International Food Research Journal 22(5): 2068-2077 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Digestibility and fermentation of tuna products added inulin by colonic microflora

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Article history

Received: 8 October 2014
Received in revised form: 12 January 2015
Accepted: 17 January 2015

Keywords

Inulin
Tuna product
Digestibility
Colonic microflora
Three-stage continuous system

Abstract

The development of functional tuna products (tuna in spring water and tuna salad cream) added 5% inulin were investigated. The aims of this study were to study the digestibility and *in vitro* fermentation by human fecal microbiota in three-stage continuous system. Both developed products showed prebiotic properties. They were resistant to artificial human saliva, partially resistant to acid conditions in the human stomach and resistant to human pancreatic α-amylase. The three-stage continuous system showed increase in beneficial bacteria. Bacterial enumeration, using a fluorescence *in situ* hybridization (FISH) technique, showed significant increases ($p < 0.05$) in bifidobacteria and lactobacilli. In contrast, the levels of bacteroides and clostridia were reduced. The highest prebiotic index (PI) scores of tuna in spring water and tuna in salad cream were 1.82 and 0.93, respectively. Short chain fatty acids (SCFA) were significant increased ($p < 0.05$), such as acetic, propionic and particular butyric acid. Butyric acid increased approximately 3 and 4 fold for tuna in salad cream and tuna in spring water, respectively.

Introduction

Microbiota refers to the inhabitation of a particular region of the body by a community of microorganisms. The gut microbiota is complexed and included 100 trillion archaeal and bacterial cells and over 1000 species (Gough *et al.*, 2011; Tremaroli and Backhed, 2012). It is increasingly being recognized that gut microbiota composition can be modified by changes in diet, for example by the introduction prebiotics aiming to improve or maintain host health (Roberfroid *et al*., 2010). It has a beneficial effect on the gut microbial balance and functional health in humans and animals. Probiotics could maintain gut microbiota during or after antibiotic treatment through receptor competition, competition for nutrients, epithelial inhibition, and mucosal adherence of pathogens (Clemente *et al*., 2012).

Prebiotics are compounds, which are resistant to metabolize by the host and reach the colon where they are preferentially utilized by selected groups of beneficial bacteria. Modulation of the human gut flora by prebiotic oligosaccharides has the potential benefit to human health by enhancing levels of 'beneficial' gut bacteria (Dandan *et al.*, 2008). Prebiotics are distinguished from other dietary fibers because of they have the special property on selective fermentation by bifidobacteria/lactobacilli within the gut microflora (Tuohy *et al.*, 2005). Currently there is great interest in the use of prebiotics in functional food such as low calories, diabetes and for well-being of gut health (Laparra and Sanz, 2010; Wichienchot and Chinachoti, 2011).

Inulin is a soluble fiber with a neutral taste and minimal influence on characteristics of a product. It is accepted as functional food ingredient for human foods and it has prebiotic property, low calorie, water-holding ability and emulsion capacity (Rubel *et al*., 2014; Mendoza *et al*., 2001). Addition of inulin in food product increased their dietary fiber content and promoted growth of *Bifidobacterium* spp. and *Lactobacillus* spp. (Komatsu *et al*., 2013; Adebola *et al*., 2013).

Meat production and consumption in the Asian countries is growing rapidly due to globalization of food industry and rapid economic growth (Nam and Lee, 2010). However meats are enrich with high protein and lipid. Meat-based functional foods have the opportunity to improve their image the needs of consumers. These changes affect of several ways to increase functional components such as addition of functional food ingredient, replacing a macronutrient and modification of the nature or bioactive compounds

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(Jimenez-Comenero, 2007). Thailand is currently the leading tuna product exporter in the world. However, canned or pouched tuna products in the market today are not serve to consumers who concern on heath. It is possible to develop healthier tuna products for gut health by addition of inulin. The aims of this research are to study the in vitro digestibility under simulated upper gut conditions and human fecal microflora changes after aseptically processed tuna products added 5% inulin were fermented in a three-stage continuous system. Prebiotic index, short chain fatty acids, vitamins and metabolites were also evaluated.

Materials and Methods

Materials

Tuna *(Thunnus tonggol)* meat was supplied by the Tropical Canning (Thailand) Public Company Limited, Songkhla, Thailand. The tuna meat was produced in accordance with the company's specification in the form of chuck and flake. Inulin (Orafti®-HP) was used for addition in standard formulae of tuna products. Inulin is food ingredient produced by BENEO-Orafti Company, Tienen, Belgium. The inulin was supplied by D.P.O. (Thailand), a local supplier of BENEO-Orafti Company in Thailand. All chemicals used were from the Sigma-Aldrich Company, Singapore otherwise specified.

Preparation of tuna products

Two tuna products (canned tuna in spring water and pouched tuna in salad cream) were prepared from standard formulae by addition of 5% (w/w) inulin. Preparation of canned tuna in spring water was done by dissolving of inulin powder in pre-heated (≤ 65°C) spring water before filling with tuna meat in can. Preparation of pouched tuna in salad cream was done by addition of inulin powder with other dried ingredients and filled in pouch (Rueangwatcharin and Wichienchot, 2014). The canned tuna in spring water and pouched tuna in salad cream were sterilized (retorting) by Tropical Canning (Thailand) Public Company Limited at 116°C for 70 min and at 114°C for 60 min, respectively. The finished products were then used for further study.

In vitro *digestibility*

Study of *in vitro* digestibility was carried out in simulated conditions of upper gastrointestinal tract. These simulated conditions composed of mouth digestion, gastric digestion and small intestine digestion at 37°C (Fässler *et al.*, 2006; Hur *et al.*, 2009; Phrukwiwattanakul *et al.*, 2014). The finished product sample (30 g) was mixed with 500 ml artificial saliva (HCl buffer, pH 6.8) containing (g/l); NaCl, 1.60; NH_4NO_3, 0.33; NH_2PO_4, 0.64; KCl, 0.20; $K_3C_6H_5O.7H_2O$, 0.31; $C_5H_3N_4O_3Na$, 0.02; H_2NCONH_2, 1.98 and $C_3H_3O_3Na$, 0.15. The mixture was added by human salivary α-amylase to obtain the final concentration of 0.33 unit/ml and then incubated at 37°C for 30 min. Samples were drawn at 1, 2, 3, 5, 10, 15, 20 and 30 min. The mixture after hydrolysis by human salivary α-amylase was boiled for 15 min, then cooled it down rapidly to inactivate enzyme. The mixture was added by HCl buffer containing (g/l): NaCl, 3.41; KCl, 0.40; $NaHCO_3$, 0.60; $CaCl_2.2H_2O$, 0.30 and adjusted to pH 2.0 using 1M HCl solution. The mixture was then added by pepsin to obtain the final concentration of 20.0 unit/ml and incubated at 37°C for 4 h. Samples were taken at 10, 20, 30, 60, 90, 120, 180 and 240 min. The mixture after hydrolysis by artificial human gastric juice was boiled for 15 min and cooled it down rapidly. The pH of mixture was readjusted to 6.9 using 1M NaOH solution. The mixture was added by porcine pancreatic α-amylase and pancreatic lipase to obtain final concentrations of 0.75, 1.6 unit/ml, respectively and incubated at 37°C for 6 h. Samples were taken at 1, 2, 3, 4, 5 and 6 h and terminated enzyme digestion by boiling for 15 min. Reducing sugar and total sugar content in sample was determined by DNS method (Robertson *et al*., 2001) and Phenol-sulphuric acid method (Dubois *et al*., 1956), respectively. Percentage hydrolysis of sample was calculated according to equation below based on reducing sugar liberated and total sugar content of the sample (Wichienchot *et al*., 2010). The tuna products added 5% inulin at before and after digestion were analyzed on contents of dietary fiber, fat and protein according to AOAC (2000).

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released (final – initial sugar) x 100}}{\text{Total sugar content - initial reducing sugar}}$$

In vitro *fecal fermentation*

Preparation of sample for fecal fermentation was done in similar conditions as described in *in vitro* digestibility study. The finished products of tuna in spring water or tuna in salad cream added 5% inulin (30 g) was mixed with 500 ml artificial saliva. The mixer was added by human salivary α-amylase and then incubated at 37°C for 1 min. The pH of mixer was adjusted to 2.0 using HCl solutions. The mixer was added by pepsin and incubated at 37°C for 2 h. The mixer was then added by porcine pancreatic α-amylase and pancreatic lipase and incubated at 37°C for 4 h. The mixer was coagulated suspension with 95% ethanol (1:4, v/v) to obtain final concentration

of 80% and stored overnight at 4°C. Precipitate was obtained by centrifugation at 12,000×g, 4°C for 30 min. The precipitate was then dried by freeze dryer and grounded in powder. The dried samples, of tuna products after digested under upper gut conditions as described above, were used for fecal fermentation in three-stage continuous system.

The culture medium for three-stage continuous system contained (g/l): starch from potato soluble, 20; peptone water, 20; tryptone, 20; yeast extract, 18; NaCl, 18; KCl, 18; mucin from porcine stomach, 16; casein from bovine milk, 12; pectin from citrus fruit, 8; xylan from birch wood, 8; arabinogalactan from larch wood, 8; $NaHCO_3$, 6; $MgSO_4.7H_2O$, 5; guar gum, 4; inulin from chicory, 4; cysteine.HCl, 3.2; KH_2PO_4, 2; K_2PO_4, 2; bile salt, 1.6; $CaCl_2.6H_2O$, 0.6; $FeSO_4.7H_2O$, 0.02; hemin, 0.2; Tween 80, 4 ml; vitamin K_1, 10 µl (Wichienchot *et al*., 2006).

Three-stage continuous system comprised of 3 glass vessels represented 3 parts of human colon. The controlled parameters were simulated to conditions in each part of human colon including food residual volume, pH value, anaerobic condition, temperature, and flow rate of food (Egert *et al*., 2006; Macfalane *et al.*, 1998). Fecal slurry was used as inoculum of colonic microflora. Fecal slurry was prepared from mixed fresh stool of 3 healthy volunteers who had no history of antibiotics treatment in the previous 3 months. Fecal slurry (10%, w/v) was prepared by diluting of mixed fresh stool with buffer containing 0.1 phosphate-buffered saline (PBS) solution, pH 7.2, and then were homogenized using a stomacher for 2 min and filtered via stomacher bags. Addition of 100 ml fecal slurry to each vessel (V1, V2 and V3) contained 180 ml, 200 ml and 220 ml of sterile culture medium, respectively. The pH set point (±0.1) of V1, V2 and V3 were 5.5, 6.2 and 6.8, respectively. The feed reservoir containing sterile medium was connected to the V1 via a peristaltic pump that was working at a flow rate of 28±2 ml/h. The entire system was maintained anaerobic by flushing with O_2-free N_2 gas and controlled at 37°C using a circulating water bath. The sterile medium in feed reservoir and vessels were magnetically stirred. The vessels were left for 24 h in batch culture mode before fresh sterile medium from feed reservoir fed into the system continuously. System was continuous fermented for 10 days until it reached steady state, i.e. the condition where there was no change in both genus and amount of microorganisms. Samples were collected at day 0 and 10 then dried sample of canned tuna in spring water or pouched tuna in salad cream added 5% inulin after digested by artificial conditions of upper gut was added in V1 for 8 g/day from day 11 to 20. Samples were collected to count numbers of microorganisms with genus Bifidobacteria, Bacteroides, Lactobacillus/Enterococcus, Clostridia and Eubacteria using fluorescence *in situ* hybridization (FISH) technique (Macfarlane *et al.*, 1998). Short chain fatty acids (SCFA) in samples were analyzed by high performance liquid chromatography (HPLC) (Rycroft, 2001). Finally, prebiotic index (PI) of each sample was calculated according to the following equation (Palframan *et al*., 2003).

$$\text{Prebiotic index (PI)} = \alpha+\beta-\gamma-\delta$$
$$\alpha=(Bif_{21}/Bif_{11})/\text{ Total}$$
$$\beta=(Lac_{21}/Lac_{11})/\text{ Total}$$
$$\gamma=(Bac_{21}/Bac_{11})/\text{ Total}$$
$$\delta=(Clos_{21}/Clos_{11})/\text{ Total}$$

Total = total numbers of eubacteria on day 21/total numbers on day 11
α = change in numbers of bifidobacteria at day 11 and 21
β, = change in numbers of lactobacilli at day 11 and 21
γ = change in numbers of bacteroides at day 11 and 21
δ = change in numbers of clostridia at day 11 and 21

Enumeration of colonic bacteria by fluorescent in situ *hybridization (FISH) technique*

Sample (375 µl) was mixed with 4% (w/v) paraformaldehyde solution (pH 7.2) and stored at 4°C over night in order to immobilize the bacterial cells. The immobilized cells were rinsed with PBS twice and dissolved into 150 µl PBS and 150 µl ethanol. The sample was stirred well and stored at -20°C for at least 1 h or until use but it should not be kept longer than 3 months. The immobilized cells were diluted to an appropriate concentration. Samples (20 µl) were transferred by pipette onto the surface of a microscope slide that was coated with TEFLON or poly-L-lysine. The slide was warmed in a slide dryer at 45°C for 10-15 min until the sample was dried. The slide was dipped into 50%, 80% and 96% (v/v) ethanol respectively for 3 min each in order to weaken the bacterial cell wall/cell membrane so that a DNA probe could conglomerate with the DNA bacteria. The slide was dried in a slide dryer again.

Then 45 µl buffer was mixed into 5µl of a specific DNA probe solution (50 mg/µl) that was suitable for the type of bacteria that had to be counted. Next, 50 µl mixture of buffer and probe was placed on a slide surface prepared earlier. The slide was incubated in a hybridization oven at suitable temperature (48°C for Bac303 and Lab158 and 50°C for Bif164, Chis150 and Eub338) for 4 h. Then the slide was immersed in wash buffer immediately at the same temperature of hybridization for 15 min. The slide was then dipped into distilled water for 2 to 3 sec, dried immediately

by blowing with cleaned air. And 5µl of antifade solution was added to the surface of the slide. The sample was enumerated on bacterial cell numbers using a fluorescence microscope for 15 fields per sample. Each bacterial count (cells/ml) was reported according to the DNA probe used in an average number of 15 counts.

Changes in human faecal microflora were assessed by fluorescent *in situ* hybridization with DNA probes. These DNA probes, were specific for hybridization to their respective 16S rRNA region predominant classes of the gut microbiota. They were manufactured and tagged with the fluorescent marker Cy3. The DNA probes used in the study were Bif164 (CATCCGGCATTACCACCC), Bac303 (CCAATGTGGGGGACCTT), Chris150 (TTATGCGGTATTAATCT(C/T)CCTTT), Lab158 (GGTATTAGCA(T/C)CTGTTTCCA) and Eub338 (GCTGCCTCCCGTAGGAGT), and were specific for bifidobacteria, bacteroides, clostridia, lactobacilli and eubacteria (represented total number), respectively (Rycroft, 2001).

Analysis of short chain fatty acids and vitamins

Samples taken from the three-stage continuous system were analyzed by HPLC with UV detector for short chain fatty acids (SCFA), including acetate, propionate and butyrate. Samples were also analyzed for vitamin B_2 and folic acid by HPLC. Samples (2 ml) from three-stage continuous system were centrifuged at 17,000×g, 4°C for 15 min to separate the microbial cells. Cell-free samples were filtered through a 0.2 µm nylon filter. Samples were analyzed for SCFA using a HPLC, Bio-Rad Aminex HPX-87 H ion exclusion column with 7.8 mm diameter and 300 mm length at 50°C. Mobile phase and flow rate were 0.005 M H_2SO_4 and 0.6 ml/min, respectively. A UV detector at 215 nm was used to determine amount of SCFA in the peak area, compared to the peak with the same retention time to respective standard using the Chemstation program. Standard concentrations of each short chain fatty acid including acetic acid, propionic acid and butyric acid were prepared at 0, 1, 10, 20, 40, 80 and 100 mM (Olano-Martin *et al*., 2000).

Vitamin B_2 and folic acid were analyzed by HPLC using Inertsil Diol column with 4.6 mm diameter and 250 mm length at 40°C. A UV detector was set to 254 nm and mobile phase [CH_3CN:H_2O:TFA (90:10:0.1)] was eluted at flow rate of 1.0 ml/min. Qualification and quantification was done by comparison of the peak area and retention time to respective standards using the Chemstation program. Standard concentrations of vimamin B_2 and folic acid were 0, 5, 25, 50, 100 mM (Hongpattarakere *et al*., 2012).

Analysis of metabolites by gas-chromatography-mass spectrometry

Samples (2 ml) from V3 were centrifuged at 17,000×g, 4°C for 15 min to separate the microbial cells. Cell-free samples were filtered through a 0.2 µm nylon filter. Samples were analyzed by gas-chromatography-mass spectrometry (GC–MS), Trace GC Ultra/ISQ MS, Thermo Scientific in a Starbilwax column (30.0 m × 0.25 mm × 0.25 µm). The temperature program began at 50°C hold for 5 min, ramp to 245°C and decreased at a rate of 7°C min^{-1} hold for 5 min. Solvent delay time was 3 min. The temperatures of the MS source and transferline were 240°C and 240°C respectively with a flow rate of 1.0 mL/min. Qualification of each metabolite was done by comparison to chemical compound in library of database.

Statistical analysis

The difference between the bacterial counts was tested for significance using ANOVA for the comparison between treatments. All statistical analyses were performed with SPSS version 17.0. The difference was considered at a statistically significant ($p<0.05$).

Results and Discussion

Effect of artificial human saliva hydrolysis

In vitro digestion models are widely used to study the structural changes, digestibility, and release of food components under simulated gastrointestinal conditions. The digestibility of tuna in spring water and tuna in salad cream added 5 % inulin was carried out *in vitro* digestion. Initially, the digestion used artificial human saliva which contained human salivary α–amylase before passing through artificial gastric juice and pancreatic α–amylase. The percentage of hydrolysis increased with increasing incubation time (Figure 1a). The degree of hydrolysis of tuna in spring water added 5% inulin at 1, 2, 3, 5, 10, 15, 20 and 30 min was 0.01, 0.02, 0.03, 0.04, 0.12, 0.66, 1.05 and 1.24%, respectively. The maximum hydrolysis of 1.24% occurred at 30 min. The degree of hydrolysis of tuna in salad cream added 5% inulin was 0.02, 0.07, 0.09, 0.12, 0.31, 0.72, 1.20 and 1.20%, respectively. It has been reported that oligosaccharides were decreased by human saliva hydrolysis using human salivary α–amylase. Both of tuna products added 5% inulin had approximate 98.8% resistant to human salivary α–amylase. However, it was lower resistant than that

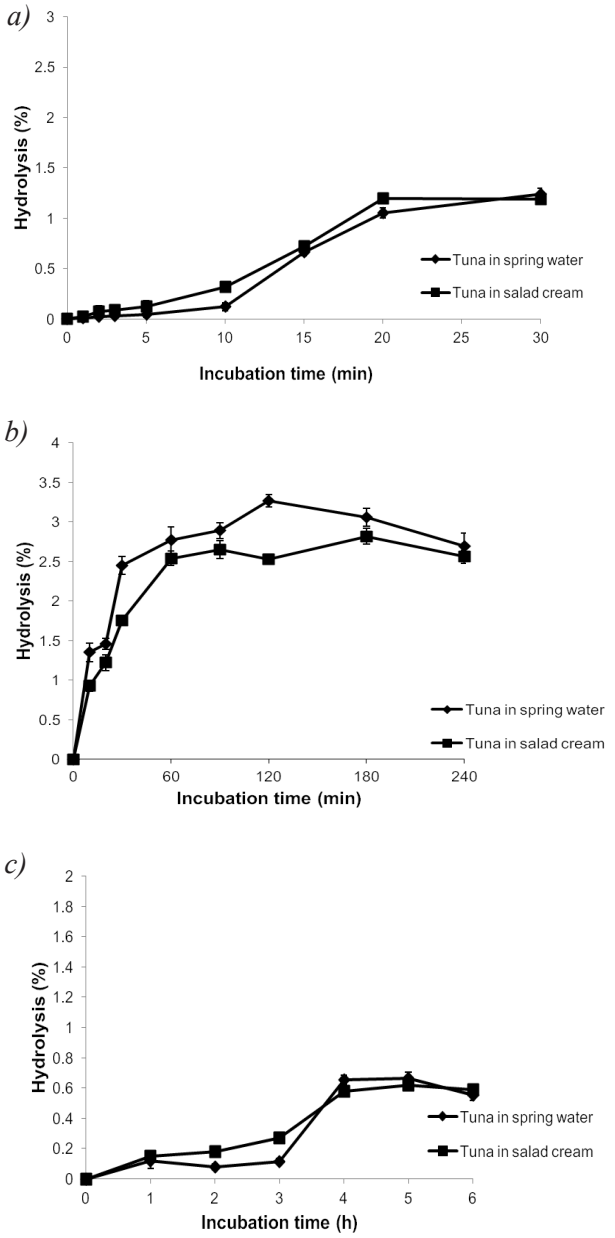

Figure 1. Percentage of hydrolysis of aseptically processed tuna products added 5% inulin by (a) human salivary α-amylase for 30 min and pH 6.8; (b) artificial gastric juice for 4 h and pH 2.0 and (c) human pancreatic α-amylase for 6 h and pH 6.9 at 37°C

of inulin in similar conditions (Thungchoho, 2012). In addition, the compositions of tuna in salad cream contained not only inulin but some carbohydrates including dietary fiber from wheat and corn flours and vegetables. So that hydrolysis is due to digestion of these carbohydrate ingredients not inulin.

Effect of hydrolysis of artificial human gastric juice

The hydrolysis of artificial human gastric juice at pH 2.0 was shown in Figure 1b. The percentage of hydrolysis increased with increasing incubation time ($p < 0.05$). The degree of hydrolysis of tuna in spring water added 5% inulin at 10, 20, 30, 60, 90, 120, 180 and 240 min was 1.35, 1.46, 2.45, 2.77, 2.89, 3.27, 3.06 and 2.69%, respectively. The maximum hydrolysis (3.27%) occurred after 120 min of incubation. In previous report, the maximum hydrolysis of pitaya oligosaccharides (4.07%) occurred after 120 min of incubation at pH 1.0 (Wichienchot *et al*., 2010). The degree of hydrolysis of tuna in salad cream added 5% inulin was 0.93%, 1.22, 1.75, 2.54, 2.65, 2.53, 2.82 and 2.56%, respectively. The results showed that the degree of hydrolysis increased rapidly within 50 min and slightly increased thereafter.

Mixed oligosaccharides obtained by 80% ethanol extraction from white-flesh dragon fruit were hydrolyzed with artificial human gastric juice and its showed resistant to the juice. The percentage of hydrolysis increased with a decreasing pH of the artificial gastric juice (Wichienchot *et al*., 2010). Tuna products added 5% inulin showed approximate 97.0 % resistant to simulated conditions in human stomach. In addition, the tuna products showed slightly higher gastric juice resistant compared to inulin which gave the maximum resistance of 91.1% (Thungchoho, 2012).

Effect of hydrolysis of human pancreatic α-amylase

Tuna in spring water and tuna in salad cream added 5% inulin could withstand human pancreatic α-amylase digestion. The maximum hydrolysis of 0.67% (tuna in spring water) and 0.62% (tuna in salad cream) was observed after 4 h incubation (Figure 1c). Tuna products with the addition of inulin showed prebiotic properties since it had high resistant to artificial human saliva, gastric juice and pancreatic α–amylase. The resistances to simulated human conditions in the mouth, stomach and small intestine were 1.24, 3.27 and 0.67% respectively. Jackfruit oligosaccharides could withstand human pancreatic α-amylase digestion which showed on maximum hydrolysis to be 9.73% (Wichienchot *et al*., 2010).

The resistance of tuna products added 5 % inulin in stimulated upper gut conditions was investigated. It were 95.86% of tuna in spring water and 95.36% of tuna in salad cream would reach the colon. It has been reported that 88% of inulin and oligofructose reach the colon, using both the ileostomy patient model and the incubation model (Cummings and Macfarlane, 2002). It has also been reported that 82.76 % of inulin would reach the colon (Thungchoho, 2012). Typically, inulin is resisted to hydrolyze by acid in stomach and by human digestive enzymes. Digestive enzymes secreted by the pancreas, and of mammals in particular, are unable to hydrolysis β-glucosidic bonds (Loo, 2006). Therefore, tuna products added 5 % inulin in this study appeared to have partial

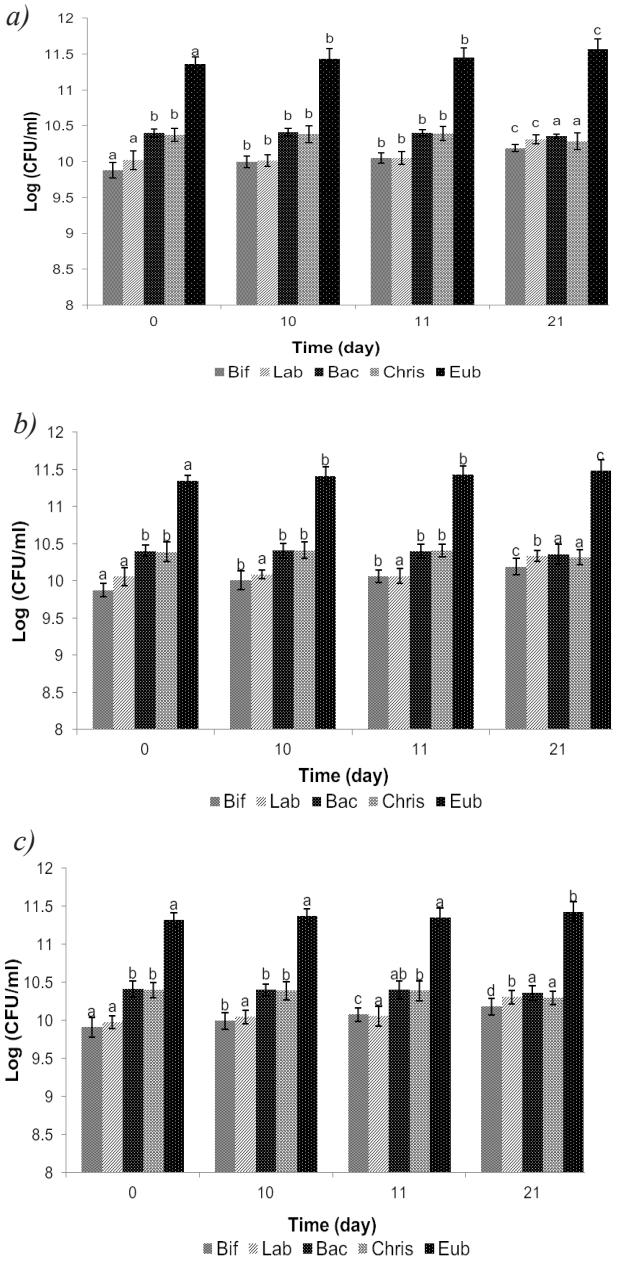

Figure 2. Numbers of faecal bacteria in three-stage continuous system in (a) vessel 1, (b) vessel 2 and (c) vessel 3 by fermentation of tuna in spring water added 5% inulin

Values with different superscipts within the same bacterial species mean it has significantly difference (p<0.05).

enzymatic resistant.

Composition of the finished tuna products

It was found that tuna in spring water added 5% inulin before digestion consisted of dietary fibre, fat and protein in the concentrations of 1.08, 0.13 and 86.07% dry basis, respectively. And after digestion though simulated conditions in upper gut, the concentrations were 1.15, 0.08, and 18.28%, respectively. Composition of tuna in salad cream added 5% inulin before digestion were 8.84, 14.17, and 28.61% (dry basis), respectively. And after digestion were 8.05, 2.18 and 5.97%, respectively. This indicated that protein is a major component that it was digested followed by fat whereas dietary fiber seemed to be less affected.

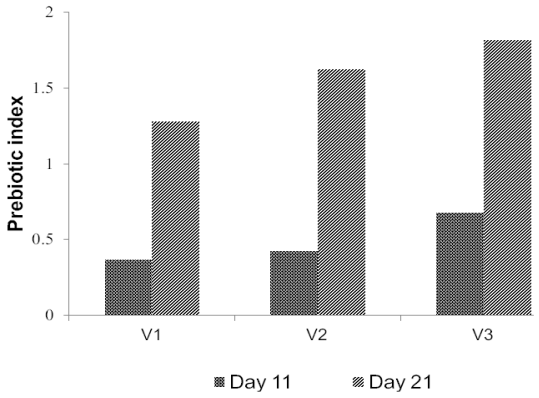

Figure 3. Prebiotic index (PI) scores of tuna in spring water added 5% inulin in vessel 1, 2 and 3 of three-stage continuous system

Bacterial change in three-stage continuous system

An *in vitro* model of human large intestine was used to investigate the effects of inulin addition in the tuna products on gut microflora. The experiments were carried out for 21 days and samples were collected at day 0, 10, 11 and 21 and specific genus of bacteria was enumerated by FISH technique. The numbers of bifidobacteria, lactobacilli, clostridia, bacteroides and eubacteria were given in Figure 2. It was found that the numbers of eubacteria were highest and it is a representative of total bacteria. Bifidobacteria and lactobacilli were measured because it is important genera due to numerous purported beneficial effects, including saccharolytic fermentation (Roberfroid *et al.*, 2010; Bouhnik *et al.*, 2004). Tuna in spring water and tuna in salad cream added 5 % inulin showed significantly (p<0.05) increase in bifidobacteria and lactobacilli. Furthermore, the clostridia and bacteroides were decreased at day 21. Many reports confirmed that inulin selectively stimulate the growth of bifidobacteria and lactobacilli in *in vitro* and *in vivo* studies (Gibson *et al.*, 2004; Hartzell *et al.*, 2013). It has been reported that highly soluble inulin (HSI) and oligofructose (OF) decreased the bacteroides, cluster I clostridia, cluster XIV clostridia and *E. coli* and increased beneficial bifidobacteria and lactobacilli (Vardakou *et al.*, 2008).

Prebiotic index (PI)

The changes in selected bacterial populations that presented prebiotic index are shown in Figures 3. The results showed that the highest prebiotic effect was found in tuna in spring water added 5% inulin. The prebiotic index (PI) of tuna in spring water added 5%

Table 1. Short chain fatty acids (SCFA) production by fermentation of tuna in spring water added 5% inulin in three-stage continuous system

Day	Acetic (µg/ml)			Propionic (µg/ml)			Butyric (µg/ml)		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
0	21.43±1.47^{b}	30.28±1.25^{c}	36.57±1.31^{c}	15.52±1.07^{b}	2.07±0.32^{b}	3.05±0.41^{b}	7.61±0.42^{c}	11.79±1.27^{c}	13.78±0.79^{d}
10	38.77±2.62^{a}	71.87±2.42^{b}	98.60±2.79^{b}	21.33±0.12^{a}	14.13±1.97^{a}	13.73±1.32^{a}	23.53±0.81^{b}	27.95±2.64^{b}	31.11±1.62^{b}
11	38.03±1.51^{a}	68.81±2.68^{b}	90.98±2.57^{b}	25.28±2.36^{a}	12.25±1.34^{a}	15.35±1.59^{a}	25.10±2.26^{b}	23.03±1.49^{b}	26.58±2.85^{c}
21	43.42±1.30^{a}	95.98±2.18^{a}	128.32±2.18^{a}	28.12±2.43^{a}	13.06±1.52^{a}	17.83±1.22^{a}	35.35±2.35^{a}	67.14±2.74^{a}	51.29±2.71^{a}

Values with different superscipts within the same column mean it has significantly difference (p<0.05)
Inoculum = 10% fecal slurry contained acetic acid, propionic acid and butyric acid at concentrations of 16.02, 2.52 and 4.58 µg/ml, respectively)

Table 2. Short chain fatty acids (SCFA) production by fermentation of tuna in salad cream in three-stage continuous system

Day	Acetic (µg/ml)			Propionic (µg/ml)			Butyric (µg/ml)		
	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3	Vessel 1	Vessel 2	Vessel 3
0	5.06±0.27^{c}	20.84±1.18^{c}	16.17±1.52^{c}	1.54±0.68^{c}	1.31±0.16^{c}	n.d.	16.08±1.28^{c}	22.22±0.32^{c}	26.76±0.72^{a}
10	59.70±1.14^{a}	68.84±1.09^{b}	68.04±2.26^{b}	4.87±1.16^{a}	5.82±0.57^{b}	10.66±0.51^{a}	57.01±2.86^{b}	42.26±0.19^{b}	34.91±0.63^{a}
11	54.11±1.62^{b}	76.10±2.35^{a}	71.07±1.76^{b}	3.12±0.57^{a}	7.65±0.39^{a}	10.70±1.03^{a}	61.45±1.34^{b}	36.25±1.41^{b}	49.28±0.17^{b}
21	52.54±2.33^{b}	75.70±1.24^{a}	93.34±3.65^{a}	2.73±0.79^{b}	3.80±0.18^{b}	4.30^{b}±0.42^{b}	94.13±3.36^{a}	66.80±2.24^{a}	65.85±0.50^{a}

Values with different superscipts within the same column mean it has significantly difference (p<0.05)
*n.d. = not detected
Inoculum = 10% fecal slurry contained 4.53 µg/ml butyric acid

inulin in vessel 1, 2 and 3 was 1.28, 1.63 and 1.82, respectively. The prebiotic index of tuna in salad cream added 5% inulin in vessels 1, 2 and 3 was 0.93, 0.73 ,and 0.77, respectively. The result give a positive prebiotic index of both tuna products added 5% inulin. Thus addition of 5% inulin to standard formulae of tuna product resulted in significantly (p<0.05) increase of beneficial colonic bacteria. It has been reported that batch fermentation of 10% inulin (w/v) for 24 h was approximately 1.0 (Ghoddusi *et al.*, 2007). The higher PI value (2.03) obtained from arabinoxylans by fecal batch culture (Vardakou *et al.*, 2008). Tuna in salad cream added 5% inulin had lower PI score than tuna in spring water added 5% inulin. This is because the inulin (fructan) content in finished product of tuna in salad cream less than in spring water. Tuna in salad cream has lower pH than tuna in spring water. So that during steam sterilization inulin in tuna in salad cream has more hydrolyzed to short chain inulin. It is resulted in tuna in salad cream added 5% inulin had high PI in V1 where low molecular weight prebiotic substance is readily taken up and metabolized by the saccharolytic bacteria. In contrary, inulin in tuna in spring water has less hydrolyzed thus higher molecular inulin could reach distal colon in V3.

Production of SCFA, vitamin B_2 and folic acid

The concentration of each short chain fatty acid (SCFA) is shown in Tables 1 and 2. Acetic, propionic and particular butyric acid were increased when fermentation time increased (p<0.05). However, no production of propionic acid in tuna in salad cream added 5% inulin. The SCFA was increased according to an increase of bifidobacteria and lactobacilli. These colonic bacterial are predominant members of the gut microbiota that are able to produce acetate and lactate which can be converted to butyrate and propionate (Duncan *et al.*, 2002). Pompei *et al.* (2008) reported that acetic and lactic acids were the major end products of fructan fermentation. And production of acid was much higher in cultures containing fructans than in the control cultures. In this study, there was no production of vitamin B_2 and folic acid during fermentation of either tuna in spring water and tuna in salad cream added 5% inulin (data not shown). However, Beitane and Ciproica (2011) found that the added inulin concentrations significantly influence the contents of vitamin B_1 and B_2 (p<0.05) in fermented milk.

Metabolites production in three-stage continuous system analyzed by GC-MS

Metabolic profile of tuna in spring water added 5% inulin after fermentation for 21 days in V3 of three-stage continuous system was investigated. The metabolites were butyric acid, butyric acid 2-methyl, acetic acid, pentanoic acid, pentanoic acid 4-methyl, piperidinone, propanoic acid, propionic acid 2-methyl, hexanoic acid and phenol 4-methyl. As the inulin were fermented over the course of 24 h, additional acetate, propionate, and butyrate were produced by the bacteria (Haenen *et al*., 2013). Surprisingly, tuna in spring water added 5% inulin after fecal fermentation produced phenols. This could be explained that tuna in spring water has slightly high content of non-digestible protein (18.28%). And after it is fermented by fecal proteolytic bacteria then phenols, indoles, amines and ammonia are generated during the catabolism of amino acids (Tamara *et al*., 2006). Metabolites of tuna in salad cream added 5% inulin were acetic acid, propionic acid, propionic acid 2-methy, butyric acid, butyric acid 2-methyl, pentanoic acid, pentanoic acid 4-methyl, hexanoic acid and piperidinone. Profile of metabolites produced during fecal fermentation depends on the composition of substrate. In this study confirmed that substrate containing high amount of non-digestible protein resulted in production of phenolics and amines derivatives.

Conclusions

This is a study of 2 standard formulae for canned tuna in spring water and pouched tuna in salad cream with added 5% inulin (w/w) on gut health benefits. In vitro digestion of simulated upper gastrointestinal conditions found that both products were significantly (p<0.05) resistant to digestive enzyme salivary α-amylase in artificial saliva, and partially resistant to acid conditions in the human stomach and resistant to human pancreatic α-amylase. The fermentability of both tuna products in three-stage continuous system showed increase in bifidobacteria and lactobacilli (p<0.05). Furthermore, the levels of bacteroides and clostridia were significantly (p<0.05) reduced. The short chain fatty acids produced were acetate, propionate and notably butyric acid which had potential for cancer prevention. The canned tuna in spring water and pouched tuna in salad cream added 5% inulin after digested by simulated upper gut conditions showed a positive prebiotic index and stimulated the growth of bifidobacteria and lactobacilli. So that these tuna products added 5% inulin showed prebiotic properties and benefits to human gut health.

Acknowledgements

This work was supported by the research grants from the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand.

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