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Nutritionally-complete formula fortified with isomalto-oligosaccharide for hemodialysis patients

Kridsada Keawyok[1], Nualpun Sirinupong[2] and Santad Wichienchot[2*]

[1]Functional Food and Nutrition Program, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand; [2]Center of Excellence in Functional Foods and Nutraceuticals, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

***Corresponding Author**: Santad Wichienchot, PhD., Center of Excellence in Functional Foods and Nutraceuticals, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

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ABSTRACT

Background: Recent studies have demonstrated that chronic kidney disease (CKD) is associated with dysbiotic gut microbiota. Therefore, the gut microbiota may be used as a biomarker and therapeutic agent to improve the health of CKD patients.

Objective: To study the probiotic selectivity of isomalto-oligosaccharide (IMO) produced from tapioca starch and its application in a novel nutritional formula developed for hemodialysis patients.

Methods: IMO was produced from tapioca starch in a pilot-scale reactor. The selectivity of IMO for probiotic strains was studied. Among six probiotic strains tested, two probiotic strains were selected for the development of a nutritionally-complete formula for CKD patients.The sensory attributes, probiotic survival and decapsulation, and the microbiological quality of the formula were evaluated.

Results: IMO showed prebiotic properties by promoting the growth of pure cultures of the probiotics tested. The IMO powder and the commercial probiotics, *Lactobacillus paracasei* and *Bifidobacterium animalis* were used as ingredients to develop a nutritional formula for CKD patient with an energy distribution from protein:carbohydrate:fat of 18.43:35.29:43.27 and a total energy per 80 g serving of 400 kcal. It was found that 89.47% of the encapsulated probiotics in the formula survived gastrointestinal tract conditions and 96.90% of the probiotics were released after exposure to phosphate-buffered saline (pH 7.2) for 6 h. The sensory evaluation of the nutritional product showed no significant difference ($p > 0.05$) in its viscosity, taste,

sweetness, saltiness and overall characteristics compared to a commercial product (Nepro®).The product also met relevant standards for the microbial quality of food products containing probiotics.

Conclusion: The nutritionally complete formula developed, fortified with a synbiotic combination of IMO and probiotics successfully met the requirements of the WHO and the Thai FDA regulation. This novel formula for CKD patients is the first reported with fortification by probiotics and an IMO prebiotic.

Keywords: chronic kidney disease, isomalto-oligosaccharide, hemodialysis patient, nutritional formula, probiotic

BACKGROUND

Complex communities of microorganisms, termed the microbiota, are present on the body surfaces of virtually all vertebrates. In the lower intestines, these organisms reach extraordinary densities and have evolved to degrade a variety of plant polysaccharides and other dietary substances including non-digestible components in the upper gut [1]. This both enhances the host's digestive efficiency and ensures a steady nutrient supply for the microbes. Metabolic efficiency appears to have acted as a potent selective force that shaped the evolution of both sides of the host-microbiota relationship. Millions of years of co-evolution, however, have forged pervasive interconnections between the physiologies of microbial communities and their hosts that extend beyond metabolic functions. Thus, the interconnections between the gut, the microbiota, and the brain have recently been studied since there is evidence that they are linked not only to metabolic syndromes, but also to degenerative diseases such as Alzheimer's and Parkinson's, which can be modulated by dietary consumption, [2, 3, 4].

The gut microbiota is essential for regulating the normal functioning of the intestinal barrier. It promotes immunological tolerance to antigens from nutrients or organisms, controls nutrient uptake and metabolism, and prevents the propagation of pathogenic organisms [5]. Hence, the concept has emerged that dysfunction of the intestinal microbiota may play a significant role in cancer, metabolic and inflammatory digestive diseases, and recently, it has been demonstrated that chronic kidney disease (CKD) is associated with dysbiosis of the gut microbiota [6, 7, 8, 9, 10]. In CKD patients, the utilization of therapies modulating the gut microbiota, such as probiotics, has emerged as an attractive strategy to reduce uremic retention solutes (URS) and to improve cardiovascular disease. Probiotics are defined by the World Health Organization (WHO) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [11]. Probiotics are being increasingly used for various pathologic conditions, such as constipation and diarrhea. However, not all probiotic strains are beneficial in all circumstances and the careful selection of specific organisms based on the desired clinical outcome is crucial.

Over recent years, considerable experimental and clinical data has reinforced the hypothesis that

probiotics have a therapeutic role in maintaining a metabolically balanced gastrointestinal (GI) tract, reducing the progression of CKD and the generation of URSs [11]. However, it has been demonstrated that in CKD patients, a compensatory mechanism occurs, due to failure of the nephrons. The aim of this mechanism is the elimination of waste products and the preservation of electrolytes and it involves the colon as a replacement excretion system. A massive urea discharge with uric acid and oxalate epithelial secretion occurs [12], which alters the colonic microenvironment and consequently affects the gut microbial population [13].

In addition to microbiota modeling in CKD, other studies have reported that hemodialysis patients should have a small but significant dietary fiber intake in order to provide an important source of fermentable carbohydrates in the colon [9,10,14]. Moreover, in such patients, altered protein assimilation in the small intestine with a consequent increase in the abundance of dietary protein bio-availability in the colon has been observed. This leads to a decreased amount of carbohydrate being available in the large intestine, favoring a switch from a saccharolytic to a proteolytic catabolism. In this mechanism, bacteria hydrolyze urea, causing a high ammonia concentration and an alkaline pH, which in turn favors proteolytic species proliferation [15]. Protein fermentation leads to the generation of different waste metabolites, such as phenols and indoles, mainly represented by *p*-cresol and indoxyl sulphate, which are known to be the main uremic toxins found in CKD patients which promote the progression of the disease.

In addition, besides being involved in metabolic processes in health and disease, the microbiota could also explain inflammatory and oxidative co-morbidities found in CKD patients [16]. Increased intestinal permeability allows bacterial translocation, which is responsible for endotoxemia. Endotoxin is a potent immune system activator which induces the inflammatory cascade and leads to low-grade systemic inflammation. Therefore, CKD is associated with dysbiosis of the gut microbiota, which in turn increases the progression of CKD [7, 9, 11].

The study aimed to develop a nutritional formula fortified with synbiotics based on isomalto-oligosaccharide (IMO) for hemodialysis patients. The selectivity of IMO by probiotic strains was investigated, and a synbiotic formula for CKD patients was developed which was then used as a component in a nutritional product. The sensory qualities of the product and its ability to release probiotics (decapsulation), probiotic survival and the microbiological quality of the formula were all evaluated.

MATERIALS AND METHODS

Preparation of isomalto-oligosaccharide from tapioca starch: IMO powder was produced in a 72-liter pilot-scale reactor by an enzymatic method, the details of which have been patented. Briefly, it was composed of three steps: liquefaction by α-amylase, saccharification by β-amylase, and transglucosylation by transglucosidase. First, tapioca starch was prepared as a slurry and gelatinized, then α-amylase was added. Beta-amylase and transglucosidase were added thereafter under optimal temperatures and reaction periods. The IMO syrup was purified by nanofiltration (MWCO 200 Da) to remove mono- and di-saccharides. Spray drying of the purified IMO syrup was performed using a laboratory spray dryer (B290 model, Buchi, Flawil, Switzerland). The equipment was operated concurrently and a spray nozzle with an orifice of 0.7 mm in diameter was

used. The flow rate (5 mL/min) of sample solution was fed into the drying chamber through a peristaltic pump and the dried product was collected. The inlet and outlet air temperature were maintained at 180 °C and 90 °C, respectively. The aspiration was maintained at 100% and cyclone air flow rate was 30 m^3/h.

Selectivity of IMO by probiotic strains

Probiotic microorganisms: The probiotic strains were carefully selected based on their potential effects on CKD, their commercial availability, and their conformance with the Thai FDA regulations relating to probiotics for food use. All the probiotics used in this study were in the form of encapsulated powders. The six probiotic strains used were:

1) *Lactobacillus acidophilus*
2) *Lactobacillus paracasei*
3) *Lactobacillus rhamnosus*
4) *Bifidobacterium longum*
5) *Bifidobacterium infantis*
6) *Bifidobacterium animalis* subsp *lactis* (BB-12®)

Cultivation media: A complex agar medium capable of supporting good growth of *Lactobacillus* was used in this study. It was prepared based on the formulation developed by de Man, Rogosa and Sharpe (MRS), containing (per liter) D-glucose (20 g), bacteriological peptone (10 g), beef extract (8 g), sodium acetate (5 g), yeast extract (4 g), dipotassium phosphate (2 g), ammonium citrate (2 g), Tween 80 (1 g), magnesium sulfate (0.2 g), manganese sulfate (0.05 g), and agar (10g) [17]. All the ingredients were thoroughly mixed and dissolved by heating for 1 min with frequent agitation until completely dissolved. Then, bromocresol purple (0.02%) was added to the agar medium. For *Bifidobacterium,* the medium was prepared using the same formula, but L-cysteine-hydrochloride (0.05%) was added. The medium was sterilized in an autoclave at 121 ºC for 15 minutes, then cooled to 45-50 ºC and dispensed onto plates. The prepared medium was stored at 2-8 °C. The color of the medium was amber with slight opalescence.

Preparation of probiotics: Three *Lactobacillus* strains, comprising *L. acidophilus, L. paracasei* and *L. rhamnosus* were sub-cultured by putting the lyophilized cultures into MRS broth and incubating them at 37 ºC for 24 h to obtain a starter culture (inoculum). A modified MRS with IMO produced from tapioca starch (hereafter, tapioca IMO) as a carbon source was also prepared. The starter culture (0.5 mL) was inoculated into the modified MRS broth (9.5 mL) and incubated at 37 ºC for 48 h. Samples were taken at 0, 12, 24, and 48 h. Serial dilution was performed using the pour-plate method. A sterile plate was inoculated with 1 mL of inoculum and sterile MRS agar was poured onto it then swirled, and incubated at 37 ºC for 48 h. The number of colonies growing on the surface of the solidified medium was counted and reported as CFU/mL.

Three strains of *Bifidobacterium* comprising *Bifidobacterium longum*, *Bifidobacterium infantis,* and *Bifidobacterium animalis* subsp. *lactis* were sub-cultured and inoculated into the MRS broth modified by the addition of L-cysteine-hydrochloride (0.05%). The medium was incubated at 37 ºC under anaerobic conditions for 48 h to obtain an inoculum.

For the preparation of modified MRS with tapioca IMO as a carbon source, the starter culture (0.5 mL) was inoculated into MRS broth modified by adding L-cysteine-hydrochloride (9.5 mL), and incubated at 37 ºC for 72 h. Samples were taken at 0, 12, 24, 48, and 72 h. Serial dilution using the pour-plate method was performed. A sterile plate was

inoculated with 1 mL of inoculum and sterile MRS agar was poured onto the plate then swirled, and incubated at 37 ºC for 72 h in an anaerobic jar. The number of colonies growing on the surface of the solidified medium was counted and reported as CFU/mL.

Determination of probiotic growth: The number of probiotics growing on the modified MRS supplemented with tapioca IMO as a carbon source was compared with control samples prepared by inoculating the starter culture (0.5 mL) into MRS broth (9.5 mL) and incubating it at 37 ºC for 48 h (*Lactobacillus*) or 72 h (*Bifidobacterium*). Samples were taken at 0, 12, 24, 48, and 72 h. Serial dilution using the pour-plate method was performed. A sterile plate was inoculated with 1 mL of inoculum and sterile MRS agar was poured onto it and swirled, then incubated at 37 ºC for either 48 h or 72 h in an anaerobic jar. The number of colonies growing on or in the solidified medium was counted and reported as CFU/mL.

The log CFU/mL was calculated using the following formula:

$$\text{Log(CFU/mL)} = \text{Log}\{\text{Number of colonies} * \text{Dilution factor}) / 1\}$$

The strains of each *Lactobacillus* and *Bifidobacterium* probiotic producing the highest numbers of colonies were selected for further use as probiotic strains based on their presumed selectivity for growth on tapioca IMO. The two probiotic strains selected were used for the development of a nutritionally complete formula for CKD patients.

Development of nutritionally complete formula: The nutritionally complete product developed for CKD patients was formulated to include the encapsulated probiotics *L. paracasei* and *B. animalis* and tapioca IMO in a synbiotic powder, which was mixed with other commercial food ingredients. The formula contained carbohydrate as an energy source providing 35-45% of the total energy requirements of the patients, representing energy in the range of 30-35 Cal/kg of ideal body weight (IBW) or about 1.8 Cal/mL [18]. The composition was developed to meet the requirements of the WHO and the Thai FDA for patients with CKD.

All the food ingredients used in this study were purchased from certified companies and the additives were food grade to ensure that they were safe for use in standard and medical food. The composition of the nutritionally complete formula for kidney patients follows: carbohydrate from tapioca IMO and maltodextrin (34% Cal); protein from milk protein isolate (18% Cal); fat from canola oil and medium-chain triglyceride (MCT) oil (48% Cal) plus trace amounts of necessary vitamins and minerals. Dry mixing was performed in a 10-kg stainless-steel V shaped mixer in a clean room. The formula developed contained two strains of encapsulated probiotics at a concentration of 7 x 10^7 CFU/g of each strain for the reduction of uremia. The nutritionally complete formula was produced in a powdered form and kept in a sterile amber container in a refrigerator before sensory evaluation.

Product sensory evaluation: The nutritionally complete formula was evaluated for its sensory attributes and compared with a commercial product (Nepro®) by 30 of semi-trained panelists using a 9-point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely). The sensory evaluation was performed in a certified sensory laboratory. Each sample was labeled with a random 3-digit code, and the samples were served

in a random order to each panelist. After sensory evaluation, the products were improved according to the comments of the panelists to arrive at the final product.

Probiotic survival in simulated gastrointestinal tract: The survival of the probiotics was evaluated in simulated GI tract conditions based on the method described by Krasaekoopt *et al.*(2004) [19]. The encapsulated probiotics were placed in a tube containing sterile simulated gastric fluid (pH 2.0) containing 3 mg/mL pepsin (Sigma, USA) and incubated at 37 °C for 3 h. The probiotics were then removed and the live cells counted. Then the probiotics were placed in a tube containing sterile simulated intestinal fluid (pH 8.0) containing 3 mg/mL pancreatin and 1% bile salt (Sigma, USA). The tubes were then incubated at 37 °C for 4 h. An aliquot (1 mL) of each probiotic strain was removed and the cells were counted by the drop-plate method on MRS agar containing 0.5% $CaCO_3$. The survival rate was calculated by comparing the number of cells after inoculation and before incubation and reported as the percentage of survival. Probiotic strains with a higher than 50% survival rate were selected for subsequent tests. The survival rate was calculated by the following equation [20]:

$$Survival = \frac{logCFU/mL\ at\ 3\ h}{log\ CFU/mL\ at\ 0\ h} \times 100$$

Probiotic decapsulation: Quantities of 1 g of the nutritional formula were transferred into 9 mL phosphate-buffered saline (PBS; pH 7.2), mixed gently and then incubated at 37 °C for 6 h. Samples were kept after exposure to PBS for 0, 1, 2, 3, 4, 5, and 6 h [21]. The cell release of the probiotics was counted using the pour plate technique in MRS agar containing 0.05% L-cysteine. The percentage of cell release was calculated by the following equation [20]:

$$Cell\ released(\%) = \frac{Released\ bacteria\ count\ at\ difference\ time\ (\log CFU/mL)}{Initial\ bacteria\ count\ (log\ CFU/mL)} \times 100$$

***Microbiological quality*:** The synbiotic product was subjected to microbiological analyses (total bacteria, mold, and yeast counts) immediately after production and during storage. The sample (1 g) being analyzed was serially diluted in 9 mL of distilled water. Using the pour-plate method, each diluent was plated out onto three different mediums in order to conduct cell counts, as follows: agar for a bacterial count, potato dextrose agar, to which 0.01% chloramphenicol had been added to inhibit bacterial growth, for a yeast count, and Sabouraud dextrose agar for a mold count. The plates were incubated at 37 °C for 48 h for bacterial growth and at 27 °C for 72 h for yeast and mold growth [22]. Microbiological quality analyses were carried out according to the following procedures; total plate count [23], yeast and mold [24], coliforms [25], *E. coli* [25], *Salmonella* spp.[26], *Staphylococcus aureus* [27], and *Clostridium perfringens* [28].

Statistical analysis: The data were expressed as mean ± SD and compared using analysis of variance (ANOVA) with Duncan's test to determine significant differences at $p < 0.05$. All statistical analyses were carried out using the SPSS statistical software (SPSS version 16, Inc., Chicago, IL) for Windows.

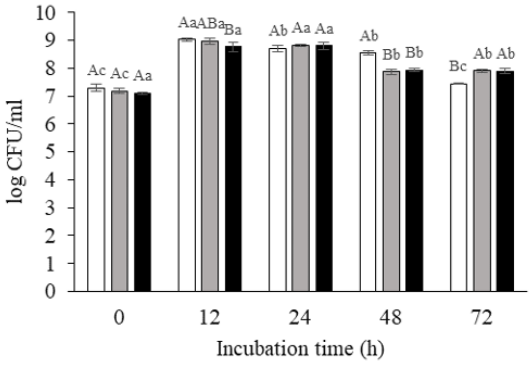
RESULTS AND DISCUSSION

Selectivity for growth of probiotic strains: The IMO was investigated for its selectivity for the growth of certain probiotic strains. Three *Lactobacillus* strains (*L. rhamnosus*, *L. paracasei* and *L. acidophilus*) were compared for their growth in a medium with different carbon sources added (D-glucose, tapioca IMO and commercial IMO). The numbers of cells of

L. rhamnosus, *L. paracasei,* and *L. acidophilus* obtained from the different carbon sources are presented in Figures 1A-C. It was found that the IMO from tapioca starch increased the growth of *L. rhamnosus* from 7.1 log CFU/mL at the time of initial incubation to a highest level of 8.9 log CFU/mL after 12 h. Growth in all the carbon sources was observed in the first 12 h of fermentation and tended to decrease thereafter. The numbers of *L paracasei* and *L. acidophilus* were highest at 12 hours with 9.2 and 9.5 log CFU/mL, respectively. Glucose as a carbon source exhibited the fastest rate of depletion since it was rapidly fermented and utilized by the probiotics.

IMO is a multifunctional molecule which exerts positive effects on human digestive health and acts as a prebiotic. Several authors have studied the positive effects induced by the administration of oligosaccharides at different dose levels. In most studies a significant increase in colonic bifidobacteria caused by long-term application of prebiotics has been demonstrated both *in vivo* and *in vitro*. Although certain groups of bacteria with. beneficial attributes can be stimulated by prebiotic carbohydrates, differing results have been reported about the possible capability of other microbes present in the GI tract to utilize them as well. IMO is an oligosaccharide compound composed of glucose

A



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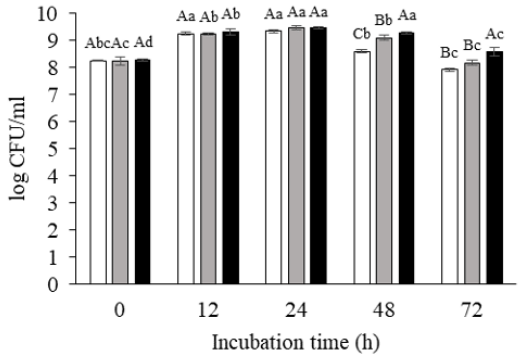

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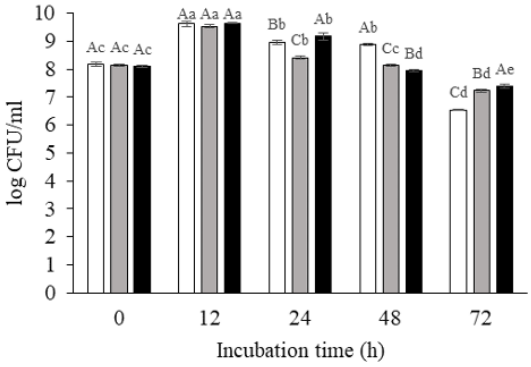

Figure 1. Growth promoting activities of probiotics: *L. rhamnosus* (A), *L paracasei* (B) and *L. acidophilus* (C) in ☐ glucose, ▨ tapioca IMO and ■ commercial IMO as carbon sources

monomers linked by α(1-6)-glycosidic bonds with various oligosaccharides, such as isomaltose, isomaltotriose, and isomaltotetraose. *Lactobacillus* strains produce oligo 1-6 glucosidase enzymes, which hydrolyze IMOs, especially isomaltose, to D-glucose [29]. Thus, the results of this study showed that IMO from tapioca was specific to promoting the growth of *L. rhamnosus*, *L. paracasei,* and *L. acidophilus*. Chen *et al*. (2011) [30] reported that IMO in MRS was able to promote the growth of *L. acidophilus.* In another recent study, the influence of IMO on the survival of *L. rhamnosus* used to produce Cheddar cheese and its effect on the texture, and sensory quality of the cheese was studied [31]. Further, the prebiotic properties of

IMO produced from rice starch were evaluated in a three-stage continuous culture system. The results showed a slight increase in beneficial bacteria, bifidobacterial, and lactobacilli while the pathogenic bacteria, clostridia and bacteroides, were significantly decreased ($p<0.05$) [32].

The growth activities of the three strains in the medium with added IMO were compared and the results are shown in Figure 2. It was found that the growth of *L. paracasei* was significantly higher ($p < 0.05$) at 10.1 log CFU/mL after 24 h incubation than the growth of *L. rhamnosus* and *L. acidophilus* at 9.1 log CFU/mL and 8.7 log CFU/mL, respectively.

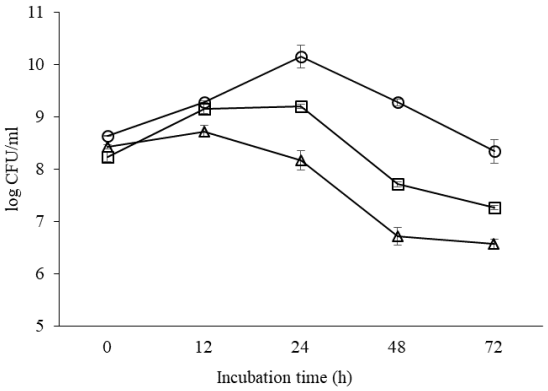

Figure 2. Growth promoting activities of probiotics: *L. rhamnosus,* *L. paracasei* and *L. acidophilus* in tapioca IMO as a carbon source

Throughout the experiment the ranking by growth from highest to lowest of the three *Lactobacillus* strains in the medium containing tapioca IMO were in the order - *L. paracasei, L. rhamnosus,* and *L. acidophilus*.

Bifidobacteria present in the dominant colonic microbiota, account for up to 25% of the culturable fecal bacteria in adults and around 80% in infants. Bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human GI disorders, such as colonic transit disorders, intestinal infections, colonic adenomas, and cancer [33]. The growth promoting activity of the three *Bifidobacterium* strains, *B. longum*, *B. animalis,* and *B.bifidum* were compared in media containing different basic (D-glucose) and prebiotic (tapioca IMO and commercial IMO) supplements. The numbers of *B. longum*, *B. animalis,* and *B. bifidum* resulting from the presence of the various carbon sources are shown in Figures 3A-C, respectively.

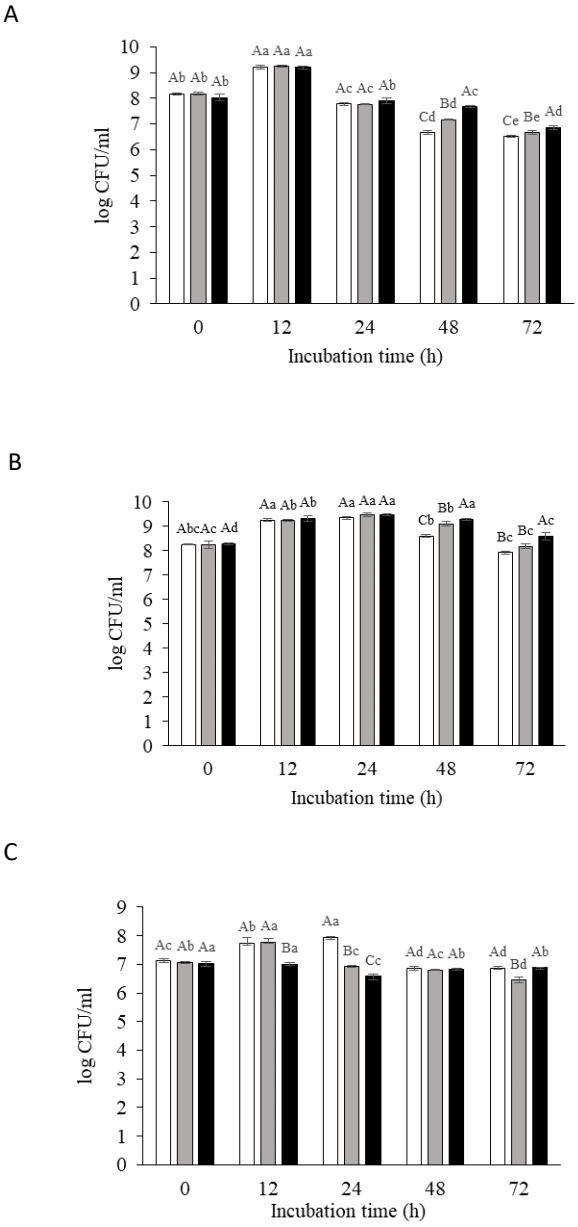

Figure 3. Growth promoting activities of probiotics: *B. longum* (A), *B. animalis* (B) and *B. bifidum* (C) in ☐ glucose, ▒ tapioca IMO and ■ commercial IMO as carbon sources

As shown in Figure 4, after 12 hours the growth of the probiotics *B. longum*, *B. animalis,* and *B. bifidum* incubated on medium supplemented with tapioca IMO had increased from 8.1, 8.0, and 7.0 log CFU/mL, to 9.2, 9.2, and 7.7 log CFU/mL, respectively, and further significant increases in growth were observed for *B. animalis* and *B. longum* as the incubation time increased to 24 h. At that time, *B. animalis* exhibited the highest growth of 9.3 log CFU/mL which was significantly ($p<0.05$) higher than that of *B. bifidum* which decreased between 12

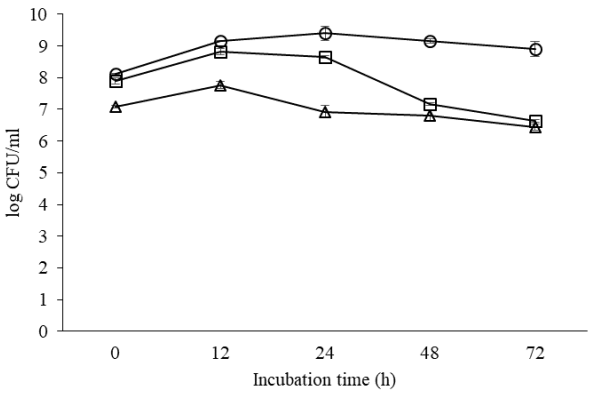

Figure 4. Growth promoting activities of probiotics: *B. longum* *B. animalis* and *B. bifidum* in tapioca IMO as a carbon source

and 24 h. However, thereafter, for all three strains the trend of growth decreased. At all times, however, the ranking from the highest to the lowest growth of the three *Bifidobacterium* strains in medium containing tapioca IMO at any given time were in the order: *B. animalis, B. longum,* and *B. bifidum*.

Nutrition analysis and nutrition labeling: The nutrition formula of the product developed for patients with CKD was designed to be consumed in an 80 g/serving diluted with water to a total volume of 220 mL, providing total energy per serving of 400 kcal or 1.8 kcal/mL. The nutritional information appearing on the product label shows that the protein:carbohydrate:fat ratio in the product is 15.43:41.29:43.27, with a total of 495 kcal contained in each 100 g of product. The micronutrient content of the product, including vitamins A, B1, B2, sodium (Na), calcium (Ca), iron (Fe), potassium (K), and phosphorus (P) were analyzed.

The amount of nutrient intake is the major factor determining the nutritional status of dialysis patients. Energy intake from carbohydrates and fats is of primary importance in maintaining good nutritional status in both healthy subjects and dialysis patients, and the IMO included in the product also serves as a source of energy for patients with renal failure. In addition, a nutritionally safe level of protein intake is also needed. In this study, the nutrition formula was developed with a high protein content by incorporating whey protein. However, if the energy intake from carbohydrates and fats is insufficient, amino acids from protein are utilized as a source of energy and increased protein intake is responsible for the accumulation of metabolic waste, such as urea and phosphates, and an acceleration of metabolic acidosis, which results in CKD patients requiring more dialysis. As described by Marcus *et al,* there is a possibility that increasing protein intake does not directly contribute to an increase in the serum albumin concentration [34]. In addition, impaired protein assimilation in uremia leads to a large influx of undigested proteins into the distal

intestine, which favors the proliferation of proteolytic bacteria [35] and fermentation of the residual protein in the colon results in the generation of potentially toxic metabolites, such as ammonia, phenols, amines, indoles, and thiols [36]. The increase of these toxins in patients with CKD may be related to increased generation by the dysbiotic microbiome or decreased elimination due to reduced kidney function.

To counteract the negative effects of protein breakdown, nutritional formulae developed in other studies have included other beneficial nutrients, such as lactulose and raffinose, which are considered to have prebiotic effects [37]. The uremic toxins, p-cresyl sulfate and indoxyl sulfate, produced by intestinal bacteria have been shown to decrease after exposure to prebiotics. In this study, the nutritionally complete formula incorporated the encapsulated probiotics, *L. paracasei* and *B. animalis*. These probiotic strains are commercially available and meet the Thai FDA regulations relating to probiotics for food use.

The guidelines issued by the Food and Agriculture Organization of the United Nations and the WHO state that it is necessary for probiotic strains to remain intact during their passage through the upper intestinal tract to ensure their health promoting effects upon entering their site of action, regardless of the delivery mode applied [38]. Further, to ensure that the minimum therapeutic level of viable probiotic microorganisms exists, a level of viable cells of at least 10^6 CFU/g should be maintained throughout the product's shelf-life [39]. Therefore, the encapsulated probiotic content of the product developed in this study is 7 x 10^7 CFU/g, which would be sufficient for the management of uremia in CKD patients.

Product sensory evaluation: The nutritional formula developed in this study and a commercial product (Nepro®) were tested for their sensory attributes by panelists using a 9-point hedonic scale. The hedonic scores showed the gustatory effects of adding different whey protein sources, whey protein concentrate (WPC) and whey protein isolate (WPI), on the sensory properties of the nutritional product formulated in this study. It was found that adding WPC or WPI resulted in no significant differences ($p > 0.05$) in appearance, color, viscosity, taste, sweetness, saltiness, bitterness or overall acceptability. When the products were compared with the commercial product (Nepro®), there were also no significant differences ($p > 0.05$) for all those attributes. The average values for the sensory attributes of the nutritional formula for CKD patients with WPC and WPI are presented in Table 1, from which it can be observed that the overall values for sensory attributes were 6.45 and 6.86, for the product with WPC and WPI, respectively.

Effect of simulated upper gut conditions on probiotic survival: The survival of the probiotics under simulated upper gut conditions was

Table 1. Sensory attributes of synbiotic formula for CKD patients using whey protein concentrate and whey protein isolate compared with a commercial product (Nepro®)

Sensory evaluation	Formula		
	WPC	WPI	CP
Appearance	6.49±1.36[b]	6.60±1.34[b]	7.62±1.29[a]
Color	6.60±1.28[b]	6.78±1.18[b]	7.72±1.21[a]
Viscosity	6.56±1.26[a]	6.92±1.14[a]	6.58±1.80[a]
Odor	6.07±1.27[c]	7.52±1.72[a]	6.64±1.78[b]
Taste	6.33±1.40[a]	6.74±1.77[a]	6.49±1.77[a]
Sweetness	6.56±1.55[a]	6.37±1.97[a]	6.74±1.63[a]
Saltiness	6.35±1.38[a]	6.74±1.30[a]	6.68±1.44[a]
Bitterness	6.96±1.38[a]	7.00±1.32[a]	6.80±1.57[a]
Oily	5.72±1.83[b]	6.50±1.70[a]	6.92±1.50[a]
Overall	6.45±1.33[a]	6.86±1.38[a]	6.90±1.47[a]

WPC = Product containing whey protein concentrate and MCT oil, WPI = Product containing whey protein isolate, CP = commercial product (Napro®)

evaluated. In order to exert beneficial effects on the host, the bacteria must be able to survive in the acidic conditions present in the stomach, and be resistant to bile acids at the beginning of the small intestine. Although many scientists agree on the importance of the probiotics' survival *in vivo*, many products available on the market do not meet this requirement. This study evaluated the survival of both the probiotic microcapsules alone as well as the encapsulated probiotics contained in the nutritional product for CKD patients. The number of bacteria was compared before and after the transit through the simulated GI tract to evaluate the protective effect of the probiotic microcapsules and the probiotic microcapsules contained in the nutritional product. The results showed that both products were able to resist degradation by *in vitro* upper gut conditions simulating those in the mouth, stomach and small intestine with survival rates of 99.88, 90.06, and 89.47%, respectively as presented in Table 2.

Several parameters have been reported that affect the survival of probiotics such as the encapsulation method, the type of wall material, pH, the number of initial probiotic cells, the probiotic strain, and the food matrix [38, 40, 41, 42]. In this study, the percentage survival of the probiotics after passing through all stages of the upper gut conditions was 89.47% and this result demonstrates the importance of protecting the probiotic bacteria by encapsulation. These data also support those reported by Priya *et al.* (2011), who observed that the GI survival of *L. acidophilus* increased when the probiotic was encapsulated. In fact, in another study, uncoated bacteria were found to be almost destroyed under GI conditions [43].

Table 2. Survival rate of probiotics under simulated upper gastrointestinal conditions

Gastrointestinal condition	Incubation time (min)	Probiotic survival (log CFU/mL)	Survival (%)
Mouth	0	6.88±0.08	100
Artificial saliva + α-amylase	2	6.87±0.09	99.88
Stomach	60	6.37±0.15	92.54
Simulated gastric fluid (SGF) + pepsin	120	6.20±0.09	90.06
Small intestine	60	6.17±0.08	89.62
Simulated intestinal fluid (SIF) + pancreatin	120	6.16±0.12	89.47

Release of encapsulated probiotics: The efficient release of viable and metabolically active cells in the intestines is one of the aims of microencapsulation [44].The ability of *Lactobacillus paracasei* and *Bifidobacterium animalis* subsp. *lactis* (BB-12®) to be released from the nutrition formula when exposed to PBS (pH 7.2) at the beginning of the incubation period (0 h) were determined to be 49.27 ± 5.84 and 54.12 ± 4.09% for the encapsulated probiotics and the product containing the probiotics, respectively, as shown in Figure 5. When the incubation time increased, the release of the cells also increased. The ability to release *Lactobacillus paracasei* and *Bifidobacterium animalis* after exposure to PBS at pH 7.2 for 4 h were 97.00 and 87.81 % of the initial population found in the encapsulated probiotics and the product containing the probiotics, respectively. After 5 – 6 h incubation, there were no significant differences ($p > 0.05$) between the cell release from the probiotics alone and those contained in the nutritional product, indicating that the product composition had no effect on the release of cells from their microcapsules after 5 h. This result is similar to that of a previous study which observed no difference in the percentage release of cells of the probiotic, *L. plantarum* microcapsules after 5 h incubation [19].

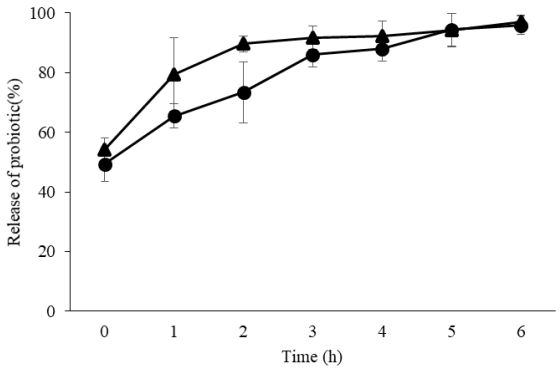

Figure 5. Percent release of ●— encapsulated probiotics and ▲— synbiotic product developed in this study

Microbiological quality of synbiotic formula for CKD patients: The synbiotic formula was evaluated for its microbiological quality. The results showed that the numbers of yeast and mold, coliform, *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus,* and *Clostridium perfringens* cells were relatively low or were not detected in the nutritive product developed in this study, and the results of the microbiological tests were therefore in conformity with the Thai FDA regulations for the microbial quality of powdered products containing probiotics.

CONCLUSION

The tapioca IMO produced showed selective properties for different strains of probiotics. Among the probiotic strains tested, *Lactobacillus paracasei* and *Bifidobacterium animalis* showed the highest promotion of growth by the tapioca IMO prebiotic. Consequently, these two strains were selected, and incorporated as synbiotic active ingredients in the nutritionally-complete formula developed for CKD patients. The product was investigated for probiotic survival and decapsulation and it was found that 89.47% of the probiotics in the products were able to tolerate the GI tract conditions, while 96.90% of the encapsulated probiotics were released after consumption. The nutritionally-complete formula developed successfully met the requirements of the WHO and the Thai FDA regulations. This is the first report of the use of a combination of selected probiotics with IMO, which represents a novel synbiotic formula for CKD patients with high acceptance in sensory evaluation comparable to that of the commercial product, Nepro®.

Competing Interests: The authors declare that there are no conflicts of interest.

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