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FFHD
Functional Foods in Health and Disease

Effect of nutritionally complete formula on gut microbiota and their metabolite in fecal batch fermentation system

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Submission Date: September 7th, 2021; **Acceptance Date:** November 17th, 2021; **Publication Date:** December 6th, 2021

Please cite this article as: Wichienchot S., Keawyok K. Effect of nutritionally complete formula on gut microbiota and their metabolite in fecal batch fermentation system. *Functional Foods in Health and Disease* 2021; 11(12): 641-658. DOI: https://www.doi.org/10.31989/ffhd.v11i12.841

ABSTRACT

Background: Emerging evidence has revealed that the gut microbiota is significantly altered, contributing to the occurrence and development of chronic kidney disease (CKD). Therefore, the target of increasing short-chain fatty acids (SCFAs) and lactic acid production and reduction of uremic toxins were interested.

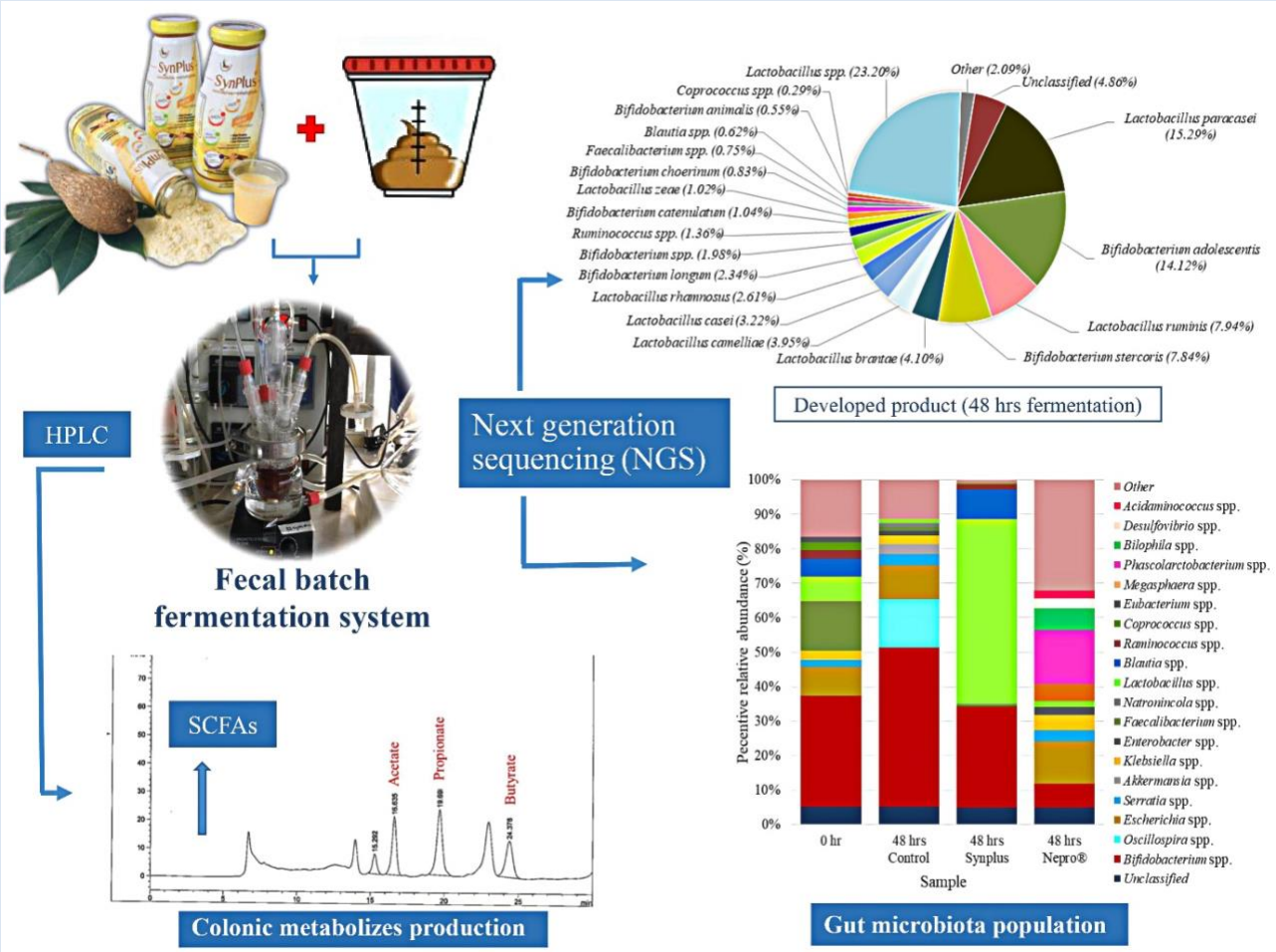
Objective: To study the effect of the nutritionally complete formula (Synplus) developed for hemodialysis patients on gut microbiota and their metabolite in *in vitro* fecal fermentation of healthy volunteers.

Methods: Fecal fermentation (*in vitro*) using batch culture in an evironment mimicking human large intestine was used to study the change of gut microbiota by next generation sequencing (NGS) during fermentation of the developed formula (Synplus), commercial formula (Nepro®) and control. The gut metabolites were determined including short-chain fatty acids (acetic, propionic, and butyric) and lactic acid. The uremic toxins *(p*-cresol and indole) were determined by high performance liquid chromatography (HPLC).

Results: The increase of *Lactobacillus* spp. (53.74%) and *Bifidobacterium* spp. (29.35%) was observed in the developed product (Synplus) compared with control at 48 hrs fermentation meanwhile these genera were decreased in a commercial product (Nepro®). Moreover, the abundance of the genus *Escherichia* spp. (12.33%) was observed in Nepro®

fermentation, with *Escherichia albertii* species which is a newly discovered pathogen of the gastrointestinal tract. Microbial metabolites produced by fecal fermentation of Synplus revealed that propionate, acetate, and butyrate increased significantly ($p<0.05$). All the samples evaluated exhibited acetate in abundance when compared to other SCFAs. Acetate was the most abundant SCFA in all samples. The concentrations of acetate for Synplus fermentation were 15.63±3.26, 147.29±2.39, 162.28±4.13 and 189.39±0.17 mM at 0, 12, 24, and 48 hrs respectively. Total SCFAs produced from Synplus was significantly increased ($p<0.05$) and higher than control and Nepro®, respectively. The concentration of *p*-cresol at 48 hrs fermentation for control, Synplus and Nepro® were 3.79±0.12, 6.31±2.37 and 11.59±0.10 µg/mL, respectively. The indole concentration of control, Synplus and Nepro® were 3.64±0.08,15.06±3.56 and 12.81±1.68 µg/mL, respectively. There were also indicated that imbalance of gut microbiota was related with the ratio of uremic toxins (indole and *p*-cresol) to SCFAs.

CONCLUSION: The synbiotic product containing prebiotic and probiotic may be used to improve gut microbiota thus reducing the risk of kidney disease.



Keywords: synbiotic, gut microbiota, uremic toxins, SCFA, CKD

INTRODUCTION

The gut microbiome and their metabolites play important roles in physiology and pathology of host [1-2]. They benefit the host in a variety of ways, such as regulating gut motility, prevent pathogen invasion, circulatory system, immunity and inflammation, producing vitamins, transforming bile acid, absorbing minerals, activating and destroying toxins, genotoxins, and possible positive effects to decrease colon cancer incidence [3]. However, changes in the composition of gut microbiota exert major consequences to the host's health, both beneficial and harmful [4]. While metabolites especially short-chain fatty acids (SCFAs) have been studied to enhance health [5-6], they also have a pH-lowering effect on the intestinal lumen. This is protective against gut pathogens and enhances the absorption of some types of nutritional factors. Recent data have shown strong associations between gut microbiota and many human diseases [7]. In the occurrence of chronic kidney disease (CKD), gut microbiota dysbiosis is observed with a growth in pathogenic bacteria compared with symbiotic bacteria [8]. However, toxic metabolites or uremic toxins like indoles [9], *p*-cresol [10-12], urea, ammonia, phenylacetylglutamine and trimethylamine N-oxide, synthesized by the gut microbiome may trigger the development and advancement of chronic kidney disease (CKD) [8]. Increasing amounts of uremic toxins causes an induce changes the intestinal pH and contributes to an alteration of intestinal permeability by affecting the tight junctions of the enterocyte [13]. An increase in the absorptive ability of the intestinal barrier which permits translocation of endotoxins as well as other products from bacteria to the circulatory system, has been reported in CKD. However, consumption of prebiotics, probiotics or synbiotics could ameliorate the altered mucus integrity, epithelial tight junction, and epithelial cells survival [14]. In addition, increasing evidence showed that SCFAs exhibited beneficial effects on CKD in both animal and clinical studies [6]. Several studies were reported that probiotics retard CKD progression, the mechanism likely would be a reduction in the generation of gut-derived nephrotoxic substances and the attenuation of systemic inflammation. Significant declines in C-reactive protein, indoxyl sulfate, and *p*-cresyl sulfate levels have often been noted when probiotics have been administered to patients with CKD [15]. Therefore, gut microbiota could be employed as a biomarker and remedial agent in improving the health of CKD patients.

The objective of this study was to evaluate the effects of the nutritionally complete formula for hemodialysis patients on gut microbiota and their metabolite in *in vitro* fecal fermentation system.

MATERIALS AND METHODS

Materials: The nutritionally complete formula for CKD patients (Synplus) was produced, comprising of encapsulated probiotics *Lactobacillus paracasei* CASEI 431® and *Bifidobacterium animalis* subsp. *lactis* BB12® (7×10^7 CFU/g of each strain) and prebiotic isomalto-oligosaccharide (IMO) produced from tapioca in a synbiotic powder form. This combination was blended together with some other other commercial food components. This formula was evaluated for its sensory attributes, probiotic survival in simulated gastrointestinal tract, probiotic decapsulation and microbiological quality as described in our previously study [16].

The commercial product of oral nutritional supplement and tube feed for hemodialysis patients (Nepro® HP) from Abbott Laboratories Ltd. was used as a commercial formula.

Preparation of samples: Before the *in vitro* fecal fermentation, Synplus powder and Nepro® were digested enzymatically, simulating the upper gastrointestinal tract (mouth, stomach, and the small intestine) digestion. The artificial human saliva used is composed of NaCl, 1.60 (g/L); NH_4NO_3, 0.33; NH_2PO_4, 0.64; KCl, 0.20; $K_3C_6H_5O{\cdot}7H_2O$, 0.31; $C_5H_3N_4O_3Na$, 0.02; H_2NCONH_2, 1.98; and $C_3H_3O_3Na$, 0.15 and 15 mL of porcine mucin (Sarkar, Goh, Singh, & Singh, 2009). Briefly, 15 g of each sample were weighed and added to 75 mL of artificial human saliva and α-amylase from human saliva (747U/90 mL). The solution was homogenized using a homogenizer (T 25 digital ULTRA-TURRAX®, IKA Works Inc., Staufen,

Germany) for 15 min at 37 °C. The pH was adjusted to 7.5 in order to mimic the condition for food digestion in the mouth. The mixture was further digested with 90 mL of simulated gastric fluid (SGF) and 1.44 g of pepsin from porcine gastric mucosa (360,000U/180 mL) according to the procedure of Jensen, Abrahamsen, Maehre, and Elvevoll (2009) with minor modifications. The pH of the samples was further adjusted to 5.5, 3.8 and 1.5 with 1 M HCl, corresponding to the incubation durations of 30, 30 and 120 min, respectively at 37 °C in a shaking incubator (ES-20/60, speed at 220 rpm, Biosan, Riga, Latvia). This mimicked the human gastric digestion. Subsequently, 180 mL of simulated intestinal fluid (SIF), 10 mM of bile salt and 9 g of pancreatin from porcine pancreas (36,000 U/360 mL) was included in the combination. The pH was modified to 5.0 for 15 min, then to 6.5 for 240 min, respectively to mimic the digestion of the small intestine. This reaction was stopped eventually through heating at 95 °C for 10 min in order to terminate the enzyme reaction. Subsequently, the solution wascooled to room temperature in an ice bath. The non-digestible samples were collected with 1 KDa MWCO dialysis bag (Spectrum Laboratories, Inc., CA, USA) accompanied by constant removal of the distilled water permeate until 72 hrs. The corresponding retentates were lyophilized andpreserved at -20 °C for the study on fecal fermentation [2].

Preparation of fecal slurry: Fresh feces were collected from three healthy volunteers. The inclusion criteria for participating healthy volunteers were male or female gender of age between 25 and 40 years old, 18.5-24.9 body mass index, and who consume a regular diet. Volunteers who were current or former smokers had a history of metabolic or gastrointestinal disorders and had consumed antibiotics for 3 months prior to the fecal sample collection were excluded. Fresh fecal will be diluted (1:10) in buffer containing 0.1 M phosphate-buffered saline (PBS) solution, pH 7.2, homogenized using a stomacher (Seward Stomacher 400 Circulator, UK) for 2 min and filtered via stomacher bags called fecal slurry. The fecal slurry was used directly as inoculum for batch culture fermentation. This trial received ethical approval no TNSU 011/2564 from Office of Human Research Ethics Committee, Thailand National Sports University, Thailand.

Fecal batch fermentation: Fecal fermentation (*in vitro*) using batch culture monitored under anaerobic condition was used to evaluate the growth of fecal bacteria during the fermentation of nutritionally complete formula (Synplus), commercial product (Nepro®) and basal medium (control). The batch culture comprised of of a water-jacket glass vessel (320 mL), controlled at 37°C, to which pre-reduced basal culture medium was added. The pH in the system was maintained by a pH-controller between 6.8 ± 0.2 throughout the experiment. Also, O_2-free N_2 gas was introduced to sparge the reaction and to sustain the anaerobic environment prior to the addition of fecal slurry and study samples. The unsupplemented medium/basal medium composed of (per liter): 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl_2.6H_2O$, 2 g $NaHCO_3$, 0.005 g haemin, 0.5 g L-cysteine HCl, 0.5 g bile salts, 2 mL Tween 80, 10 mL vitamin K, and 4 mL of 0.025% (w/v) resazurin solution. The added medium was regulated att pH 7.0 by addingg 1 mol l^{-1} HCl and maintained at 37°C under anaerobic condition [17-18]. A 10% (w/v) fecal slurry will be prepared using fresh feces from three healthy volunteers and pre-reduced (0.1 mol l^{-1}) phosphate buffer (pH 7.0) will be mixed in a stomacher for 2 min. The soluble of each sample will be added to give a final concentration of 1% (w/v). The detail of samples used in *in vitro* fecal fermentation as follow:

Vessel 1: fecal slurry + basal medium

Vessel 2: fecal slurry + basal medium + Synplus

Vessel 3: fecal slurry + basal medium + Nepro®

Each of the experimental vessels were continuously stirred using a magnetic stirrer with the temperature maintained at 37°C using a circulating water bath. The pH of the batch culture was automatically controlled at pH

6.8 ± 0.2 using a pH controller. Five mililiters of each sample was drawn from individual vessels during 0, 12, 24 and 48 hrs incubation period for the evaluation of SCFAs and uremic toxins. Short chain fatty acids (SCFA) and the uremic toxins (*p*-cresol and indole) were analyzed by HPLC [19]. The gut microbiota of each sample was evaluated by next generation sequencing (NGS).

PCR amplification, Illumina sequencing: Samples (375 µl) from each vessel at an interval during the fermentation periodwere aliquoted with 1,125 mL of filtered 4% (w/v) paraformaldehyde solution of pH 7.2 and kept at 4°C for not less than 4 hrs. The bacteria cells were fixed and washed 2 times in a filtered PBS, followed bycentrifugation at 13,000 ×g for 10 min. The cell pellets were suspended into sterile PBS (150 µl) and added 95% ethanol (150 µl). These samples were mixed and stored at -20°C not less than 1 hr or until utilized but it was not kept longer than 3 months [20]. The composition of gut microbes was examined by tag-encoded 16S rRNA gene MiSeq-based (Illumina, San Diego, CA, USA) high throughput sequencing as earlier reported [21]. Briefly, the V3-V4 region of the 16S rRNA gene was magnified by PCR by primers suitable with the Nextera Index Kit (Illumina). Amplified fragments together with adapters and tags were purified and quantified before Illumina sequencing. The nucleotide variations were detected and calculated for the percentage of mutations using basic variance detection. This analysis was performed by the Office of Scientific Instrument and Testing, Prince of Songkla University, Songkhla, Thailand.

Analysis of short-chain fatty acids and lactic acid:

From the batch culture, samples were collected and analyzed using HPLC with a UV detector for SCFAs. (Macfarlane *et al.*, 1998). Two mililters of each of the samples from the fecal fermentation were centrifuged at 17,000×g for 15 min to separate and eliminate particulate materials. The supernatant was then filtered by a 0.2-micrometer nylon filter paper. The short chain fatty acids were analyzed by an Aminex HPX-87H ion exclusion column (BIO-RAD, USA*)* consisting of 7.8 mm diameter and 300 mm length at 50°C. Also, the UV detector was fixed at 215 nm. The concentration of each of the SCFAs were determined by comparing the under peak area to the standards using Chemstation software (CHEM32 version, USA). Lactic acid, acetic acid, propionic acid, and butyric acid with concentrations of 10, 20, 40, 80, 100 and 250 mM were used as standards in the analysis [22]. External calibration curves of acetic, propionic, and butyric were employed as a standard for the quantification of SCFAs in the sample.

Analysis of p-cresol and indole: Uremic toxins including phenols (*p*-cresol) and indole in the samples were determined by high-performance liquid chromatography (HPLC) with a UV detector. Two milileters of each sample were centrifuged at 17,000 ×g for 15 min for the removal of particulate materials and to separate the cells. The supernatant was then filtered through a 0.2-micrometer nylon filter paper. Phenols (*p*-cresol) and indole in feces were analyzed using an Eclipse Plus C18 column (4.6mm×250mm i.d., 5 µm) at a flow rate of 0.5 ml/min. The mobile phase was a mixture of solvents A (water) 50% and B (acetonitrile) 50% and column was maintained at 35 °C. The UV detector was fixed at 220 nm which aided in estimating the concentration of each kind of peak area for urmic toxin by comparing to the standards using Chemstation software (CHEM32 version, USA).

Statistical analysis: All analyses were carried out in triplicate and reported as means ± standard deviation. Data were analyzed by analysis of variance (ANOVA) and statistical significance with Duncan's multiple range test ($p < 0.05$) using SPSS (version 14.0).

RESULT AND DISCUSSION

Gut microbiota profile: The effect of samples on the growth modulation of microbiota by fecal fermentation in batch culture was determined by next-generation sequencing (NGS). The result of gut microbiota classified in genus level as showed in Figure 1.

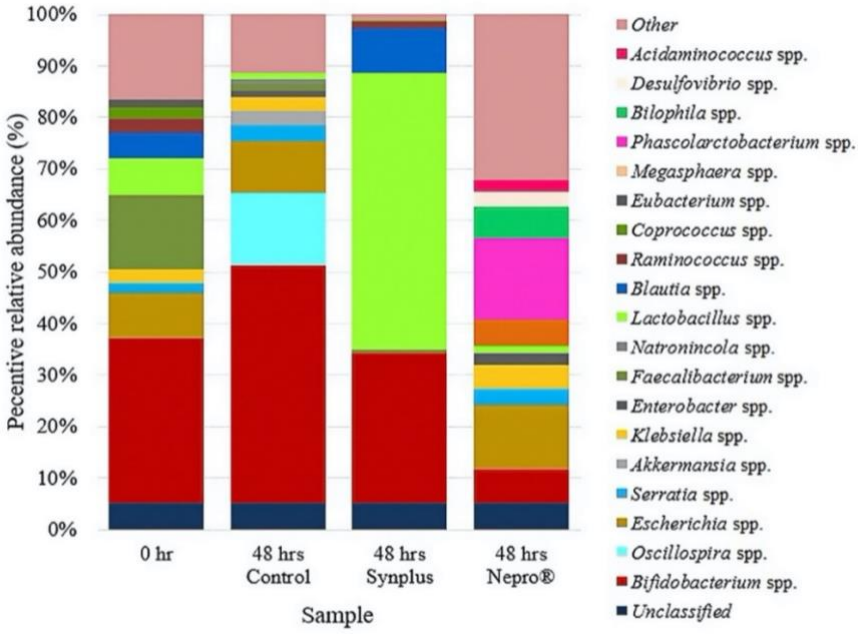


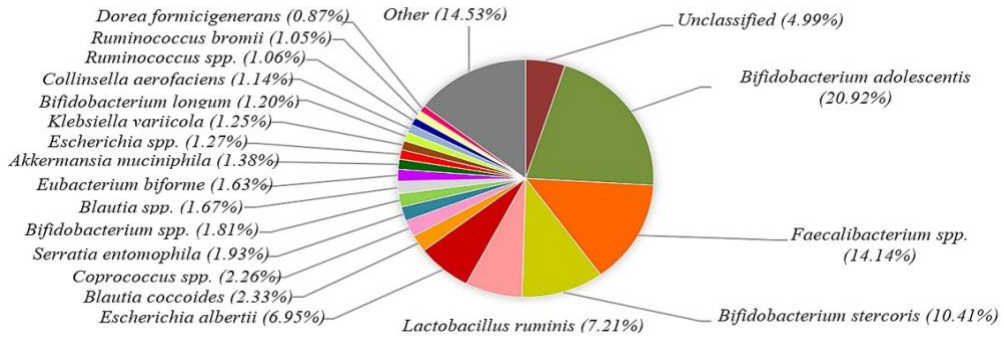
Figure 1. Bar chart of the gut microbiota population classified at the genus level by fecal inoculum from feces of healthy volunteers in *in vitro* batch fermentation at baseline (0 hr), 48 hrs of the basal medium as control, 48 hrs of nutritionally complete formula (Synplus) and 48 hrs of commercial product (Nepro®).

These results were found that the population of *Bifidobacterium* spp. (32.26%) had the highest amount of microbiota at the baseline (0 hr fermentation). Moreover, the top ten of the microbiota proportions at baseline were *Faecalibacterium* spp. (14.39%), *Escherichia* spp. (8.49%), *Lactobacillus* spp. (7.08%), *Blautia* spp. (5.16%), *Klebsiella* spp. (2.79%), *Raminococcus* spp. (2.52%), *Coprococcus* spp. (2.26%), *Serratia* spp. (1.93%), and *Eubacterium* spp. (1.63%), respectively. After the difference samples, control, Synplus and Nepro® were fermented by *in vitro* fecal batch fermentation system for 48 hrs and followed by the microbiota investigation. The results were demonstrated that both vessels which contained Synplus and Nepro® showed difference microbiota profile when compare with baseline and control at the same time. The microbiota profile of Synplus showed abundance of *Lactobacillus* spp. by 53.74% of reads classified to genus level. Other microbiota compositions were *Bifidobacterium* spp. (29.35%), *Blautia* spp. (8.63%), *Raminococcus* spp. (1.43%), *Faecalibacterium* spp. (0.75%) *Coprococcus* spp. (0.25%) and another genus (1.11%), respectively. On the other hand, the microbiota profile of Synplus was also difference when compared with Nepro® at 48 hrs fermentation. Top genus of Nepro® fermentation were *Phascolarctobacterium* spp. (15.72%), *Escherichia* spp. (12.33%), *Bifidobacterium* spp. (6.78%), *Bilophila* spp. (6.28%), *Megasphaera* spp. (4.96%), *Klebsiella* spp. (4.55%), *Serratia* spp. (3.22%), *Desulfovibrio* spp. (2.93%), *Acidaminococcus* spp. (2.25%), *Enterobacter* spp. (2.24), *Lactobacillus* spp. (1.67%) and another genus (32.11%). These results indicated that the difference of culture medium showed effect to the growth of bacterial or change in the microbiota profile. Interestingly, Synplus fermentation at 48 hrs showed an abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. and had the highest when compared with other samples. Previously studies

reported that probiotic like *Lactobacillus* spp. and *Bifidobacterium* spp. enhanced an antimicrobial property by stimulating antimicrobial peptides (AMPs) production, intestinal pH reduction, and defensins production, which inhibit pathobionts overgrowth [14]. On the other hand, *Lactobacillus* spp. and *Bifidobacterium* spp. were decreased with an abundance of *Phascolarctobacterium* spp. and *Escherichia* spp. Some studies had been reported that high colonization rate and abundance of *Phascolarctobacterium* in the human gut can produce high concentration of acetic acid. This genus is positively correlated with the metabolic status and positive emotions in the host [23-25]. The genus *Escherichia* spp. is classified in the group of proteolytic bacteria and potential harmful for host especially the species of *Escherichia albertii*, a newly discovered enteric pathogen that has been linked to sporadic infections in humans [26] which was found in this study.

Figure 2 showed the gut microbiota population classified at the species level by fecal inoculum from feces of healthy volunteers in *in vitro* batch fermentation at 0 hr or the baseline (a), at 48 hrs of the basal medium as control (b), nutritionally complete formula (Synplus) (c) and commercial product (Nepro®) (d). The gut microbiota bacteria classified at the species level by fecal inoculum at the baseline or 0 hr before fermentation was showed in Figure 2(a). This result was found that the population of *Bifidobacterium adolescentis* (20.92%) had the highest amount of microbiota at the baseline. Moreover, the top ten of the proportions were *Faecalibacterium* spp. (14.14%) *Bifidobacterium stercoris* (10.41%) *Lactobacillus ruminis* (7.21%) *Escherichia albertii* (6.95%) *Blautia coccoides* (2.33%) *Coprococcus* spp. (2.26%) *Serratia entomophila* (1.93%) *Bifidobacterium* spp. (1.81%) *Blautia* spp. (1.67%) and *Eubacterium biforme* (1.63%). It is noteworthy that the result was showed an abundance in probiotic bacteria which consisted of *Bifidobacterium adolescentis, Bifidobacterium stercoris and Lactobacillus ruminis* probably because of fecal were used for inoculum received from three healthy volunteers. However, our studies are also evaluated for gut microbiota of CKD patients in separated article. The benefits of some probiotics were studied. It was found that *Bifidobacterium adolescentis* produced GABA and displays anti-inflammatory effects by means of *in vitro* studies [27-28]. Morover, *Bifidobacterium adolescentis* can regulate the *Proteobacteria* to *Bacteroidetes* ratio in the gut microbiota and inhibit NF-κB activation in the colon [29]. However, the gut microbiota profiles of each treatment were changed after 48 hrs for *in vitro* batch fermentation. Pie chart of the gut microbiota population from *in vitro* batch fermentation at 48 hrs were classified at the species level for baseline at 0 hr (a), the basal medium as control (b), Synplus (c) and Nepro® (d) as showed in Figure 2. The modulation of microbiota by fecal fermentation with Synplus product contained probiotics (*Lactobacillus paracasei* and *Bifidobacterium animalis*) and prebiotic (isomalto-oligosaccharide) obviously showed an abundance of *Lactobacillus paracasei* (15.29%) as showed Figure 2(c). The main microbiota compositions were composed of *Bifidobacterium adolescentis* (14.12%) *Lactobacillus ruminis* (7.94%), *Bifidobacterium stercoris* (7.84%), *Lactobacillus brantae* (4.10%), *Lactobacillus camelliae* (3.95%), *Lactobacillus casei* (3.22%), *Lactobacillus rhamnosus* (2.61%), *Bifidobacterium longum* (2.34%) and *Ruminococcus spp*. (1.36%). It was showed high relative abundance of the genus of *Lactobacillus* spp. approximately 53%. Moreover, this phenomenon may occur by the overgrowth the genus of *Lactobacillus* spp. in mixed-fecal culture system where it can out-compete by inhibiting the growth of other gut microbiota [30]. Likewise, the previous studies were reported that *Lactobacillus* spp. and *Bifidobacterium* spp. and some other probiotics can enhance an antimicrobial property by stimulating antimicrobial peptides (AMPs) production, reduction of intestinal pH, and defensins production, which inhibit pathobionts overgrowth [13]. This reason was consistent and supported with the results of the phenomenon in this study.

(a)



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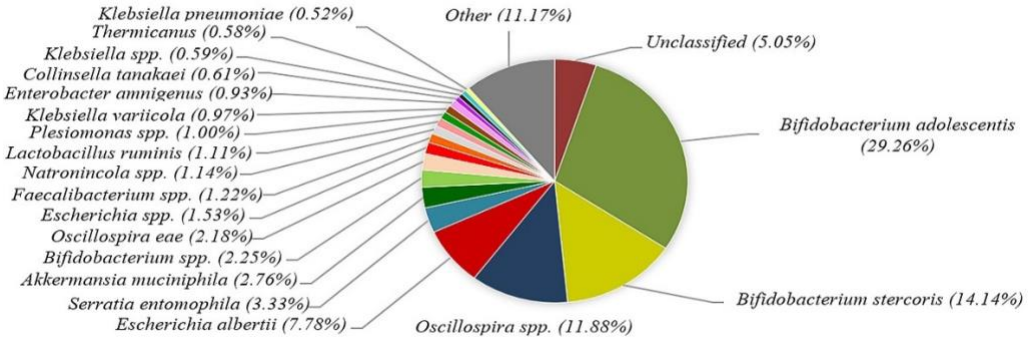

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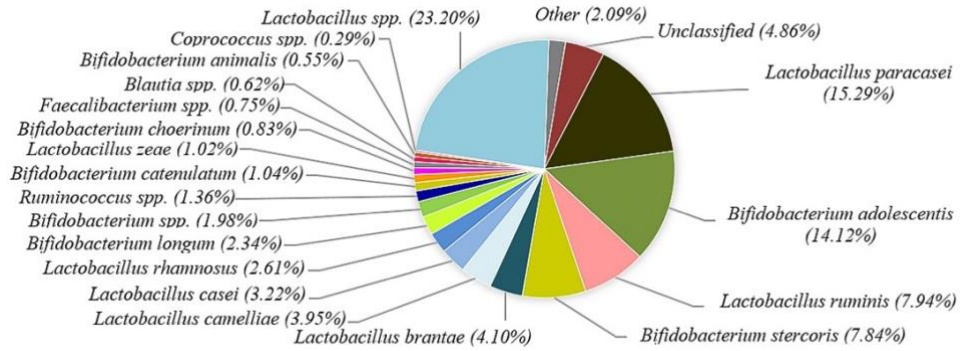


(d)

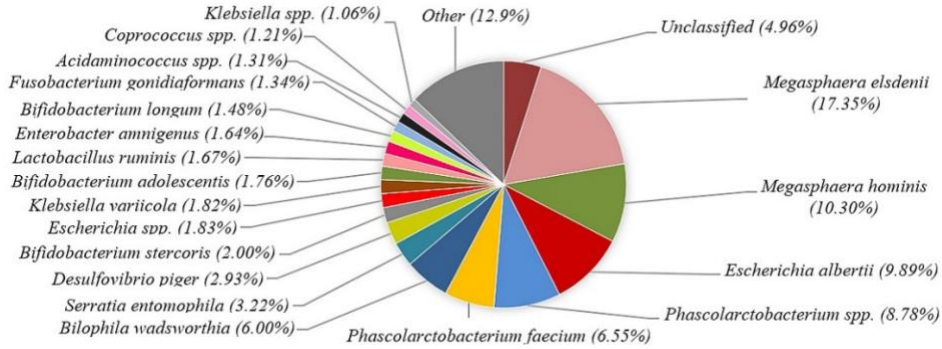


Figure 2. Pie chart of the gut microbiota population classified at the species level by fecal inoculum from feces of healthy volunteers in *in vitro* batch fermentation at baseline (0 hr) (a); 48 hrs of the basal medium as control (b); nutritionally complete formula (Synplus) (c); and commercial product (Nepro®) (d).

The previously studies were demonstrated that the settlement of *Bifidobacterium* and *Lactobacillus* in the gastrointestinal tract improved barrier function and prevented pathogen invasion [31]. Recent studies revealed that probiotics like *Bifidobacterium* and *Lactobacillus* exhibited the potential to alievate diminished kidney function in patients with CKD, and to also reduce numerous harmful biological activities of uremia [32]. Several studies were reported that probiotics delayed the progression of CKD. The mechanism may be due to a decrease in the synthesis of nephrotoxic substances derived from the gut, and the depletion of systemic inflammatory disease. Administration of one synbiotic capsule to CKD patients for the period of eight weeks promoted the abundance of *Bifidobacterium* and sustained *Lactobacillus* populations [33]. The use of the supplements incorporated with probiotics three times a day for the period of four weeks, resulted into for four weeks with probiotics given three times a day led to an appreciable reduction in plasma *p*-cresol levels in patients with stage 3 and 4 CKD [33]. However, supplementation with 15g of the symbiotic product per day substantially lowered amounts of serum *p*-cresol levels and reduction in levels of indoxyl sulfate in patients with moderate to severe CKD managed for 18 weeks [34]. Moreover, previous studies have identified gut microbiota as one of the important mediators of systemic inflammation and may contribute to renal failure risks. The normal gut microbiota can protect the kidney whereas gut dysbiosis can facilitate CKD development [35]. Thus, the result from this study indicated that Synplus is a product which promote growth of probiotic bacteria and probably show positive effect for CKD patients. On the other hand, the modulation of microbiota by fecal fermentation with the commercial product Nepro® was obviously difference with another sample as showed in Figure 2 (d). The top ten of microbiota population for Nepro® were *Megasphaera elsdenii* (17.35%), *Megasphaera hominis* (10.30%), *Escherichia albertii* (9.89%), *Phascolarctobacterium* spp. (8.78%), *Phascolarctobacterium faecium* (6.55%), *Bilophila wadsworthia* (6.00%), *Serratia entomophila* (3.22%), *Desulfovibrio piger* (2.93%), *Bifidobacterium stercoris* (2.00%) and *Escherichia* spp. (1.83%), respectively. It was noteworthy that not presented the species of *Lactobacillus* in the top ten of microbiota profile while, *Megasphaera elsdenii*, *Megasphaera hominis* and *Escherichia albertii* were abundance in the first three of microbiota profile. The growth of *Escherichia* spp. had a potential harmful for host especially the specie of *Escherichia albertii* which has been related to spontaneous infections among humans [26]. The overgrowth in the gastrointestinal tracts of *Escherichia* spp. may contribute to uremic toxins production and lead to accumulation in circulatory system in CKD patients [36]. However, the clinical trial and the molecular connection between intestinal microbiota-acquired metabolites, mechanism or signaling pathways, and renal dysfunction and chronic kidney disease remain to be elucidated [6].

Production of short-chain fatty acids and lactic acid: Short-chain fatty acids (SCFAs) are the main end products of bacterial fermentation through the microbiota from complex polysaccharides [37], such as indigestible dietary fibers including inulin, oligosaccharide [38] and epithelial-derived mucus as endogenous substares in the human colon [39]. These SCFAs are known to have wide-ranging influence on host physiology, including managing energy metabolism, immunity, anti-inflammation, as well as blood pressure by identifying their appropriate receptors and suppressing HDACs [5]. Kidney disease is often linked to malnutrition, circulatory system, hypertension or hypotension, inflammation, immunity, and oxidative stress that may be averted by SCFAs. In addition, several evidence has highlighted that SCFAs demonstrated positive effects on CKD in both experimental animals [40-42] and clinical trials [43-46]. Interestingly, the beneficial effects of SCFAs on diabetic nephropathy, the primary cause of end-stage renal disease (ESRD) globally, should be given more attention due to the beneficial effects of

SCFAs on the immunomodulation and in balancing of energy metabolism [6].

This study did not only investigate altered intestinal microbiota of healthy volunteers by *in vitro* fecal fermentation in batch culture but also evaluated the effect of synbiotic which contain in the developed product on SCFAs concentration. The interventional of prebiotic and probiotics microorganisms stimulated an increasing of SCFAs production [14]. It was related to this study, the result was found that lactic acid and SCFAs such as acetic, propionic, and butyric acids were produced during the fermentation period. SCFAs concentration at 0, 12, 24 and 48 hrs fermentation of each sample were evaluated and presented in Table 1. Synthesis of microbial metabolites by fecal fermentation of Synplus showed that acetate, propionate, and butyrate increased significantly (p<0.05) as the time of fermentation increased. Acetate was evaluated and observed as the most abundant of SCFAs in all samples of this study. The concentration of acetate for Synplus sample were 15.63±3.26, 147.29±2.39, 162.28±4.13 and 189.39±0.17 mM at 0, 12, 24, and 48 hrs fermentation, respectively. Interestingly, the concentration of acetate and lactate of Synplus fermentation were significantly increased and significantly highest (p<0.05) when compared with control and Nepro®. This result was related to the abundant growth of *Lactobacillus* spp. and *Bifidobacterium* spp., the SCFAs production bacteria as mentioned above in Figure 1. *Lactobacillus* spp. is the one genera of lactic acid bacteria (LAB) that ferment sugars and other carbohydrates present in foods that are indigestible by the host in the small intestine to produce primarily lactic acid [47-48]. Moreover, various components of the gut microbiota may produce lactate, a major short chain hydroxyl fatty acid found in the intestinal lumen. This may be further transformed into other SCFAs via a subclass of bacterial species that ferment lactate [48]. This is positive result which several studies have been shown the effect of SCFAs to be nephroprotective and health benefits for normal population by several pathways, including maintenance of the gut barrier integrity, modulation of glucose and lipid metabolism, regulation of the immune system and the inflammatory and antioxidant response [49-50].

Table 1. Effects of nutritionally complete formula on lactic acid and SCFAs production in fecal batch fermentation.

SCFA (mM)	Sample	Fecal fermentation time (hrs)			
		0	12	24	48
Lactic	Control	2.26±0.04^{Ab}	2.39±0.01^{Bab}	2.27±0.18^{Bb}	2.52±0.01^{Ba}
	Synplus	2.70±0.25^{Ac}	33.45±0.20^{Ab}	33.78±0.60^{Ab}	36.22±0.38^{Aa}
	Nepro®	2.54±0.27^{Aa}	2.41±0.02^{Ba}	2.31±0.05^{Ba}	2.42±0.03^{Ba}
Acetic	Control	23.18±1.87^{Ac}	45.62±0.80^{Cb}	45.44±1.00^{Cb}	55.94±1.58^{Ca}
	Synplus	15.63±3.26^{Ad}	147.29±2.39^{Ac}	162.28±4.13^{Ab}	189.39±0.17^{Aa}
	Nepro®	15.98±8.41^{Ac}	85.25±9.57^{Ba}	71.32±2.51^{Bb}	68.00±5.06^{Bb}
Propionic	Control	ND^{c}	14.43±0.74^{Bb}	14.81±0.30^{Cb}	20.04±0.13^{Ca}
	Synplus	ND^{c}	20.71±2.80^{Ab}	28.41±3.41^{Ba}	30.75±0.72^{Ba}
	Nepro®	ND^{d}	24.09±0.50^{Ac}	51.45±2.09^{Ab}	58.31±4.27^{Aa}
Butyric	Control	5.08±1.58^{Ac}	7.22±1.08^{Ab}	10.28±1.93^{Ab}	18.15±3.31^{Aa}
	Synplus	5.46± 1.03^{Ad}	8.03±0.15^{Ac}	12.64±1.37^{Ab}	17.85±1.03^{Aa}
	Nepro®	5.62±0.83^{Ab}	7.93±0.11^{Ab}	12.96±10.74^{Ab}	24.38±10.71^{Aa}

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

Short chain fatty acids are regarded as aliphatic carboxylic acids of low carbon number (C2–6) that are produced during colonic fermentation of dietary fiber or via protein catabolism (creating branch-chained forms), with acetate (C2), propionate (C3) and butyrate (C4) the predominant contributors to total SCFA content [51]. Thus, total SCFAs from this study were calculated by combined the concentrations of acetic, propionic, and butyric acids for report. Effects of difference samples on total SCFAs production in fecal batch fermentation of control, Synplus and Nepro® was showed in Figure 3. The result presented that the total SCFAs was significantly increased (p<0.05) by fermentation time increased. Interestingly, total SCFAs from Synplus fermentation was significantly highest (p<0.05) when compared with other samples. This phenomenon was elucidated by due to Synplus fermentation was composited of oligosaccharide and the encapsulated probiotics *Lactobacillus paracasei* CASEI 431® and *Bifidobacterium animalis* subsp. *lactis* BB12®. The concentration of total SCFAs for Synplus fermentation were 21.10±2.72, 176.06±1.57, 203.34±8.91 and 238.01±1.92 mM at 0, 12, 24, and 48 h, respectively. In addition, total SCFAs concentration of Synplus fermentation showed significantly increased (p<0.05) and higher than control and Nepro® since 12 hrs of the fermentation time. Likewise, for the Nepro® fermentation, the total SCFAs was significantly increased by fermentation time increased (p<0.05) and the concentration were 21.62±9.10, 117.27±9.17, 135.75±6.13 and 150.71±1.37 mM at 0, 12, 24, and 48 hrs, respectively. It is worth noting that total SCFAs of Synplus and Nepro® were significantly increased (p<0.05).

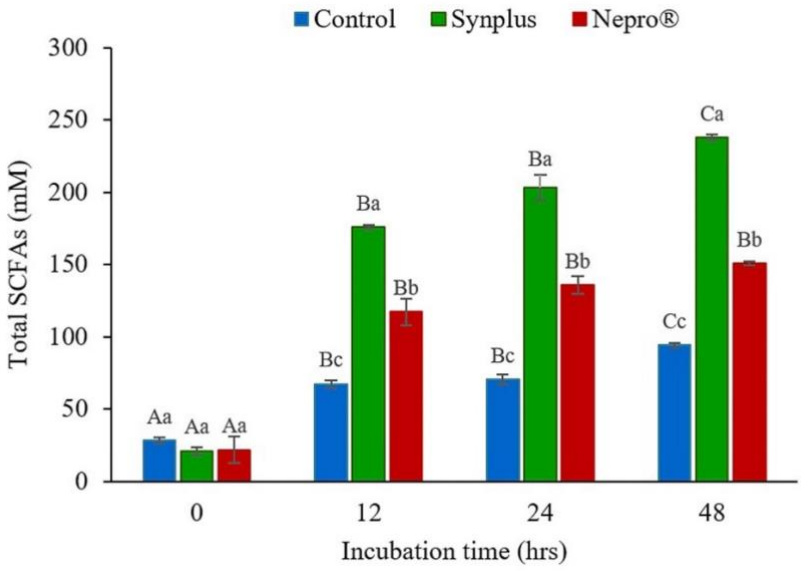


Figure 3. Effects of nutritionally complete formula on SCFAs production in fecal batch fermentation.

Different capital letters above the bars indicate significant difference between samples and lowercase letters indicate significant difference between incubation times (p<0.05).

Short chain fatty acids have been studied as products of fermentation from indigestible carbohydrates by the gut [37], including dietary fibers or oligosaccharides [38]. Previous studies in rodent models reported increased numbers of *Lactobacillus* spp. and *Bifidobacterium* spp. as a result of dietary intervention with IMO [52]. It is possible that Synplus had been added prebiotic IMO in the product composition led to abundance of *Lactobacillus* spp. and *Bifidobacterium* spp. result in an encouraged to significantly increasing (p<0.05) of their metabolites. Animal and cell culture studies involving supplementation reported that SCFAs exhibited the ability to alievate several progressive factors which are believed to support the progression of CKD by decreasing inflammatory and fibrotic responses [51]. Moreover, a variety of experimental and clinical trials were reported the positive associated of SCFAs on CKD progression such as decreasing of uremic toxin and

microbiota balance, mucosal effect, immunity, and host metabolism [14]. In addition, decreased intake of fermentable dietary fiber may lead to a great depletion in the abundance of SCFA-producing bacteria, a situation which may partly contribute to a decrease in the regulatory T lymphocytes along with CKD-related systemic inflammation [4]. However, Effects of Synplus product on lactate and SCFAs production in animal and clinical trial need to be an administration for additional data and confirm.

Production of p-cresol and indole: The effect of the nutritionally complete formula (Synplus) on production of metabolites including *p*-cresol (Figure 4a) and indole (Figure 4b) in batch fermentation using feces of healthy volunteers were studied. The result was showed that *p*-cresol concentration significantly highest ($p<0.05$) at 48 hrs fermentation for Nepro®. While no significant difference ($p>0.05$) for control and Synplus. However, at 48 hrs of fermentation were found that the *p*-cresol concentration of control, Synplus and Nepro® were 3.79±0.12, 6.31±2.37 and 11.59±0.10 µg/ml, respectively. Interestingly, the *p*-cresol concentration of Nepro® was significant increased ($p<0.05$) since 12 hrs of fermentation and significant difference ($p<0.05$) when compared with control and Synplus. On the other hand, the result showed difference for indole production. It was found at 24 hrs for Synplus fermentation showed significant highest ($p<0.05$) of indole concentration. The concentration of indole at 24 hrs incubation for control, Synplus and Nepro® were 4.93±0.06, 15.75±0.98 and 12.13±0.80 µg/ml, respectively. In addition, it was found that at 48 hrs of Synplus fermentation gave a high level of indole concentration and significant difference ($p<0.05$) when compared with control, but no significant difference ($p>0.05$) when compared with Nepro®. The concentration of indole at 48 hrs incubation for control, Synplus and Nepro® were 3.64±0.08, 15.06±3.56 and 12.81±1.68 µg/ml, respectively.

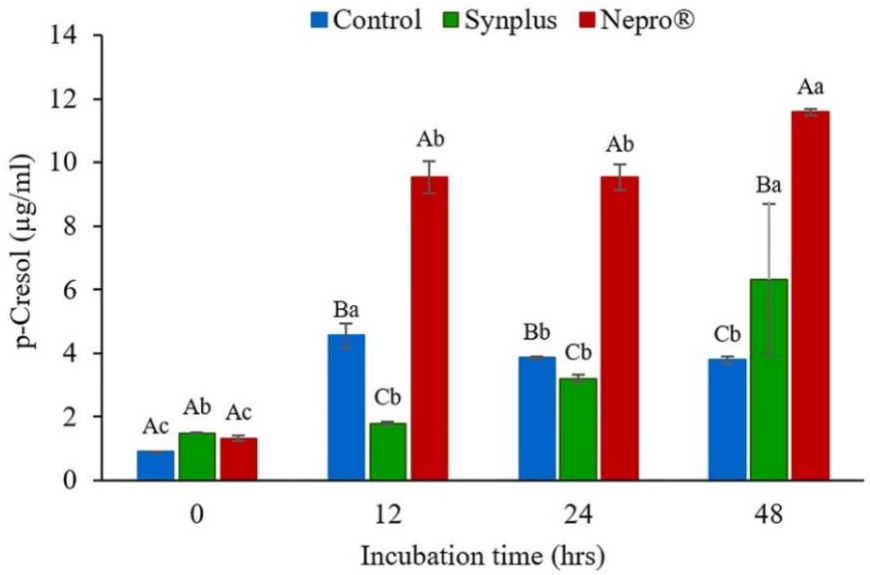
A significant ($p<0.05$) increase in the amounts of indole with *p*-cresol in the Synplus and Nepro® samples probably because both samples were products containing high protein. The nutrition information of Synplus product was reported that protein containing up to 18% of total calories [16] and 14% of total calories for Nepro®. As we known protein as a source for uremic toxin generation. The samples were compared during with vessel containing protein (Synplus and Nepro®) and without (control) at 24 and 48 hrs in fecal batch fermentation have significantly higher concentrations of indole and *p*-cresol as Figure 4a and Figure 4b. This result agrees with previous studies by Wang *et al*. [53], where they investigated the effects of prebiotics on the proteolysis and their potential in counteracting the adverse effects of a high-protein diet. They were found volatile organic compounds (VOCs) such asindole, phenol as well as *p*-cresol were increased during fermentation. The protein diet affected the production of indole and *p*-cresol [53].

From this study, Synplus and Nepro® led to the production of highest concentration of indole and *p*-cresol. This was probably because both products contain high levels of aromatic amino acids, which are the primary raw materials for bacteria to synthesize phenolic and indolic compounds [53]. Protein metabolism by the gut bacteria is potentially harmful because of the produced toxic subtances including ammonia, amines, *p*-cresol, and indole [53]. Phenolic compounds (phenol and *p*-cresol) are microbial metabolites generated from tyrosine [54-55]. These compounds present cytotoxicity and enhances paracellular permeability *in vitro* [56] and promote skin cancer [57]. *p*-Cresol demonstrates cytotoxicity and genotoxicity, hence reducing endothelial barrier activity *in vitro* [58]. *p*-Cresyl sulfate, a sulfate-conjugate of *p*-cresol, inhibits Th1-type cellular immune responses in mice [59]. The increased amount could be linked to chronic kidney disease-related events likecardiovascular disease [60-61]. Indoles are aromatic categories of compounds consisting of a pyrrole ring. Tryptophan metabolism through bacterial tryptophanase produces more than 600 indoles in the colon, which are aassimilated, and sulfate conjugated in the liver [62]. Indoxyl sulfate (IS) levels in the serum are inversely correlated with the degree of kidney function [63] and could be utilized to predict CKD progression [64]. In addition, tyrosine metabolism, phenylalanine, and tryptophan synthesize phenol, indole, *p*-cresol, and

skatole, which are likely carcinogens; phenol and *p*-cresol may slow down intestinal epithelial barrier function *in vitro* [65-67].

Even though, the results from this study were demonstrated that Synplus product promotes to increasing of indole and *p*-cresol production in fecal batch culture fermentation by fecal inoculum. However, not only that compound but it was found that significantly elevated concentrations of SCFAs production for fermentation with a sample containing prebiotics and probiotic (Synplus). Therefore, the ratio of uremic (indole and *p*-cresol) and SCFAs (acetic, propionic and butyric) production were calculated and reported in Table 2.

(a)



(b)

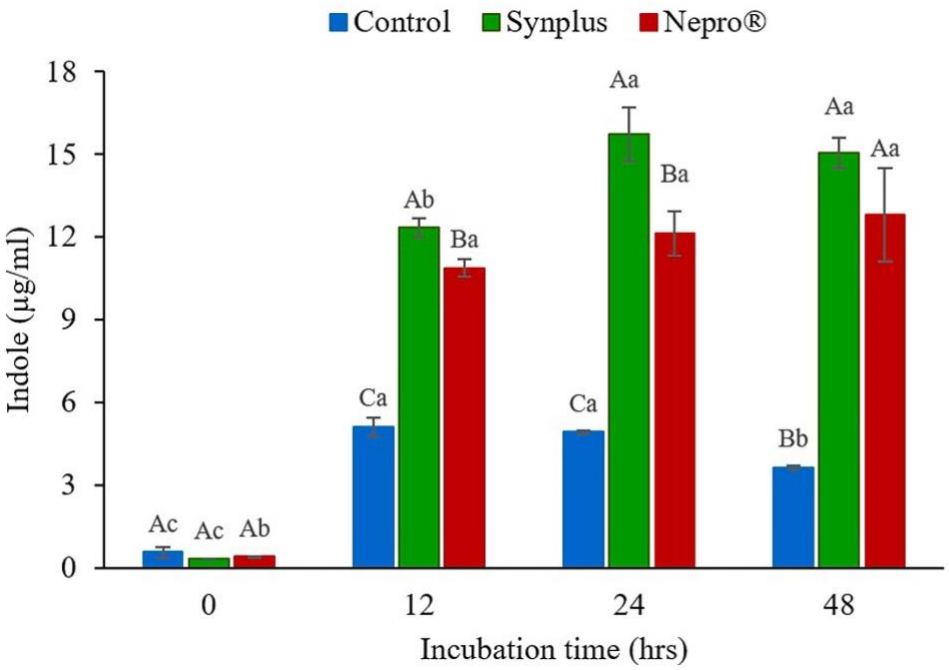


Figure 4. Effects of nutritionally complete formula on uremic toxins production; *p*-cresol (a) and indole (b) in fecal batch fermentation by fecal inoculum from healthy volunteers. Different capital letters above the bars indicate significant difference between samples and lowercase letters indicate significant difference between incubation times ($p<0.05$).

The ratio of uremic (indole and *p*-cresol, μM) and SCFAs (mM) especially at 48 hrs of fermentation showed uremic to SCFAs ratio were 1:1.42, 1:1.27 and 1:0.74 for control, Synplus and Nepro® respectively. It was indicated that even though indole and *p*-cresol significantly lower ($p<0.05$) in the control sample but in term of uremic to SCFAs ratio almost close with Synplus. Synplus fermentation showed significant highest *(* $p<0.05$*)* of SCFAs then refer to uremic (μM) to SCFAs (mM) ratio showed at 48 hrs was 1: 1. 27. This implies that even though indole and *p*-cresol concentration increased but also increasing of SCFAs for Synplus fermentation. Enhanced consumption of prebiotics, with the possibility of reaching the colon and leading to particular modulations in the composition and/or activity in the gut microbiome, may lessen the adverse effects of gut microbial proteolysis in patients consuming diets with high-protein or food formula with high-protein [51]. Thus, this effect needs to be confirmed *in vivo*, and there is a chance that consumption at a higher dosage may exhibit more beneficial gut modulation and health outcomest. This study also showed the significance of diet or nutrient on the metabolic effectsassociated with CKD by the activity of gut mocrobiota. This relation therefore suggests that diet helps to modulatethe gut bacteria, which in turn could mitigate potential risks of kidney disease in normal populations.

Table 2. The ratio of uremic (indole and *p*-cresol) and SCFAs (acetic, propionic and butyric) production in fecal batch fermentation.

Fecal fermentation time (hrs)	Sample	Uremic: SCFA (indole and *p*-cresol) : (acetic, propionic and butyric)(μM : mM)
0	Control	13.25:28.27 (1:2.13)
	Synplus	16.54:21.09 (1:1.27)
	Nepro®	15.71:21.61 (1:1.37)
12	Control	85.93:67.28 (1:0.78)
	Synplus	121.99:176.00 (1:1.44)
	Nepro®	181.18:117.27 (1:0.64)
24	Control	77.78:70.55 (1:0.90)
	Synplus	164.04:203.34 (1:1.23)
	Nepro®	212.95:135.75 (1:0.63)
48	Control	66.17:94.15 (1:1.42)
	Synplus	186.39:238.01 (1:1.27)
	Nepro®	202.22:150.71 (1:0.74)

CONCLUSION

The nutritionally complete formula (Synplus) showed alter and/ or maintain the beneficial gut microbiota and their metabolite in *in vitro* fecal fermentation in batch culture using healthy volunteers' feces. The Synplus fermentation was showed an abundance the genus of *Lactobacillus* spp. (53.74%) and *Bifidobacterium* spp. (29. 35%) while the genus *Escherichia* spp. (12. 33%) was observed in Nepro® fermentation especially, the specie of *Escherichia albertii*. The generation of microbial metabolites by fecal fermentation of Synplus demonstrated that acetate,

propionate, and butyrate significantly (p<0.05) increased as fermentation time increased. Total SCFA concentration of Synplus was significantly highest by 238.01±1.92 mM at 48 hrs of fermentation (p<0.05). In addition, total SCFAs concentration of Synplus fermentation showed significantly increased (p<0.05) and higher than control and Nepro® at 12 hrs and throughout of the fermentation time. Moreover, the concentration of *p*-cresol for Synplus (6.31±2.37 µg/ml) were significantly lower than Nepro® (11.59±0.10 µg/ml) at 48 hrs fermentation, and no significant different in indole. However, both metabolites remain significantly higher than control. The study was also indicated that imbalance of gut microbiota was related with the ratio of uremic (indole and *p*-cresol) to SCFAs. Thus, it can be said that synbiotic formula change in the gut microbiota and benefits on health which may be used to reduce the risk of kidney disease in normal population by stimulate production of SCFAs and suppression of uremic toxin released.

List of Abbreviations: CKD: chronic kidney disease, SCFAs: short-chain fatty acids, NGS: next generation sequencing, HPLC: high performance liquid chromatography, IMO: isomalto-oligosaccharide, GABA: Gamma aminobutyric acid, SGF: simulated gastric fluid, SIF: simulated intestinal fluid, PBS: phosphate-buffered saline, AMPs: antimicrobial peptides, ESRD: end-stage renal disease, VOCs: volatile organic compounds, IS: Indoxyl sulfate.

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

Author's Contributions: All authors contributed to this study and the writing of the paper.

Acknowledgements and Funding: This work was financially supported by Faculty of Sports and Health Science, Thailand National Sports University Yala Campus, Yala, Thailand.

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