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Prebiotic properties of isomaltooligosaccharides from cassava as a potential ingredient in high-protein drinks for athletes

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ABSTRACT

Background: Studies show that prebiotics can improve the health of athletes. Isomaltooligosaccharide (IMO) is a food ingredient containing prebiotic properties.

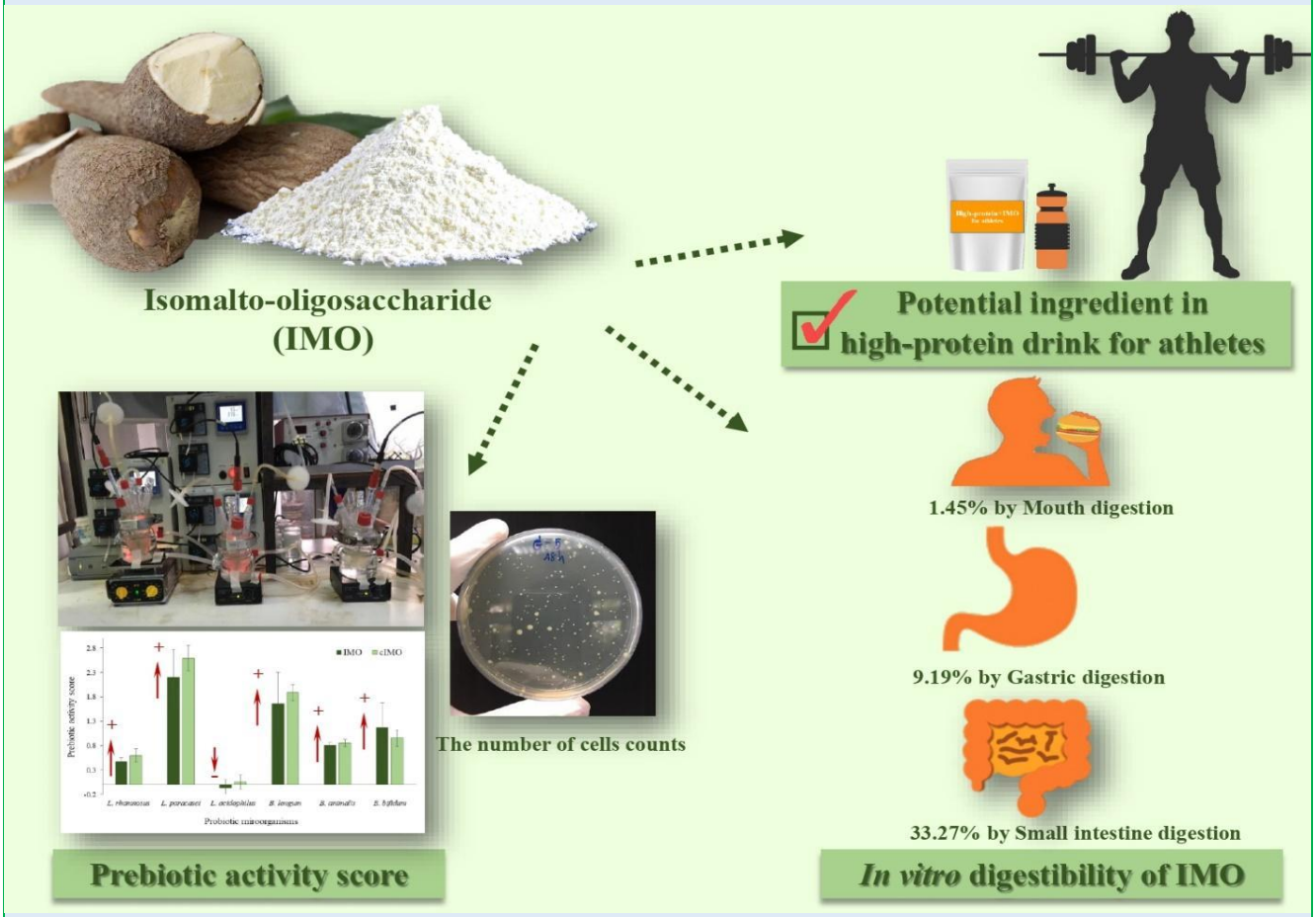
Objectives: The purpose of the study is to examine the prebiotic properties of isomaltooligosaccharides based on digestibility of *in vitro* under-simulated upper-gut conditions and a prebiotic activity score. Additionally, the study explores the potential to use IMO as an ingredient for high protein drinks.

Methods: IMO powder from cassava starch was prepared through enzymatic methods. The prebiotic properties of IMO were evaluated based on *in vitro* digestibility and a prebiotic activity score. Researchers assessed the digestibility of *in vitro* in simulated upper-gastrointestinal-tract conditions, consisting of mouth digestion, gastric digestion, and small-intestine digestion. The study calculated the probiotic activity score according to the number of growing beneficial and harmful bacteria. Finally, researchers determined the potential to use IMO as an ingredient for developing high-protein drink products.

Results: The digestion of IMO by simulated salivary fluid using human salivary α-amylase for 2 min, artificial human gastric juice at pH 2.0 for two hours, intestinal fluid with pancreatic α-amylase (0.75 unit/mL), and pancreatic lipase (1.6 unit/mL) for two hours with a pH of 6.9 and a temperature of 37 ºC were 1.54±0.33%, 9.19±0.64%, and 33.27±4.09%,

respectively. Comparing the results with commercial isomaltooligosaccharide (cIMO), researchers found that the percentage of digestion differed significantly. Prebiotic activity scores of IMO for *L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *L. acidophilus* LA 5, *B. longum* DSM 219, *B. animalis* subsp. BB12® and *B. bifidum* BB536 were 0.477±0.07, 2.197±0.58, -0.058±0.16, 1.660±0.63, 0.801±0.59 and 1.179±0.05, respectively. Notably, the results were not significant when compared to cIMO. Researchers measured the nutritional formula in a high-protein drink containing IMO at 40 g (30 g protein) and the total serving at 148 kcal. For macronutrient distribution, the ratio of protein, carbohydrate, and fat in the product is 81:19:0. Micronutrients were added, comprising of 0-50% Thai RDI. Finally, the product also met relevant standards for the microbial quality of food products in powdered form.

Conclusion: IMO from cassava was partially resistant to *in vitro* digestion under simulated upper-gastrointestinal conditions and promoted the growth of probiotic bacteria. Moreover, powdered IMO can be used as an ingredient for high-protein drink products.



Keywords: prebiotic, probiotic, high protein drinking, athletes, IMO

BACKGROUND

Functional oligosaccharides are active prebiotics that derive either from natural plants or from carbohydrate raw materials, mainly starch generated through enzymatic reactions [1]. Many oligosaccharides are produced from starch, such as gentiooligosaccharides, cyclodextrins, maltooligosaccharides and isomalto-oligosaccharide [2]. Isomaltooligosaccharides (IMO), known as branched oligosaccharides, have been synthesized from starch for combined prebiotic functional properties, including low-calorie content and low sugar [3]. Furthermore, IMO is also included in the list of Foods for Specified Health Use (FOSHU) [4]. We can see IMOs in beverages, both healthy drinks and soft drinks, meal replacement for weight control, energy drinks, yogurt, frozen product, sweeteners, cookies biscuit and chocolate, coffee, bread, jams, and tofu. The most dominant property of IMO is its prebiotic lineament, leading to better gut health, absorption of minerals, cholesterol control, and immune-system function, as well as prevention and resistance to several diseases [3]. Our previous studies found that there are prebiotic properties in IMO, as it promotes the growth of the probiotics tested in pure cultures [5].

IMO has been shown to reduce gastrointestinal symptoms, upper respiratory tract symptoms, and infection episodes. Consequently, it may provide an advantage to the athlete in increasing the numbers of healthy training days and completed races through increasing both qualitative and quantitative probiotics. Overall, from the studies in animals and humans, there is growing evidence indicating that gut microbiota composition plays an essential role in host physiology and physical performance [6]. Recent studies of probiotics and prebiotics have shown improved health for athletes [7-11]. Apart from direct benefits, some studies show that probiotics and prebiotics might indirectly benefit athletes by maintaining gastrointestinal function and improving gut-barrier function, nutrient absorption, and performance recovery [12]. These benefits help to prevent the immunosuppressive effects and respiratory tract infections caused by intense exercise. As a result, susceptibility to illness is reduced and athletic performance is improved [13].

Moreover, probiotics and prebiotics may improve athletic performance by enhancing training adaptations, attenuating physiological responses during post-exercise recovery periods, and improving mood following intense exercise. Research allows for a growing understanding of the composition and metabolic activity of the gut microbiota, which can be modulated by greater dietary intake following exercise (e.g., increased protein intake in resistance-trained athletes) [14]. The alterations in response to dietary protein components and microbial metabolites significantly affected health in both directions. Some of the protein metabolites are associated with the development of colon cancer and inflammatory bowel diseases. These protein metabolites include ammonia, amines, and gases such as hydrogen, sulfide, and methane-which are cytotoxins, genotoxins, and carcinogens [14]. The protein intake of athletes along with these prebiotic components is essential. Thus, the development of high-protein drinks containing prebiotics for athletes will be beneficial for their overall health.

Thus, this research aims to study the digestibility of IMO's *in vitro* under simulated upper-gut conditions and record the prebiotic activity score. Furthermore, research explored the potential use of IMOs in high-protein drinks.

MATERIALS AND METHODS

All the studies were performed under ethical approval; no TNSU-SCI 012/2565 from the Office of Research Ethics Committee at Thailand National Sports University in Thailand. The materials and methods were conducted as follows:

Preparation of isomaltooligosaccharide powder: In this study, isomaltooligosaccharide (IMO) powder from cassava starch was prepared through enzymatic methods. The three-step method involved liquefaction of

α-amylase, Kleistase E5NC), saccharification (β-amylase, Fungamyl) and transglycosylation (Transglucosidase L "Amano"). The cassava starch was dissolved in water to obtain a 30% (w/v, dry basis) starch slurry. Trans-glucosidase (1 U g/L) and α-Amylase (3 U g/L) were added and maintained at 60 °C for six hours. The sample solution from the reactor was purified by nanofiltration (MWCO 200 Da) after an enzymatic reaction and sprayed dry into IMO powder. IMO powder was kept at -20 °C for use in this study. All the methods used followed the protocol of previous work (adapted from Chock-chaisawasdee *et al.)* [15]. However, we received this raw material from our previous study. The IMO was composed of total carbohydrate 95.90%, including Glucose (1.88%), Maltose (13.42%), Iso-maltose (28.05%), Panose (7.90%), and other oligosaccharides (44.63%).

Prebiotic property of IMO; In vitro digestibility: The *in vitro* digestibility of IMO was carried out under simulated conditions of the upper gastrointestinal tract. The simulated conditions consisted of mouth, gastric, and small intestine digestion at 37°C [16]. A sample consisted of 500 mL of 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. To obtain the final concentration of 0.33 unit/mL, human salivary α-amylase was added to the mixture and incubated at 37°C for two minutes. Samples were taken for digestible evaluation at 0, 0.5, 1.0, 1.5, and 2.0 min in order. After hydrolysis by human salivary α-amylase, the mixture solution was boiled for 15 min before cooling rapidly to inactivate the enzyme. HCl buffer containing (g/L) NaCl, 3.41; KCl, 0.40; $NaHCO_3$, 0.60; $CaCl_2$, $2H_2O$, and 0.30 was added to the mixture and then adjusted to pH 2.0 by using 1M HCl solution to obtain the final concentration of 20.0 unit/mL. The mixture was added by pepsin and then incubated at 37°C for two hours. The sampling was performed at 0, 30, 60, 90, and 120 minutes. After hydrolysis using artificial human gastric juice, the mixture solution was boiled for 15 min and rapidly cooled down again. The pH was readjusted to 6.9 using 1M NaOH solution to simulate small intestine digestion. After that, researchers added porcine pancreatic α-amylase and pancreatic lipase to the mixture to obtain final concentrations of 0.75, 1.6 unit/ml, respectively, and then incubated at 37°C for two hours. Sampling was done at 0, 30, 60, 90, and 120 minutes before boiling for 15 min to terminate enzyme digestion. Researchers found the DNS method [17] and phenol-sulphuric acid method [18] reduced total sugar content in the sample. Each experiment was done at least three times, and the results were compared with commercial isomaltooligosaccharide (cIMO). According to the equation below (1), the hydrolysis percentage of each sample was calculated based on the model's reducing sugar liberated and total sugar content [19].

$$Hydrolysis(\%) = \frac{Reducing\ sugar\ released\ (final - initial\ sugar)\ x100}{Total\ sugar\ content - initial\ reducing\ sugar} \quad (1)$$

Prebiotic activity; Microorganisms: To be considered a prebiotic, a food ingredient must stimulate or selectively change the composition and activity in the gut flora, resulting in greater health and well-being [20]. In this study, six probiotic strains were used for prebiotic property evaluation as follows:

1. *Lactobacillus rhamnosus* LGG®
2. *Lactobacillus paracasei* CASEI 431®
3. *Lactobacillus acidophilus* LA 5®
4. *Bifidobacterium longum* DSM 219
5. *Bifidobacterium animalis* subsp. BB12®
6. *Bifidobacterium bifidum* BB536

Moreover, prebiotics had little to no ability in increasing the growth of potentially harmful bacteria. As we know *Escherichia coli* (*E. coli*) is a bacterium that generally lives in the intestines of healthy people, a type of bacteria that causes both minor and severe illness.

Therefore, this study used *Escherichia coli* (*E. coli*) to represent harmful bacteria for prebiotic property evaluation.

Cultivation media: MRS agar was used as a complex medium, based on the formulation developed by de Man, Rogosa, and Sharpe to provide a medium that would support the tremendous growth of *Lactobacilli*. The formula of the medium in one liter of distilled water contains ammonium citrate (2 g), agar (10 g), bacteriological peptone (10 g), beef extract (8 g), D-glucose (20 g), dipotassium phosphate (2 g), magnesium sulfate (0.2 g), manganese sulfate (0.05 g), sodium acetate (5 g), Tween 80 (1 g), and yeast extract (4 g). All ingredients were mixed well and dissolved by heating. This mixture was boiled for one minute until completely dissolved. Subsequently, researchers added bromocresol purple (0.02 %) to the agar media. *Bifidobacterium* media used the same formula, but L-cysteine-hydrochloride (0.05 %) was added. *Escherichia coli* (*E. coli*) was cultured in casamino acid-peptone-glucose (CPG), which contains 0.1% casamino acid, 1% peptone, and 1% glucose.

The media was sterilized for 15 minutes in an autoclave at 121 ºC, then cooled down to 45-50 ºC, and dispensed into plates. The prepared medium was kept at 2-8 °C. The media's color was amber for MRS and cream-colored for CPG with slight opalescence on both.

Determination of microorganism's growth: By placing the lyophilized culture in the MRS broth and then incubating at 37 ºC for 24 hours, *Lactobacillus* strains, composed of *L. acidophilus* LA 5®, *L. paracasei* CASEI 431® and *L. rhamnosus* LGG®, were subcultured to obtain a starter culture (inoculum). The arrangement of modified MRS consisted of new carbon sources to substitute glucose with isomaltooligosaccharide (IMO) produced from cassava starch. Then, 100 mL of modified MRS was added to the water-jacket glass vessel with a stirrer and temperature control. The media was controlled under anaerobic conditions with nitrogen flushing overnight. Each 10-mL (10%) starter culture was inoculated in modified MRS broth and incubated at 37 ºC for 48 hours. Samples were taken from each vessel at 0 h (initial) and 48 hours. The pour-plate method was utilized in a serial dilution. The sample (1 mL) was inoculated on an empty plate. A sterile MRS agar was poured, mixed, and incubated at 37 ºC for 48 hours in an anaerobic jar. The number of colonies growing on the surface of the medium was counted as CFU/ml and reported.

Bifidobacterium, composed of *Bifidobacterium longum* DSM 219, *Bifidobacterium bifidum* BB536, and *Bifidobacterium lactis* subsp. BB12®, were subcultured and inoculated in the modified MRS broth by adding L-cysteine-hydrochloride (0.05 %). The medium was incubated at 37 ºC under anaerobic conditions for 48 hours in the water-jacket glass vessel to obtain a starter culture (inoculum). Samples were also analyzed and reported simultaneously (0 hours and 48 hours incubation time).

Researchers prepared the modified CPG with new carbon sources to substitute glucose utilizing IMO. The starter culture (10 ml) of *Escherichia coli* (*E. coli*) was inoculated in modified MRS broth incubated at the same condition (under the anaerobic condition at 37 ºC for 48 hours). At 0 hours (initial) and 48 hours, samples were taken from the vessel. Researchers used a serial dilution made with the pour-plate method. A 1-mL inoculum was inoculated on an empty plate. A sterile CPG agar was poured, mixed, and then incubated at 37 ºC for 48 hours in an anaerobic jar. The number of colonies growing on the surface of the medium was counted as CFU/ml and reported.

Researchers replicated each experiment at least three times. The log CFU/mL at any given time was calculated finally using the equation (2) as follows:

$$\text{Log (CFU/mL)} = \text{Log } \{(\text{Number of colonies} * \text{Dilution factor})/0.1\} \quad (2)$$

Prebiotic activity score: A quantitative score reflected the estimated prebiotic activity, which supports the selective growth of lactobacilli and bifidobacteria, as reported by Zhang *et al.* [20]. This score is calculated based on the following equation:

$$\text{Prebiotic activity score} = \left\lfloor \frac{\left(probiotic\ log\ CFU\ ml^{-1}\ on\ the\ prebiotic\ at 48\ hrs - probiotic\ log\ CFU\ ml^{-1}\ on\ the\ prebiotic\ at\ 0\ hr\right)}{\left(probiotic\ log\ CFU\ ml^{-1}\ on\ glucose\ at 48\ hrs - probiotic\ log\ CFU\ ml^{-1}\ on\ glucose\ at\ 0\ hr\right)} \right\rfloor - \left\lfloor \frac{\left(enteric\ log\ CFU\ ml^{-1}\ on\ the\ prebiotic\ at 48\ hrs\ enteric\ log\ CFU\ ml^{-1}\ on\ the\ prebiotic\ at\ 0\ hr\right)}{\left(enteric\ log\ CFU\ ml^{-1}\ on\ glucose\ at\ 48\ hrs - enteric\ log\ CFU\ ml^{-1}\ on\ glucose\ at\ 0\ hr\right)} \right\rfloor \quad (3)$$

The equation above defines prebiotic activity score. If IMOs can be selectively metabolized by probiotics but not by other intestinal bacteria, a positive prebiotic activity score will be counted. A higher score can reflect the higher prebiotic activity. Using Eq. (3), the prebiotic activity score of a particular oligosaccharide can be defined relative to any given probiotic strain [20].

The number of colonies that grew on modified MRS with IMO for each probiotic microorganism was used to calculate the prebiotic activity score, and finally, the results were compared with commercial isomalto-oligosaccharide (cIMO).

The potential use of IMO as an ingredient for high protein drinking for athletes; Product development and packaging design: The target product properties for this study were protein content (more than 70%) and the determination of health benefits. Researchers converted a high-protein drink containing a prebiotic into powdered form. It included macronutrients (carbohydrates, fats and protein), some minerals, and prebiotic IMO's, which were purchased from commercial food companies. The product consisted of protein (78%), carbohydrate (IMO powder) (20%), and vitamin and mineral premix (30%) of Thai Recommended Daily Intakes (vitamin A, vitamin D, vitamin E, vitamin K, vitamin B1, vitamin B2, niacin, vitamin B6, vitamin B12, vitamin C, iron, calcium, phosphorus, magnesium, iodine, potassium, selenium).

The packaging containing prebiotics for athletes contained 40g of prebiotic powder, holding around 30g of protein. The information on the product label included the product brand, nutrition information, product property, ingredients manufacturer, and information needed for the product. Moreover, the manufacturers utilized salient aluminum foil bags to attract attention.

Nutrition analysis: The SGS (Thailand) Co., Ltd., Songkhla, Thailand analyzed the finished product of this high-protein drink.

Macronutrients: The product contained the following macronutrients**:** protein [In-house method SOP LBAG-14002 based on AOAC (2019) 990.03, 992.15, 992.23], total fat [AOAC (2019) 996.06, GC/FID], saturated fat [AOAC (2019) 996.06, GC/FID], cholesterol [In-house method SOP LBCH-00259 based on AOAC (2019) 994.10, GC/FID], carbohydrate [Method of Analysis for Nutrition Labeling (1993)], energy [Method of Analysis for Nutrition Labeling (1993)], total sugar [In-house method SOP LBLC-17001 based on AOAC(2019) 982.14, HPLC-RI] and dietary fiber [In-house method LBAG-99103 based on AOAC (2019) 985.29].

Minerals: The minerals in the products included sodium, calcium, and iron [In-house method SOP No. LBCH-13532 based on AOAC (2019) 999.10 and 2011.14].

Vitamin: Researchers found the composition of vitamins in the product to include vitamin A (In-house method SOP LBFD-99076 based on Bull. Dept. Med. Sci. Vol.37 No.1

Jan-March 1995: P. 57-64), B1 [In-house method SOP LBFD-00089 based on AOAC (2019) 942.23], and B2 [In-house method SOP LBFD-00084 based on AOAC (2019) 970.65].

Microbiological quality: Microbiological analyses were conducted with a prebiotic product both after production and during storage. The study called for a serially diluted sample (1 g) dissolved in distilled water (9 mL). Using the pour-plate method, each diluent was put on an agar plate for the bacterial count, and potato dextrose agar with 0.01% chloramphenicol was added to inhibit bacterial growth for yeast count. For mold counts, researchers added Sabouraud dextrose. The plates were incubated to grow bacteria at 37°C for 48 hours and at 27°C for three days to grow yeasts and mold (Amankwah *et al*., 2009). Researchers assessed the microbiological quality according to the following: Total Plate Count (FDA BMA, 2001); Yeast & Mold (FDA BMA, 2001); Coliforms (FDA BMA, 20017); *E.coli* (FDA BMA, 2017); *Salmonella* spp. (FDA BMA, 2016) *Staphylococus aureus* (FDA BMA, 2016) and *Clostridium perfringens* (FDA BMA, 2001).

Statistical analysis: The measure ± standard deviation of three independent determinations constituted the statistical analysis. The mean values (the significant level at $p < 0.05$) were compared to a one-way analysis of variance (ANOVA) followed by Duncan's new multiple-range tests. The study applied SPSS software (version 18) for all statistical analyses.

RESULTS AND DISCUSSION

In vitro digestibility of IMO: Prebiotics are non-digestible oligosaccharides that resist digestion in the small intestine and are fermented by beneficial intestinal microbiota to support human health [21]. Therefore, when examining the prebiotic effects of diverse candidates *in vitro*, it is essential to verify whether or not they are decomposed by digestive enzymes in the mouth, stomach, or small intestine of the human body [22]. *In vitro* digestion models are mainly applied to study the structural changes, digestibility, and food component discharge under simulated gastrointestinal conditions [16]. Therefore, in this study, the digestibility of isomalto-oligosaccharide produced from cassava was carried out *in vitro* to confirm prebiotic properties. Firstly, artificial human saliva containing human salivary α–amylase was used for digestion before passing through artificial gastric juice and pancreatic α–amylase. The results displayed that increasing incubation time for both samples can raise the percentage of hydrolysis ($p < 0.05$), as it is showed in Figure 1(a). In addition, the digested IMO with salivary fluid simulating the human salivary α-amylase condition was not a significant difference when compared with commercial isomaltooligosaccharide (cIMO) ($p > 0.05$). At 0, 0.5, 1, 1.5, and 2.0 minutes, the degrees of hydrolysis of IMO were 0, 0.27±0.10, 0.37±0.07, 1.36±0.14, and 1.54±0.33%, respectively. The maximum hydrolysis of 1.54±0.33% happened at two minutes, and human salivary α–amylase reduced the number of oligosaccharides. A Similar result occurred in the indigestible tuna, with 5% inulin through *in vitro* digestion. It was found in the sample that there was significant ($p < 0.05$) resistance to the digestive enzyme, salivary α-amylase, in artificial saliva [16].

Figure 1(b) represents the hydrolysis of artificial human gastric juice at pH 2.0. The percent of hydrolysis

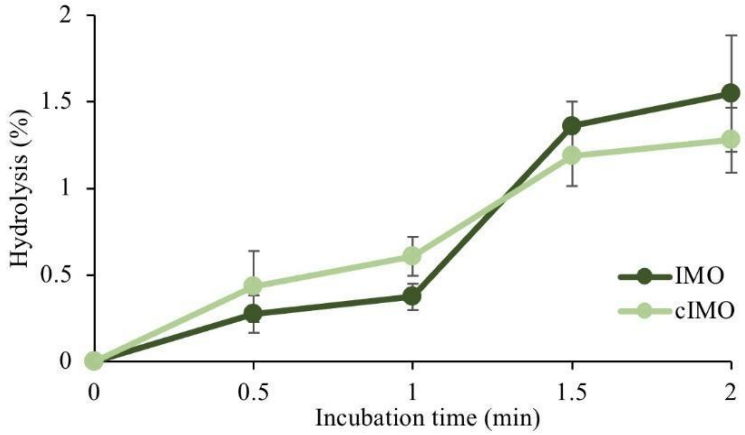
also increased with increasing incubation time. The results were akin to the digestion of IMO with simulated salivary fluid. The degree of hydrolysis of IMO using artificial human gastric juice at 0, 30, 60, 90, and 120 minutes was 0, 5.38±1.26, 8.14±0.68, 8.59±0.75 and 9.19±0.64%, respectively. The maximum hydrolysis (9.19±0.64%) occurred at 120 min of incubation. In contrast, the degree of hydrolysis of cIMO with artificial human gastric juice at 0, 30, 60, 90, and 120 minutes was 0, 5.97±0.54, 6.10±1.01, 5.90±1.14 and 7.01±0.97%, respectively. Experimental results showed that the degree of hydrolysis for both samples slightly increased after 30 minutes and stayed relatively constant. Although the degree of hydrolysis, when compared with cIMO at the initial incubation, was not significantly different (p > 0.05), after 60 minutes, the degree of hydrolysis was substantially higher preceding the end of incubation. The maximum hydrolysis of pitaya oligosaccharides (4.07%) in the previous report occurred after 120 minutes of incubation at pH 1.0 [19], while the total hydrolysis of tuna in spring water (3.27%), with 5% inulin added, occurred after 120 minutes of incubation at pH 2.0 [16].

These results showed that IMO from cassava had approximately 90.81% resistance with stimulated gastric fluid containing pepsin (20.0 unit/ml) for two hours, a pH of 2.0, and a temperature of 37 ^{o}C. Artificial human gastric juice hydrolyzed oligosaccharides consisting of 80% ethanol. The oligosaccharides showed some resistance to the fluid. The pH of the artificial gastric juice decreased, and the percentage of hydrolysis increased [19]. In contrast, tuna products with 5% inulin added displayed approximately 97.0% resistance to simulated conditions in the human stomach. Additionally, in tuna products, gastric juice resistance was slightly higher when compared to insulin, which gave a maximum resistance of 91.1% [16].

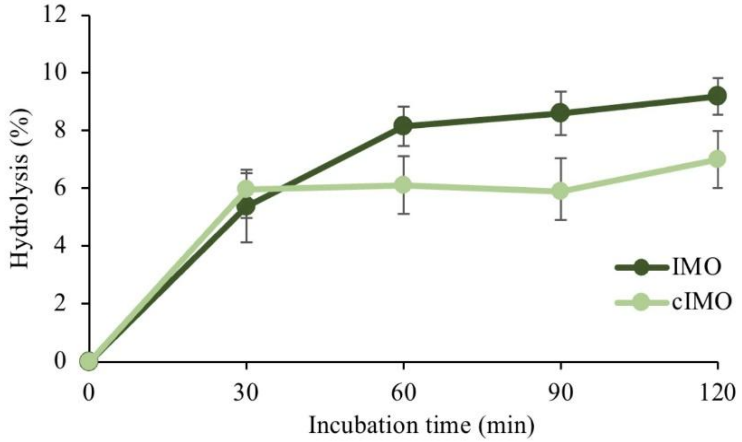
IMO hydrolysis was determined with simulated intestinal fluid containing pancreatic α-amylase (0.75 unit/ml) and pancreatic lipase (1.6 unit/mL) for two hours at a pH of 6.9 and temperature of 37 ^{o}C. The results showed that the maximum percentage of hydrolysis (33.27%) was reached at 120 minutes of incubation. The degree of hydrolysis of isomaltoligosaccharides from cassava at 0, 30, 60, 90, and 120 minutes was 0, 14.73±2.70, 20.14±3.87, 33.01 ±4.03 and 33.27±4.09%, respectively. Considering the maximum hydrolysis was 9.73%, Jackfruit oligosaccharides are shown to be resistant to human pancreatic α-amylase digestion [19].

The results revealed partial IMO from cassava starch as it was resistant to digestive enzymes in the gastrointestinal tract, owing to the steady reduction of sugar in the presence of digestive enzymes and the pH conditions mentioned above. When reacting with simulated oral, gastric, and intestinal medium sequentially, salivary α-amylase and gastric pepsin hydrolyzed partial IMO under pH conditions of 7.0, 3.0, and 7.0, respectively. This result demonstrated that reducing sugar content of IMO was slight increase after sequential in vitro digestion. Accordingly, researchers calculated the hydrolysis (DH, %) of IMO at 1.54, 9.19, and 33.27.

(a)



(b)



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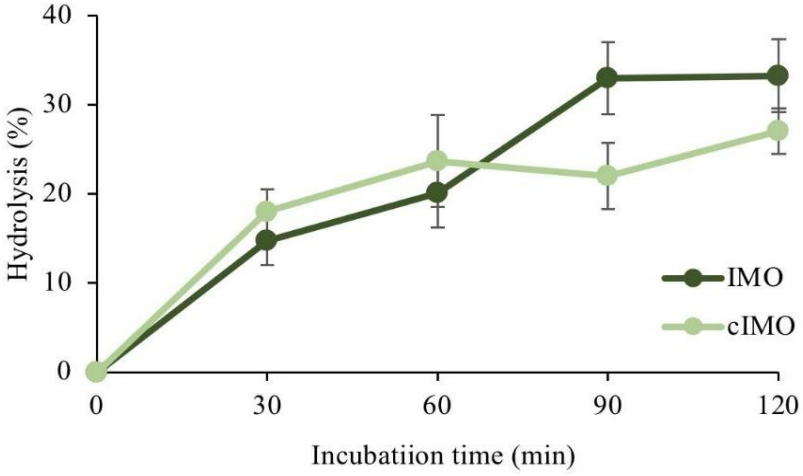

Figure 1. Percentage of hydrolysis of IMO and cIMO by (a) stimulated salivary fluid with human salivary α-amylase (0.33 unit/mL) for 2 min at pH 7.0, by (b) simulated gastric fluid with pepsin (20.0 unit/mL) for 2 hrs with a pH of 2.0 and a temperature of 37 °C, and by (c) simulated intestinal fluid with pancreatic α-amylase (0.75 unit/mL) and pancreatic lipase (1.6 unit/mL) for 2 hrs with a pH of 6.9 and a temperature of 37 °C.

As we know, IMO is poorly digestible, in that it is resistant to digestion in the stomach and small intestine. However, it can be partially broken down in the colon by bacterial species (mainly bifidobacteria and lactobacilli), acting as prebiotic dietary fiber. The synthesis process of IMO from starch involves hydrolysis. The carbohydrate is converted from α-(1,4)-linked α-D-glucooligosaccharides using α-amylase and pullulanase to α-(1,6)-linked oligosaccharides using α-transglucosidase. The easily digestible glucose with alpha 1-4 linkages was converted into glucose molecules with 1-6 linkages, making them resistant to hydrolysis in the gastrointestinal tract. The probiotic bacteria secreted extracellular enzymes to metabolize α-1,4 and α-1,6 glycosidic bonds of IMO, resulting in the production of small organic acids with health benefits, such as acetate, propionate, butyrate, and lactate [23]. In addition, animal and human studies consistently demonstrated the partial digestibility and prebiotic properties of isomaltooligosaccharides for many natural plants [21].

Selectivity for growth of probiotic strains and prebiotic activity score: Oligosaccharides were not digested in the small intestine. They pass through to the colon where they are fermented by *Bifidobacterium* and *Lactobacillus*, leading to healthy levels of bacteria in the gut. Therefore, IMOs are at least partially fermented by bacteria in the colon and may, hence, encourage the growth of bacterial subpopulations. This study evaluated the degree of selectivity among probiotic strains. Table 1 showed the growth activities of probiotics and *E. coli* in a specific broth with different added carbon sources (glucose, cassava IMO, and commercial IMO) at initial (0hr) and 48-hour incubation times. Researchers found that the numbers of cells for all probiotics and *E. coli* significantly increased at 48 hours. At the time of initial incubation, from 8.09±0.06 log CFU/mL to the highest level of 9.17±0.06 log CFU/mL at 48 hours, the fermented IMO from cassava increased the growth of *B. animalis* subsp. BB12®, while the degree of cell growth in broth with added IMO for *L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *L. acidophilus* LA 5, *B. longum* DSM 219 and *B. bifidum* BB536 were 7.94±0.05, 9.14±0.10, 8.12±0.04, 7.92±0.04 and 6.84±0.09, respectively.

Furthermore, when comparing the growth numbers of probiotics and *E. coli* with different carbon sources in broth, results showed significant differences for some strains. The growth numbers of *L. acidophilus* LA 5 and *B. longum* DSM 219 in broth with added IMO were significantly higher when compared with growth numbers in broth with added cIMO. However, the results showed no significant difference for *L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *B. animalis* subsp. BB12® and *B. bifidum* BB536 for the growth in both broths with added IMO or cIMO. The extent of probiotic growth may not confirm the selectivity growth property; therefore, the probiotic activity score must be considered.

In addition, the results show that *E. coli* had the highest growth in broth with glucose carbon sources, as shown in Table 1. The degree of cell growth at 48 hours with fermented *E. coli* in broth with added glucose, IMO, and cIMO was 7.64±0.03, 7.16±0.01, and 7.13±0.01, respectively.

Prebiotic activity scores from IMO and cIMO fermentation with probiotics bacteria (*L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *L. acidophilus* LA 5, *B. longum* DSM 219, *B. animalis* subsp. BB12® and *B. bifidum* BB536), were gained from values of cell density (Table 1) by using the above equation (3) and are shown in Figure 2. Researchers found that the highest prebiotic activity scores on MRS broth with added IMO fermented with *L. paracasei* CASEI 431® was 2.59±0.25. However, for each

strain, other probiotics produced different prebiotic activity scores from the fermentation of MRS broth with added IMO. The score for *L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *L. acidophilus* LA 5, *B. longum* DSM 219, *B. animalis* subsp. BB12®, and *B. bifidum* BB536 were 0.47±0.07, -0.05±0.16, 1.66±0.63, 0.80±0.63 and 1.17±0.50, respectively. Furthermore, it is worth noting that prebiotic activity scores from the fermentation of MRS broth with added IMO and cIMO exhibited no significant difference for each strain.

Table 1. Growth activities of probiotics and *E. coli* in broth with added difference carbon sources (glucose, cassava IMO and commercial IMO) at 0 and 48 h of incubation time.

Microorganisms	Incubation time					
	0 h			48 h		
	Glucose	IMO	cIMO	Glucose	IMO	cIMO
L. rhamnosus	7.35±0.18^{c}	7.30±0.06^{c}	7.14±0.04^{d}	8.56±0.06^{a}	7.94±0.05^{b}	7.91±0.04^{b}
L. paracasei	8.21±0.06^{c}	8.27±0.13^{c}	8.27±0.06^{c}	8.60±0.09^{b}	9.14±0.10^{a}	9.29±0.06^{a}
L. acidophilus	8.16±0.08^{b}	8.13±0.05^{b}	8.08±0.03^{b}	8.87±0.02^{a}	8.12±0.04^{b}	7.93±0.04^{b}
B. longum	7.16±0.20^{b}	7.75±0.11^{ab}	7.61±0.09^{b}	7.72±0.10^{ab}	7.92±0.04^{a}	7.80±0.16^{ab}
B. animalis	8.08±0.07^{c}	8.09±0.06^{c}	8.08±0.05^{c}	9.39±0.04^{a}	9.17±0.06^{b}	9.28±0.07^{ab}
B. bifidum	7.12±0.05^{a}	7.09±0.05^{a}	7.10±0.03^{a}	6.90±0.06^{b}	6.84±0.09^{b}	6.88±0.04^{b}
E. coli	7.12±0.01^{b}	7.12±0.03^{b}	7.11±0.06^{b}	7.64±0.03^{a}	7.16±0.01^{b}	7.13±0.01^{b}

Different lowercase letters above the numbers indicate a significant difference between samples and incubation time ($p < 0.05$).

Like the previous report, the prebiotic activity score for *L. paracasei* 1195 fermentation with commercial prebiotic FOS product-NutraFlora P-95 was 0.447. However, it was lower than another FOS product-Raftilose P95 (0.99). Furthermore, pectic oligosaccharide (POS) fermentation from citrus peel pectin has a prebiotic activity score of 0.41 for *L. paracasei* LPC-37 and 0.92 for *B. bifidum* ATCC 29521 [20]. In the research summary, the metabolic diversity of carbohydrate fermentation with individual strains and species of bacteria is substantially different following phylogenetic diversity in the genus. According to this, genetic coding for these metabolic systems may be present or absent in different strains [20]. In addition, using prebiotics with probiotic bacteria requires specific enzyme hydrolysis and transport systems that conform to the particular prebiotic [20].

The prebiotic is a good carbohydrate source that enhances the growth of beneficial bacteria [24]. Moreover, the field of prebiotics has substantially advanced in the past few years, stimulated by burgeoning knowledge in the role of human microbiota in health and disease [25]. Current evidence suggests a relationship between the balance of intestinal microbiota and exercise. These changes in the gut microbiota may strengthen physical performance by enhancing training adaptations, attenuating physiological responses during post-exercise recovery periods, and improving mood after intense exercise [26-27]. Researchers investigated several purported benefits of probiotics on athletic performance. These benefits included the impact of probiotics on oxidative stress, inflammatory response, sports performance, immune-system function, and upper respiratory tract infection (URTI) [28].

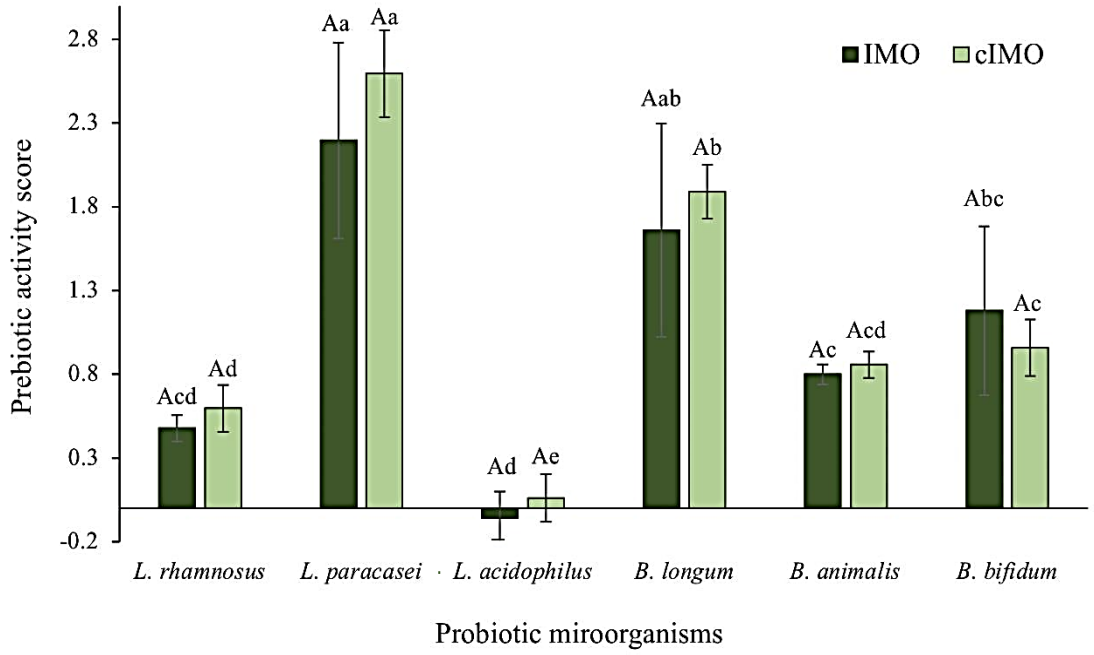

Figure 2. Prebiotic activity scores of IMO and cIMO for *L. rhamnosus* LGG®, *L. paracasei* CASEI 431®, *L. acidophilus* LA 5, *B. longum* DSM 219, *B. animalis* subsp. BB12® and *B. bifidum* BB536. Different uppercase letters above the bars display a significant difference between samples while lowercase letters indicate a significant difference between each microorganism ($p < 0.05$).

An imbalance in gut microbiota may limit the training performance of athletes, leading to poor competitiveness, a decline in overall well-being, fatigue [29], depression, and anxiety [30]. Therefore, problems with a high prevalence of upper respiratory (URTI) and gastrointestinal (GI) tract infections are well-documented and pertain to increased permeability of the gastro-intestinal epithelial wall, mucous thickness disruption, and subsequent higher rates of bacterial translocation [31]. The cause of GI symptoms may be linked to physiological, mechanical, or nutritional effects. As the blood supply to the GI tract decreases by 60 to 70% at exercise intensities of 70% of maximum oxygen consumption (VO_2max), this decrease in blood supply may also reduce mesenteric blood flow [30]. Increasing evidence supports the idea that maintaining a healthy balance of beneficial bacteria in the microbiome can support immunity, leading to fewer illnesses and sustaining optimal health. Moreover, prebiotic and probiotic supplements can abate gastrointestinal distress in overtrained athletes [32]. Probiotics enhance athletic performance by increasing modulation of the microbiota and metabolites [28], causing greater diversity in the microbiota, increasing beneficial bacteria, decreasing pathogens and extending high performance by reducing muscle tension [33-35]. For this reason, these supplements have a fascinating impact on athletic performance and overall health.

This study demonstrates that the isomaltooligosaccharides in cassava resist digestive enzymes in the gastrointestinal tract and promote the growth of probiotic bacteria. Therefore, the health benefits associated with isomaltooligosaccharides must be further studied.

The potential to use IMO as an ingredient for high protein drinking for athletes; Product design: The Sports Nutrition Society recommends a daily protein intake for athletes ranging from 1.4 to 2.0 g of protein per kg of body weight [36]. A commonly suggested dose of protein is 1–2 scoops (25–50 grams) per day. Thus, researchers used a high-protein drink with prebiotics for athletes, measuring the contents at 40 g /serving (30 g protein) diluted with water. The total volume recommended is 250 mL, and the total energy per serving is 148 kcal or 0.6 kcal/mL. The nutritional information declared on the product label was compared to Thai Recommended Daily Intakes (RDI) in the product.

The content information on the product label included the product brand, nutrition information, ingredient, manufacturer, product property, and information needed for the product. The information in the packaging also claimed that the drink contains high protein, high fiber, and low fat. In addition, the product's packaging is designed with a convenient size and beautiful color. However, this study is the first step in using IMO as an ingredient for high-protein drinks. Hence, the pilot scale or commercial production may constitute a future study.

Nutrition analysis: In addition to exercise training, the proportion and time of dietary intake greatly influenced athlete's energy expenditure and exercise performance [37]. Protein is an essential part of any athlete's diet. However, several studies reported that high-protein drinks may hinder performance in endurance sports as well as gut microbiota diversity in athletes who receive lower amounts of energy, carbohydrates, and dietary fiber [9,14]. Furthermore, in resistance sports, athletes who follow a high-protein, low-carbohydrate, and high-fat diet present a decrease of SCFA-producing commensal bacteria. In intervention studies, high-protein diets (animal protein), reduced concentrations of fecal butyrate and butyrate-producing bacteria, such as *Bifidobacteria* spp., *Roseburia* spp., and *E.* rectale [38-40].

Moreover, the results of five male volunteers who consumed high amounts of the animal protein indicated that fecal sulfide production relates to meat intake [41]; notably, hydrogen sulfide is a compound that is associated with ulcerative colitis. Additionally, undigested proteins in the distal intestine, which prefer the proliferation of proteolytic bacteria and the fermentation of the residual protein in the colon, generate potentially toxic metabolites, such as ammonia, phenols, amines, indoles, and thiols [42]. To counteract the adverse effects of protein breakdown, other beneficial nutrients, such as inulin, lactulose, and raffinose, which are assumed to have prebiotic effects, were added to the nutrition formula [43]. Therefore, protein supplements that contain prebiotics could help to alleviate gut microbiota issues in the future population [44]. The mixture of whey protein with probiotic IMO lent to the product formula's high-protein content.

Researchers evaluated the high-protein drink's nutritional composition, as shown in Table 2. The protein, carbohydrate, and fat content were 74.16, 17.1, and 0.64 g/100g, respectively. The micronutrient distribution ratio for protein, carbohydrate, and fat is 81:19:0, and the total calories of the product added up to 376 kcal/100g or 148.4 kcal/serving. The product's micro-nutrient contents, including vitamin A (as retinol), vitamin B1 (thiamine), vitamin B2 (riboflavin), sodium (Na), calcium (Ca), and iron (Fe), were analyzed, as shown in Table 2.

Table 2. Nutrition composition in high-protein drinks for athletes' product

Items	Nutrient/100 g	Nutrient/ per serving	Percent Thai RDI per serving
Total Energy	371 kcal	148.4 kcal	-
Protein	74.16 g	29.66 g	-
Total fat	0.64 g	0.25 g	0%
Saturated fat	0.29 g	0.11 g	0%
Cholesterol	3.89 mg	1.55 mg	0%
Carbohydrate	17.10 g	6.84 g	2%
Total Sugar	1.50 g	0.6 g	-
Dietary fiber	3.37 g	1.35 g	5%
Moisture	5.60 g	2.24 g	-
Sodium (Na)	128 mg	51.20 mg	3%
Calcium (Ca)	306 mg	122.40 mg	15%
Iron (Fe)	0.70 mg	0.28 mg	Less than 2%
Vitamin A (as Retinol)	Not detected	Not detected	0%
Vitamin B1 (Thiamine)	0.98 mg	0.39 mg	25%
Vitamin B2 (Riboflavin)	2.06 mg	0.82 mg	50%

Researchers compared the product composition with Thai recommended daily intakes for those 6 years of age and older, based on a 2,000-kcal diet. Researchers set the percentage of vitamins and minerals at about 0-50% of Thai RDI. In addition, the composition of dietary fiber in a 100 g product was 3.37 g. The current recommended dietary fiber intake for adults in most European countries and in the US is between 30–35 g per day for men and between 25–32g per day for women [45]. By contrast Thai RDI recommended dietary fiber intake for those 6 years of age and older is 25g. For this study, the product's serving size contained dietary fiber of 1.35 g/serving or 5% when compared with Thai RDI. Therefore, if the consumer follows a commonly suggested dose of 2 scoops/day (2 packs), they should receive 2.70 g or about 10% Thai RDI of dietary fiber intake per day. As we know, dietary fiber and prebiotics have the potential to enhance athletic performance and general human gut health [7].

Microbiological quality of high-protein drink product: Protein powder is one of the most popular nutritional supplements on the market, especially among fitness fanatics. Maintaining the microbiological quality of these products to meet customer specifications is challenging. Bacteria responsible for foodborne diseases can be seen as a warning for when consuming products because the bacteria are seen in many products [46]. Therefore, evaluating the microbiological quality of high-protein drinks is necessary. Table 3 provides information on the numbers of coliform, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus,* and *Samonella sp.* cells. It was found that the quality was relatively low or not detected in the nutritive product developed in this study. Thus, the results of the microbiological tests corresponded to Thai FDA regulations concerning the microbial quality of powdered products. The previous studies reported that several factors may influence the risk of microbiological contamination in the product. These factors include high ambient temperatures, poor hygiene, and the improper storage and refrigeration of foods [46]. Therefore, future studies should track the manufacturing of the product, storage, and transportation.

Table 3. Microbiological quality of high-protein drink product.

Items	Specification	Resulted	Units
Total plate count	Less than 10	< 10	CFU/g
Yeast & Mold count	Less than 10	< 10	CFU/g
Coliform	Less than 3	< 3	MPN/g
Clostridium perfringens	Less than 10	< 10	CFU/g
Staphylococus aureus	Less than 3	< 3	MPN/g
Escherichia coli	Less than 3	< 3	MPN/g
Salmonella sp. (25g)	Not detected	Not detected	Per 25g

CONCLUSION

The results of the study suggested that partial IMO from cassava starch is resistant to digestive enzymes in the gastrointestinal tract, allowing for the slow release of sugar in digestive enzymes and in specific pH conditions. Moreover, IMO showed a significantly high prebiotic score for *Lactobacillus paracasei* CASEI 431®, which is reputed as the most promising prebiotic candidate. The high-protein drink was successfully developed and met the requirements of the Thai FDA regulations. The product was designed with a convenient size and met standards regarding microbial quality for powdered products. This is the first study exploring the use of selected protein alongside IMO, resulting in a novel product for athletes. However, shelf life, sensory evaluation, the pilot plant, and clinical trials of the product require further study.

List of Abbreviations: IMO: isomaltooligosaccharide, cIMO: commercial isomaltooligosaccharide, FOS: fructooligosaccharide, L: liter, mL: milliliter, Kcal: kilocalorie, g: gram, MWCO: molecular weight cutoff, CPG: casamino acid-peptone-glucose, CFU: colony forming unit, GI: gastrointestinal, URTI: upper respiratory tract infections, DH: degree of hydrolysis, POS: pectic oligosaccharide.

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