C for 16 hrs (overnight). Subsequently, 1% (w/w) precipitated cells (Section 2.2) were added into the incubated mixture, with *B. lactis* Bb-12 roughly 10^{14} CFU/g, and the ice cream was made using an ice cream maker (National). Later, the ice cream sample was placed into 50 ml plastic cup and frozen at -25°C overnight for hardening (Chaikham and Rattasena, 2017).

2.5 Gut model experiment

Basal nutrient medium and pancreatic solution were prepared (Table 1) and sterilized at 121°C for 15 mins. The basal nutrient medium was acidified with 37% (conc.) hydrochloric acid to pH 2. The acidified basal nutrient medium and pancreatic solution were then used for feeding into the dynamic gut model with 1 ml/min flow rate. Four sterile fermentation vessels which consisted of the stomach (1st vessel), small intestine (2nd vessel), proximal colon (3rd vessel) and distal colon (4th vessel) were set up and operated according to the running-setup protocol of Chaikham *et al*. (2012) with some modifications. The system temperature was set at 37°C, by means of a circulating water bath, and culture pH was maintained at 5.6-5.9 and 6.6-6.9 in proximal and distal colon vessels using a pH controller with the automated addition of 0.25 M hydrochloric acid and 0.1 M sodium hydroxide. Each colon vessel was inoculated with 30 mL of fecal slurry from five healthy donors which were prepared using pre-reduced 0.1 M phosphate buffer saline (pH 7) plus 2 g of sodium thioglycolate (reducing agent) and then mixed in a stomacher for 5 mins. Fecal samples were obtained from three male and two female volunteers with a mean age of 25 years who had not taken antibiotics for at least 6 months prior to providing the sample and had no history of a gastrointestinal disorder. Various treatment compositions were fed continuously into the model for 9 weeks, as shown in Table 2. The basal period (2 weeks) was intended to modulate the microbiome in the different colons to the prevailing conditions in order to obtain a population that resembles the *in vivo* situation in terms of either community composition or metabolic activity (Chaikham *et al*., 2016). For collecting the fermented samples in both colon vessels, after 2 days feeding, 20 mL of colon fluids were withdrawn daily for assessments of microflora metabolites and populations.

2.6 Determinations of short-chain fatty acids and lactic acid

A 1-mL colon fluid was centrifuged with the rotary speed of 4,500 rpm at 4°C for 15 mins and then the supernatant was filtered through a 0.20-µm nylon filter (Vertical, Bangkok, Thailand). Afterward, 20 µL of filtrate was injected onto an HPLC system (Lachrom, Poole, United Kingdom) equipped with a refractive index detector (Lachrom L-7490) and an automatic injector (Lachrom L-7200). The column used was a RezexROA-Organic Acid-H+ column (300 × 7.80 mm, Phenomenex, Cheshire, United Kingdom). The column

Table 1. Medium compositions for *in vitro* experimental feeding.

Media	Amounts of substances in 1 L distilled water
Feeding medium	1 g arabinogalactan, 2 g pectin, 1 g xylan, 3 g potato starch, 0.4 g *D*(+)-glucose, 3 g yeast extract, 1 g peptone water, 4 g mucin from bovine submaxillary gland, 0.5 g of *L*-cysteine and distill water
Pancreatic solution	12 g Oxgall, 25 g sodium hydrogen carbonate, 1.8 g pancreatin from porcine pancreas and distilled water

Table 2. Differences in feeding compositions of low-fat ice cream.

Experimental running	Feeding compositions
Basal (14 days)	150 ml basal nutrient medium
Treatment 1 (7 days)	150 ml basal nutrient medium + 50 g low-fat ice cream (1% gelatin)
Washout 1 (7 days)	150 ml basal nutrient medium
Treatment 2 (7 days)	150 ml basal nutrient medium + 50 g low-fat ice cream (1% gelatin) + *B. lactis* Bb-12 (~ 10^{14} CFU/g)
Washout 2 (7 days)	150 ml basal nutrient medium
Treatment 3 (7 days)	150 ml basal nutrient medium + 50 g low-fat ice cream (0.5% gelatin and 0.5% KMN)
Washout 3 (7 days)	150 ml basal nutrient medium
Treatment 4 (7 days)	150 ml basal nutrient medium + 50 g low-fat ice cream (0.5% gelatin and 0.5%KMN) + *B. lactis* Bb-12 (~ 10^{14} CFU/g)

eISSN: 2550-2166

was then left in the oven at 84°C and 0.0025 mM sulfuric acid was used as a mobile phase at a flow rate of 0.5 mL/min. Data were acquired using JCL6000 software (Jones Chromatography, Wales, United Kingdom) and quantification of the samples was carried out using calibration curves of acetic, propionic, butyric and lactic acids.

2.7 Investigation of colon microflora diversities

Briefly, the colon fluids were appropriately diluted with 0.1 (w/v) sterile peptone water and spread-plated on five selective media as follows: RB-agar for bifidobacteria (anaerobic environment, 37°C, 96 hrs), LAMVAB agar for lactobacilli (anaerobic environment, 37°C, 72 hrs), MacConkey agar for fecal coliforms (aerobic environment, 42°C, 24 hrs), TSC agar plus *C. perfringens* selective supplement for clostridia (anaerobic environment, 37°C, 24 hrs) and BHI agar for total anaerobes (anaerobic environment, 37°C, 24 hrs). Later, all plated media were incubated under different conditions before colony counting (Chaikham *et al*., 2012).

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) patterns of general colon bacteria were analyzed following the modified protocol of Chaikham *et al.* (2012). Accordingly, PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE buffer solution [pH 7.4; 20 mMTris (hydroxymethyl) aminomethane, 10 mM sodium acetate and 0.5 mM EDTA]. PCR-DGGE fingerprints were made using a Bio-Rad D Gene System (Bio-Rad Laboratories Pty., Ltd., Gladesville, New South Wales, Australia) with a denaturant gradient between 45% to 60%. Different DNA patterns and densities were grouped and calculated using a BioNumerics software v.2.0 (Applied Maths, Sint-Martens-Latem, Belgium).

2.8 Determinations of ammonia and biogenic amines

Ammonia concentration in colon fluids was determined according to the method as described by Chaikham *et al.* (2012). A 1-mL colon fluid was added with one teaspoon of magnesium oxide and the mixture was then distilled using a Kjeldahl Apparatus Vapodest 30 S (Gerhardt, Königswinter, Germany). The released ammonia gas was entrapped with boric acid solution before titration with 0.02 M hydrochloric acid. The ammonia concentration was calculated and expressed as gram ammonia per 1 L colon fluid (g/L).

Cadaverine, putrescine, methylamine and tyramine were determined using the modified HPLC method as described by Tosukhowong *et al.* (2011). A total of five milliliters of colon fluids were well-mixed with 35 mL of 10% TCA solution before centrifugation at 4,000 rpm for 15 mins. One milliliter supernatant was mixed with 0.2 mL of 2 M sodium hydroxide and 0.3 mL of saturated sodium bicarbonate and then allowed to stand at room temperature for 30 mins. A 2 mL of 10 mg/mL dansyl chloride solution was added into the mixture before incubation at 40°C for 30 mins. After that, 0.1 µL of 25% ammonia was added to stop the reaction and centrifuged at 4,000 rpm for 10 mins. The supernatant was filtered through a 0.20-µm nylon membrane filter and 20 µL filtrate was injected into a Shimadzu HPLC system (CL-10 ADVP, Shimadzu, Kyoto, Japan). Separation of biogenic amines was achieved using a C18 column (YMC-Pack ODS-AM, 5 µm, 4.6 mm ID × 250 mm; YMC, Kyoto Japan). The mobile phase was a mixture of 0.1% acetic acid (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) with a flow rate of 1.0 mL/min. The gradient system of the mobile phase commenced from 0 min (50% A and 50% B) to 30 mins (10% A and 90% B), 10 mins (50% A and 50% B) and maintained at this state to 10 mins. The temperature of the column was set at 40°C and UV detection was at 254 nm. The peak area of each component was determined and converted to concentration.

2.9 Statistical analysis

Five replicates were performed, and the mean value was calculated. Analysis of variance (ANOVA) was used to carry out the variation and significance of difference. Determination of significant differences within treatment means was done using Duncan's multiple range tests at 95%. Pearson correlation coefficient was used for grouping the DNA patterns on PCR-DGGE fingerprints.

3. Results and discussion

3.1 Short-chain fatty acids and lactic acid

The results in Table 3 showed the concentrations of acetic acid, propionic acid, butyric acid, total SCFA and lactic acid presented in proximal and distal colon vessels following treatments of basal nutrient medium (Table 1) along with low-fat ice cream, *B. lactis* Bb-12, KMN and combination with both *B. lactis* Bb-12 and KMN (Table 2). The gut model was left for long-term running with periodical washouts following 7 days of different treatments, as can be observed from Table 2. Regarding SCFA, it was noticed that acetic acid concentrations were of the highest levels compared to propionic and butyric acids in both colon vessels ($P \leq 0.05$). This complied to the population data from various studies showing the greatest acetic acid production among the three types of SCFA, where ratios of acetic, propionic and butyric acids production in large intestinal follow an approximate ratio of 3:1:1 (Topping and Clifton, 2001). Probiotic-low-fat ice cream (Treatment 2) also

Table 3. Quantities of short-chain fatty acids and lactic acid in colon vessels after supplementation with difference treatments of low-fat ice cream.

Metabolites (g/L)	Experimental periods							
	Basal	Treatment 1	Washout 1	Treatment 2	Washout 2	Treatment 3	Washout 3	Treatment 4
Proximal colon								
Acetic acid	0.63 ± 0.08^{CDf}	1.29 ± 0.09^{CDc}	0.92 ± 0.10^{Bc}	1.37 ± 0.14^{CDbc}	1.10 ± 0.05^{Dd}	1.57 ± 0.14^{CDb}	1.13 ± 0.04^{Dd}	1.83 ± 0.10^{Da}
Propionic acid	0.34 ± 0.04^{Fe}	0.58 ± 0.07^{EFcd}	0.45 ± 0.06^{Cd}	0.70 ± 0.11^{EFbc}	0.54 ± 0.03^{Ed}	0.84 ± 0.05^{Eb}	0.50 ± 0.08^{EFd}	0.97 ± 0.05^{Fa}
Butyric acid	0.29 ± 0.06^{Fe}	0.47 ± 0.05^{Fcd}	0.41 ± 0.07^{Cd}	0.72 ± 0.03^{Fb}	0.60 ± 0.08^{Fcd}	0.75 ± 0.02^{Fb}	0.52 ± 0.03^{Fc}	0.90 ± 0.04^{Fa}
Total SCFA	1.35 ± 0.04^{Bg}	2.57 ± 0.12^{Bd}	1.91 ± 0.10^{Af}	2.97 ± 0.12^{Bc}	2.43 ± 0.07^{Bd}	3.36 ± 0.09^{Bb}	2.27 ± 0.08^{Be}	3.89 ± 0.13^{Ba}
Lactic acid	0.54 ± 0.10^{Df}	1.15 ± 0.07^{Dcd}	0.83 ± 0.08^{Be}	1.23 ± 0.05^{Dbc}	0.81 ± 0.10^{Ee}	1.30 ± 0.17^{Db}	0.98 ± 0.11^{Dde}	1.74 ± 0.16^{Da}
Distal colon								
Acetic acid	0.72 ± 0.06^{Cf}	1.46 ± 0.12^{Ccd}	0.87 ± 0.09^{Be}	1.53 ± 0.14^{Cc}	1.35 ± 0.10^{Cd}	1.78 ± 0.11^{Cb}	1.28 ± 0.09^{Cd}	2.10 ± 0.16^{Ca}
Propionic acid	0.40 ± 0.01^{Ee}	0.71 ± 0.08^{Ec}	0.50 ± 0.04^{Cd}	0.82 ± 0.09^{EFbc}	0.58 ± 0.05^{Fd}	0.92 ± 0.08^{Eb}	0.57 ± 0.09^{EFd}	1.17 ± 0.10^{Ea}
Butyric acid	0.37± 0.03^{EFe}	0.58 ± 0.08^{EFcd}	0.49 ± 0.06^{Cd}	0.85 ± 0.07^{Eb}	0.56 ± 0.07^{Fcd}	0.91 ± 0.07^{Eb}	0.60 ± 0.02^{Ec}	1.25 ± 0.08^{Ea}
Total SCFA	1.55 ± 0.07^{Ag}	2.94 ± 0.14^{Ad}	1.95 ± 0.11^{Af}	3.36 ± 0.08^{Ac}	2.64 ± 0.12^{Ae}	3.80 ± 0.13^{Ab}	2.61 ± 0.10^{Ae}	4.73 ± 0.15^{Aa}
Lactic acid	0.69 ± 0.12^{CDd}	1.39 ± 0.10^{Cb}	0.90 ± 0.15^{Bcd}	1.45 ± 0.09^{Cb}	0.85 ± 0.08^{Ecd}	1.49 ± 0.15^{Db}	1.02 ± 0.08^{Dc}	1.95 ± 0.13^{CDa}

Values are expressed as mean values ± standard deviation (n = 5). Means in the same rows with the same lowercase letters and means in the same columns with the same uppercase letters indicate no significant difference ($P > 0.05$).

significantly increased ($P \leq 0.05$) the levels of all SCFA compared to pure low-fat ice cream (Treatment 1). Similarly, in the study of Chaikham and Rattanasena (2017) on the effect of low-fat ice cream supplementation with probiotics (*Lactobacillus casei* 01 and *Lactobacillus acidophilus* LA5) on human colonic microflora communities, probiotic along with low-fat ice cream was shown to boost the production of SCFA, as demonstrated by the increase of all SCFA (acetate, butyrate and propionate) in both proximal and distal colon vessels of the human colon simulator compared to pure low-fat ice cream. However, the concentration of total SCFA was significantly higher ($P \leq 0.05$) in the presence of low-fat ice cream plus KMN (Treatment 3) during fermentation, expressed as 3.36±0.09 and 3.80±0.13 g/L in respective proximal and distal colon vessels, when compared to treatments of low-fat ice cream alone and the latter in combination with *B. lactis* Bb-12.

In this case, the simulated colon treatment incorporating the addition of *B. lactis* Bb-12 supplemented low-fat ice cream together with KMN (Treatment 4), was proven to be the treatment combination that can most effectively modulate the composition of all SCFA studied in this research ($P \leq 0.05$). A significant increase in SCFA concentrations was established in proximal and distal colon vessels, of 3.89±0.13 and 4.73±0.15 g/L, respectively, compared to probiotic-low-fat ice cream, low-fat ice cream with KMN and pure low-fat ice cream. There was currently insufficient research on the synergistic effects of KMN on the human colon microbiome, many dietary fibers, including pectin, have been used as prebiotics to deliver beneficiary effects to alter the gut microbial population and improve host health (Woods and Gorbach, 2001). Pectin is readily soluble in water and initiates gel formation within the gastrointestinal tract, enhancing gut microflora fermentation processes. Gel matrices increase the surface area available for enzymatic reactions and bacteria-mediated degradation processes of undigested food substances (Gibson, 2004). SCFA formed through food and nutrient fermentation by the gut microbiota confer numerous health benefits to the host. SCFA serve as nutrients for colonic epithelium, modulators of pH within intracellular and colon environments, regulators of cell volume and as enhancers of metabolic processes, such as ion transport, cell proliferation and mineral absorption (Cook and Sellin, 1998). There are a number of scientific evidence supporting the roles of SCFA as major regulators of colonic health and are known to exhibit preventative effects against gastrointestinal disorders, cancer, anti-cardiovascular diseases and suppress pathogen growth (Van Immerseel *et al*., 2010; den Besten *et al*., 2013).

Here, the impact of various treatments on lactic acid concentration in the human gut reactor was observed in

Table 3. While the control treatment (basal period) contained lowest concentrations of 0.54±0.10 and 0.69±0.12 g/L lactic acid in both proximal and distal colon vessels, respectively, all treatments in the presence of low-fat ice cream demonstrated a significant increase in lactic acid in both compartments ($P \leq 0.05$). Noticeably, while lactic acid concentrations increased with treatments of low-fat ice cream with *B. lactis* Bb-12, and of low-fat ice cream with KMN, the greatest increase in concentrations of lactic acid observed in both reactor vessels were accounted by treatments of low-fat ice cream in combination with *B. lactis* Bb-12 and KMN, which were1.74±0.16 g/L in proximal colon and 1.95±0.13 g/L in distal colon. Some of the factors contributing to higher levels of lactic acid can be accounted by the ingredients of low-fat ice cream, including sucrose and cornstarch, which served as carbohydrate substrates of colon microbiota metabolism, fermentation, and increased synthesis of lactic acid and SCFA. It is known that lactic acid is one of the major end-products of *Bifidobacterium* fermentation, along with other strains of genera *Lactobacillus* (Biavati, 2001; Pokusaeva *et al*., 2011). Thus, the inoculation of probiotics allows for increased substrate utilization to increase the yields such organic acids (Chaikham *et al*., 2012; Chaikham and Apichartsrangkoon, 2014; Chaikham and Rattanasena, 2017). Although little research has been conducted on the prebiotic properties of KMN, results suggested increased metabolic by-product yields of the simulated gut model in its presence. The presence of pectin in KMN may have contributed to this effect.

Comparing the levels of SCFA and lactic acid within the two colon compartments, the concentrations of all organic acids were significantly higher ($P \leq 0.05$) for all treatment compositions in the distal colon compared to the proximal colon. Overall, a more significant rise in levels was observed for SCFA as opposed to lactic acid. These results suggested the presence of unabsorbed organic acids, as digestion of the treatments proceed from simulated proximal (ascending and transverse) to distal (descending) colons (Chaikham *et al*., 2012). Similar trends of increase in acetate, butyrate, propionate and branched acid levels in ascending, transverse and descending colon compartments could be observed in the report of Van de Wiele *et al*. (2006) on *in vitro* prebiotic effects of longer-degree polymerized inulin-type fructans. In healthy humans, the production rate of SCFA in the proximal colon was higher than that of the distal colon due to high substrate concentration. However, the SCFA in the distal colon was higher than that of the proximal colon due to the accumulation of SCFA in the colon (MacFarlane *et al*., 1992). Subsequent depletion is defined by lower levels of SCFA production, from ~250 mM SCFA/kg fecal/48 hrs in the proximal colon to ~50 mM SCFA/kg fecal/48 hrs in the distal colon (MacFarlane and Gibson, 1995). While fermentation is predominant in the human proximal colon, trace amounts (~5%) of SCFA unabsorbed by colonocytes are transported and deposited along distal regions, and ultimately expelled via fecal matter excretion (Topping and Clifton, 2001).

3.2 Colon microbial populations and PCR-DGCE fingerprints

Enumeration and subsequent colony count determinations of specific bacterial strains and total anaerobes were conducted to observe the changes in *in vitro* gut microbial compositions under varied treatment compositions of low-fat ice cream, washout period and basal conditions. The colony units were expressed in terms of log CFU/mL colon fluid, as can be observed from Table 4. Compared to basal microbial concentrations, it was found that all treatments of low-fat ice cream (Treatments 2, 3 and 4) in both colon vessels significantly increased ($P \leq 0.05$) levels of colonic lactobacilli by approximately~1.3–1.9 log CFU/mL (proximal colon) and ~1.0–1.8 log CFU/ml (distal colon), and of bifidobacteria by ~1.3–2.4 log CFU/mL (proximal colon) and ~1.0–2.0 log CFU/mL (distal colon) as compared to basal treatment (Treatment 1). According to Chaikham and Rattanasena (2017), ingredients of ice cream provide carbon and nitrogen sources, whereas probiotic inoculation increases the utilization capacity of those aforementioned fermentation substrates.

Although supplementation of low-fat ice cream with *B. lactis* Bb-12, KMN and both in combination significantly increased ($P \leq 0.05$) lactobacilli prevalence in the gut reactor compared to pure low-fat ice cream, there was no significant difference ($P > 0.05$) in the quantities of lactobacilli among the supplementation with probiotic or KMN on their own within the proximal colon compartment (~7.9–8.0 log CFU/ml). In the distal colon vessel, however, significant increases were primarily observed in treatments containing *B. lactis* Bb-12 (Treatments 2 and 4). Combined supplementation of low-fat ice cream, probiotic and KMN yielded the highest distal colon population of beneficial lactobacilli (8.24±0.10 log CFU/ml). Similar trends were also observed for bifidobacteria levels in both vessels, which can be accounted for by direct inoculation of *B. lactis* Bb -12 in the basal nutrient medium. The greater prevalence of lactobacilli and bifidobacteria suggested synergistic effects of *B. lactis* Bb-12, and potential prebiotic effects of KMN on the growth of beneficial colon bacteria. Similarly, a study by Bianchi *et al.* (2014) showed

Table 4. Microflora communities in colon vessels after supplementation with difference treatments of low-fat ice cream.

Microbiome (log CFU/ml)	Experimental periods							
	Basal	Treatment 1	Washout 1	Treatment 2	Washout 2	Treatment 3	Washout 3	Treatment 4
Proximal colon								
Bifidobacteria	6.47 ± 0.07^{Ff}	7.75 ± 0.11^{Ac}	7.13 ± 0.04^{Ce}	8.60 ± 0.12^{Ba}	7.15 ± 0.09^{Ede}	8.13 ± 0.11^{Bb}	7.20 ± 0.03^{Ed}	8.90 ± 0.28^{Aa}
Lactobacilli	6.15 ± 0.08^{Gd}	7.40 ± 0.07^{BCb}	6.74 ± 0.05^{Ec}	7.92 ± 0.06^{Ca}	6.81 ± 0.10^{Fc}	7.90 ± 0.06^{Ca}	6.75 ± 0.15^{Fc}	8.01 ± 0.17^{Ba}
Fecal coliforms	7.89 ± 0.10^{Ca}	6.76 ± 0.09^{Eb}	8.03 ± 0.12^{Ba}	6.50 ± 0.05^{Gc}	7.95 ± 0.03^{Ba}	6.53 ± 0.04^{Gc}	7.82 ± 0.10^{Ba}	6.18 ± 0.04^{Ed}
Clostridia	7.50 ± 0.05^{Db}	7.14 ± 0.12^{Dc}	7.95 ± 0.09^{Ba}	6.95 ± 0.10^{Fcd}	7.86 ± 0.04^{Ca}	6.90 ± 0.08^{Fd}	7.85 ± 0.05^{Ba}	6.27 ± 0.13^{Ee}
Total anaerobes	8.14 ± 0.07^{Bb}	7.62 ± 0.20^{ABc}	8.45 ± 0.18^{Aa}	7.43 ± 0.02^{Dc}	8.20 ± 0.09^{Aab}	7.59 ± 0.14^{Dc}	8.30 ± 0.21^{Aa}	6.99 ± 0.07^{Cd}
Distal colon								
Bifidobacteria	6.85 ± 0.11^{Ef}	7.90 ± 0.25^{Ad}	6.96 ± 0.10^{Df}	8.84 ± 0.09^{Ab}	7.37 ± 0.06^{De}	8.44 ± 0.05^{Ac}	7.30 ± 0.08^{DEe}	8.93 ± 0.15^{Aa}
Lactobacilli	6.42 ± 0.05^{Fe}	7.43 ± 0.12^{BCc}	6.83 ± 0.13^{DEd}	8.12 ± 0.15^{Cab}	6.90 ± 0.14^{Fd}	7.98 ± 0.10^{BCb}	6.82 ± 0.05^{Fd}	8.24 ± 0.10^{Ba}
Fecal coliforms	8.01 ± 0.28^{BCa}	6.51 ± 0.07^{Fc}	7.95 ± 0.08^{Ba}	6.15 ± 0.07^{Hd}	7.83 ± 0.10^{Ca}	6.14 ± 0.04^{Hd}	7.49 ± 0.11^{CDb}	6.05 ± 0.05^{Fe}
Clostridia	8.20 ± 0.15^{Ba}	6.95 ± 0.10^{DEd}	8.03 ± 0.08^{Bab}	6.23 ± 0.13^{He}	7.90 ± 0.12^{BCb}	6.01 ± 0.10^{Hef}	7.63 ± 0.05^{Cc}	6.00 ± 0.04^{Ff}
Total anaerobes	8.58 ± 0.17^{Aa}	7.35 ± 0.04^{Cc}	8.40 ± 0.20^{Aa}	7.11 ± 0.06^{Ed}	8.12 ± 0.03^{Ab}	7.15 ± 0.07^{Ed}	8.10 ± 0.06^{Ab}	6.80 ± 0.09^{De}

Values are expressed as mean values ± standard deviation (n = 5). Means in the same rows with the same lowercase letters and means in the same columns with the same uppercase letters indicate no significant difference (P > 0.05).

enhanced prevalence and diversity of *Lactobacillus* sp. communities after feeding prebiotic and probiotic-supplemented vegetable beverages into a Simulator of the Human Intestinal Microbial and Ecosystem or SHIME reactor. According to some other previous studies, pectic oligosaccharides and pectin derived from orange and lemon peel, sugar beet pulp and kiwi fruit were observed to have excellent overall and bifidogenic prebiotic properties, as proven by increased probiotic populations and enhanced SCFA formation (Hotchkiss *et al*., 2003; Parkar *et al*., 2010; Gómez *et al*., 2014; Gómez *et al*., 2016).

Observing from the trends of change in the compositions of the simulated gut microbiome, the results in Table 4 elucidated a significant decrease (P≤0.05) in the populations of fecal coliforms, clostridia and total anaerobes upon treatment with varied compositions of low-fat ice cream compared to basal conditions (control) in proximal and distal colon vessels. While feeding of the gut reactor with low-fat ice cream resulted in the least diminishing effects on harmful bacteria populations, there were no significant differences (P>0.05) between reduced levels of aforementioned bacterium upon separate supplementations of low-fat ice cream with *B. lactis* Bb-12 and with KMN in proximal and distal colon vessels. Combined supplementation of probiotic and KMN, however, resulted in the greatest reduction in levels of clostridia, total anaerobes and fecal coliforms in both vessels. Such effects following supplementation with KMN (Treatment 3) and the latter in combination with *B. lactis* Bb-12 (Treatment 4) were not of significant difference (P>0.05). Clear correlations between increased levels of beneficial gut bacteria and organic acids (Table 3) in the simulated gastrointestinal environment can be explained by their immune-modulatory and regulative functions, and natural inhibitory effects of common gut pathogens, including *Staphylococcus aureus*, *Clostridium botulinum*, *Listeria monocytogenes*, and some harmful strains of *Escherichia coli* (Maslowski and Mackay, 2011; Chaikham *et al*., 2013). Chaikham *et al*. (2012) reported that the probiotics or/and prebiotics possibly produced low-molecular-weight antimicrobial substances (i.e. lactic acid, various SCFA and hydrogen peroxide) to inhibit the pathogenic bacteria. The study of Fooks and Gibson (2003) have shown enhanced antimicrobial effects, specifically inhibition of gut pathogens, *E. coli* and *Campylobacter jejuni*, by synbiotic treatments of *Lactobacillus plantarum* 0407 with oligofructose and *Bifidobacterium bifidum* with a mixture of oligofructose and xylo-oligosaccharides within an anaerobic fermentation system containing human feces. On a similar context, feeding of mice with probiotic *Lactobacillus helveticus* M92 and various kinds of prebiotics were shown to increase gastrointestinal populations of lactic acid bacteria, while the reduction in the levels of enterobacteria and sulphite-reducing clostridia were observed (Frece *et al*., 2009).

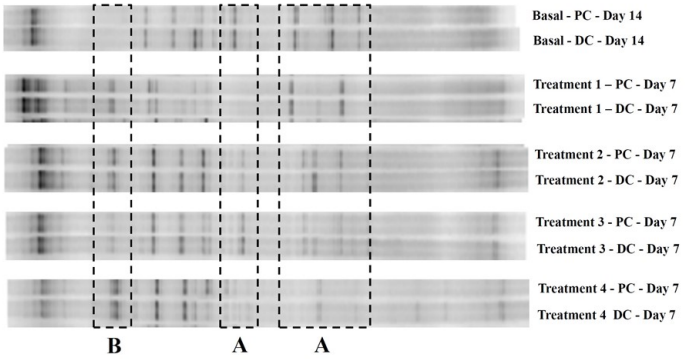

Figure 1. PCR-DGGE patterns of general colon bacteria obtained from proximal colon (PC) and distal colon (DC) compartments after feeding with different compositions of low-fat ice creams.

General colon microbial composition and population profiles were analyzed via PCR-DGCE fingerprinting of extracted bacterial DNA at Day 7 after feeding (Figure 1). Feeding of the gut reactor with pure low-fat ice cream, probiotic-supplemented and KMN supplemented low-fat ice cream has led to clear differences in PCR-DCGE profile patterns compared to the basal condition. While differing band intensities and formation of new band positions were observed in all treatments on the 7th day, the most drastic changes in band patterns occurred following treatment with combined supplementation of *B. lactis* Bb-12 and KMN in low-fat ice cream (Treatment 4). With reference to the treatment, intensities of bacterial rRNA also clearly decreased in zone A, while some bands in zone B were intensified. Generation of such profiles validated the synergism of combined effects of KMN and *B. lactis* Bb-12 supplementation on low-fat ice cream in modulating the gut microbiota. Patterns were comparable to PCR-DCGE profiles in the study of probiotic supplementations of low -fat ice cream involving a human gut reactor by Chaikham and Rattanasena (2017), where treatments of *L. casei* 01 and *L. acidophilus* LA5 strains resulted in considerable beneficial adjustments of the large intestinal microbial ecosystem. According to Bianchi *et al.* (2014), synbiotic vegetable beverages were found to induce changes in the composition and structure of microbial communities, especially lactobacilli, within stimulated colon compartments as observed from the increased number of bands in PCR-DCGE fingerprints. Similarly, Van Zanten *et al.* (2012) found that synbiotic combinations of *B. lactis* Bl-04 with melibiose, xylobiose, raffinose and maltotriose were able to shift predominant gut bacteria populations and increase their SCFA production in the colonic model system, thus may potentially be capable of manipulating the human gut microbiome. Synbiotic yogurt containing *B. lactis* Bb-12 and inulin could increase levels of bifidobacteria and reduce clostridia populations in a study of the human intestine by Palaria *et al*. (2012), using real-time PCR amplification (RT-PCR) techniques to quantify specific bacterial populations before and after yogurt treatment.

3.3 Ammonia and biogenic amines

Many strains of colon bacteria utilize urease, a metabolic enzyme, in converting nitrogenous compounds, including protein, peptides, amino acids and urea, into ammonia and biogenic amines (BA). BA are often formed via amino acid decarboxylation present in their biosynthetic pathways by various types of bacteria such as *Enterococcus*, *Escherichia* and *Morganella* strains (Mäkivuokko *et al*., 2010). Dairy products are prone to contain high levels of BAin the colon compartments due to its rich and balanced chemical composition, providing suitable conditions for microbial growth (Linares *et al*., 2011; Benkerroum, 2016). Table 5 portrays ammonia and BA formation in the colon vessels prior to and after low-fat ice cream addition. Evidently, basal concentrations of toxic nitrogenous compounds were lowest compared to low-fat ice cream treatments ($P \leq 0.05$) in both colon vessels. Highest levels of ammonia and BA accumulation were observed in the treatment of pure low-fat ice cream (Treatment 1), while cadaverine was presented at highest levels compared to other specified BA for all treatments in both colon vessels. Pure low-fat ice cream contained skim milk and gelatin, thus may serve as major protein sources for gut bacteria in the metabolic process of substrate utilization.

Following separate treatments of low-fat ice cream with *B. lactis* Bb-12 (Treatment 2) and with KMN (Treatment 3), levels of toxic metabolites were significantly reduced ($P \leq 0.05$) as compared to Treatment 1. Combined treatments of low-fat ice cream supplemented with *B. lactis* Bb-12 and KMN (Treatment 4) contributed to a further significant diminution ($P \leq 0.05$) in toxic compounds from previous treatments (~0.3 g/L and ~0.7–0.9 g/L reductions in ammonia and total BA compared to Treatment 1, respectively), although levels were still higher than basal condition (control). This may be due to the inhibitory effect of probiotic and/or KMN on the growth of BA producing bacteria. Accumulation was also greater in the distal colon vessel of the gut reactor, regardless of treatment conditions, resulting from prolonged fermentation similarly observed in the study of Chaikham and Rattanasena (2017). On the other hand, Tir Touil Meddah *et al.* (2001) observed an overall decline in ammonium concentration during SHIME treatment with *Bifidobacterium longum* whey retentate. The findings of Mäkeläinen, Forssten, Saarinen *et al.* (2010) demonstrated an increase in tyramine and cadaverine concentrations following semi-continuous colon model supplementation of some specific oligosaccharides,

Table 5. Concentrations of ammonia and biogenic amines in colon vessels after supplementation with difference treatments of low-fat ice cream.

Toxic compounds (g/L)	Experimental periods							
	Basal	Treatment 1	Washout 1	Treatment 2	Washout 2	Treatment 3	Washout 3	Treatment 4
Proximal colon								
Ammonia	0.58 ± 0.02^{Cd}	1.23 ± 0.05^{Da}	0.62 ± 0.01^{Bd}	1.15 ± 0.03^{Cb}	0.60 ± 0.06^{Cd}	1.11 ± 0.02^{Db}	0.59 ± 0.04^{Cd}	0.97 ± 0.01^{Dc}
Cadaverine	0.43 ± 0.02^{Dd}	0.75 ± 0.02^{Fa}	0.51 ± 0.04^{Cc}	0.63 ± 0.01^{Eb}	0.49 ± 0.02^{Dc}	0.62 ± 0.07^{Eb}	0.44 ± 0.03^{Ecd}	0.50 ± 0.05^{Fc}
Putrescine	0.26 ± 0.03^{Fd}	0.43 ± 0.07^{IJa}	0.28 ± 0.03^{Ecd}	0.35 ± 0.01^{Hb}	0.30 ± 0.04^{Ebc}	0.35 ± 0.05^{Hb}	0.30 ± 0.05^{FGbc}	0.31 ± 0.03^{HIbc}
Methylamine	0.25 ± 0.01^{Fd}	0.45 ± 0.04^{Ia}	0.30 ± 0.02^{Ec}	0.38 ± 0.05^{Hab}	0.27 ± 0.04^{Fcd}	0.35 ± 0.02^{Hb}	0.28 ± 0.03^{Gcd}	0.27 ± 0.02^{Icd}
Tyramine	0.19 ± 0.04^{Gd}	0.37 ± 0.05^{Ja}	0.22 ± 0.02^{Fcd}	0.32 ± 0.04^{Ha}	0.21 ± 0.01^{Gd}	0.30 ± 0.03^{Ha}	0.25 ± 0.01^{Gb}	0.24 ± 0.01^{Jbc}
Total biogenic amines	1.22 ± 0.03^{Bd}	2.13 ± 0.04^{Ba}	1.41 ± 0.03^{Ac}	1.76 ± 0.03^{Bb}	1.38 ± 0.05^{Bc}	1.70 ± 0.04^{Bb}	1.37 ± 0.02^{Bc}	1.42 ± 0.03^{Bc}
Distal colon								
Ammonia	0.64 ± 0.05^{Cd}	1.46 ± 0.08^{Ca}	0.63 ± 0.06^{Bd}	1.23 ± 0.07^{Cbc}	0.63 ± 0.02^{Cd}	1.25 ± 0.01^{Cb}	0.60 ± 0.05^{Cd}	1.17 ± 0.03^{Cc}
Cadaverine	0.47 ± 0.03^{De}	0.89 ± 0.05^{Ea}	0.57 ± 0.03^{Cd}	0.74 ± 0.03^{Db}	0.48 ± 0.04^{De}	0.73 ± 0.05^{Ebc}	0.51 ± 0.03^{De}	0.65 ± 0.04^{Ec}
Putrescine	0.32 ± 0.04^{Ec}	0.63 ± 0.01^{Ga}	0.40 ± 0.04^{Dbc}	0.50 ± 0.07^{Fb}	0.37 ± 0.05^{Ec}	0.46 ± 0.03^{FGb}	0.38 ± 0.04^{EFc}	0.34 ± 0.05^{GHc}
Methylamine	0.30 ± 0.01^{Ed}	0.59 ± 0.03^{Ha}	0.36 ± 0.02^{Dc}	0.44 ± 0.06^{Gb}	0.35 ± 0.02^{EFc}	0.43 ± 0.03^{Gb}	0.40 ± 0.07^{EFbc}	0.37 ± 0.01^{Gc}
Tyramine	0.26 ± 0.05^{EFc}	0.53 ± 0.09^{HIa}	0.29 ± 0.03^{Ec}	0.47 ± 0.05^{Ga}	0.33 ± 0.03^{EFbc}	0.50 ± 0.04^{Fa}	0.36 ± 0.01^{Fb}	0.33 ± 0.03^{GHbc}
Total biogenic amines	1.40 ± 0.04^{Af}	2.75 ± 0.05^{Aa}	1.70 ± 0.02^{Ad}	2.24 ± 0.05^{Ab}	1.62 ± 0.04^{Ae}	2.20 ± 0.03^{Ab}	1.73 ± 0.04^{Ad}	1.81 ± 0.02^{Ac}

Values are expressed as mean values ± standard deviation (n = 5). Means in the same rows with the same lowercase letters and means in the same columns with the same uppercase letters indicate no significant difference ($P > 0.05$).

while total BA levels remained mostly unchanged compared to baseline conditions. Additionally, Mäkeläinen, Ottman, Forssten *et al.* (2010) showed that probiotic *B. lactis* Bi-07 supplementation alone, or in combination with polydextrose, significantly increased the production of cadaverine and spermine, while treatments of galacto-oligosaccharide showed complete suppression and reduction in the levels of specific BA in the colon simulations. In the report of Mäkivuokko *et al.* (2010), *L. acidophilus* NCFM^{TM} plus lactitol lowered cadaverine, putrescine and total BA concentrations in the colon simulator.

While many species of probiotic bacteria are known for their therapeutic and dietetic effects, some strains of lactic acid bacteria and bifidobacteria also have the ability to decarboxylate amino acids into toxic nitrogenous metabolites (Walstra *et al.*, 2006; Chalarampopoulos and Rastall, 2009; Buňková *et al*., 2011). Although the level of BA formation by bifidobacteria is relatively low, it is contrastive to pre-existing beneficial dietary effects (Buňková *et al*., 2011). BA threatens the wellbeing of consumers by contributing to increased toxicity potential of dairy products (Lorencová *et al*., 2014). Chronic putrefaction from long-term protein fermentation in the colon increases the risks of colon cancer through secretions of ammonia and biogenic amines, which encourage neoplastic growth of colon epithelium and stimulate the production of co-carcinogenic phenols (Ouwehand *et al*., 2005). Hence, measures to reduce secretion of toxic nitrogenous compounds and bacterial proteolytic activity must be undertaken to confirm health benefits upon administration of probiotic and KMN supplemented low-fat ice cream.

4. Conclusion

Our findings demonstrated significant modulations of the simulated colon microbiota following feeding of the human gut reactor with KMN and *B. lactis* Bb-12 supplemented low-fat ice cream. The formation of beneficial SCFA (acetic, butyric and propionic acids) increased, along with lactic acid concentrations in both proximal and distal colon vessels, by Treatments 2-4 as compared to Treatment 1. Both KMN and *B. lactis* Bb-12 suppressed the ammonia formation and BA formation in the colon. The greater increase in populations of bifidobacteria and lactobacilli, along with declination in the levels of clostridia and fecal coliforms, were also

observed in combined treatments of KMN and *B. lactis* Bb-12 compared to control, pure low-fat ice cream and separate supplementations of the latter. Changes in colon microbial diversity were supported by the formation of new patterns from generated PCR-DCGE fingerprints. In summary, KMN was able to boost the effects of probiotic-low-fat ice cream in improving the gut microbiota and their metabolites.

Conflict of interest statement

No conflicts of interest exist in this study.

Acknowledgements

This project was supported by the Office of the Higher Education Commission and Thailand Research Fund (Grant No. TRG5880002). A special thanks is also given to Mahidol and Phranakhon Si Ayutthaya Rajabhat Universities for scientific facilities.

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eISSN: 2550-2166