Enzyme Treatment Enhances Release of Prebiotic Oligosaccharides from Palm Kernel Expeller

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Heat and enzyme treatments were used to increase the prebiotic oligosaccharides from palm kernel expeller (PKE), and the prebiotic efficacy of three types of PKE-extracts, namely raw PKE-extract (PKE_{RAW}), enzyme-treated PKE-extract (PKE_{ENZ}), and steam + enzyme-treated PKE-extract ($SPKE_{ENZ}$) was evaluated *in vitro* using three strains of *Lactobacillus* (*L. brevis* I 218, *L. salivarius* I 24 and *L. gallinarum* I 16), and *in vivo* using Sprague-Dawley rats as an animal model. Results of the *in vitro* study showed that the PKE-extracts were able to support the growth of *Lactobacillus sp.* strains. However, their growth varied significantly among strains and PKE-extracts ($P<0.05$), with *L. brevis* I 218 recording the highest growth compared to the other two strains, and the highest growth in the steam plus enzyme ($SPKE_{ENZ}$) extract. Results of the *in vivo* study reaffirmed that all the PKE-extracts tested can support growth of beneficial bacteria (*Lactobacillus and Bifidobacterium*), but only $SPKE_{ENZ}$ treatment group had significantly higher *Lactobacillus* and *Bifidobacterium* counts and lower population of *E. coli* compared to the control. It was demonstrated that PKE is a potential source of prebiotic, which may be used to effectively improve host health and wellbeing by modulating the host gut microflora, and by proper pre-treatment, the release of prebiotic oligosaccharides from PKE can be enhanced.

Keywords: Palm kernel expeller; Oligosaccharides; Gut microflora; Prebiotic

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INTRODUCTION

Palm kernel expeller (PKE) is an agro-industrial byproduct that contains a high level of non-starch polysaccharides (NSPs), mainly in the form of mannan (Dusterhoft *et al.* 1991). This extremely hard, highly crystalline, and water insoluble mannan is the main drawback for its utilization in the feed industry, particularly for monogastric animals such as broiler chickens, swine, and fish. This limitation has prompted many researchers to focus on the application of fungal cultures and/or use of enzymes to hydrolyze the mannan fiber to improve the nutritive quality of PKE. However, results obtained thus far have been inconsistent and not very encouraging (Ng *et al.* 2002; Iyayi and Davies 2005; Saenphoom *et al.* 2011). Many studies testing the effect of enzyme treatment on PKE and on the growth of broiler chickens were conducted (Saenphoom *et al*. 2011; 2013). The same studies reported that, upon enzyme treatment, total reducing

sugar (mainly mannose) increased many folds, but the additional sugar in the treated PKE did not improve the growth performance of the broiler chickens. The authors postulated that pre-treating PKE with enzyme primarily releases mannose and mannose-based oligosaccharides, and these are not readily absorbed and utilized as energy source but could be a source of prebiotic. Other previous studies reported that inclusion of PKE decreased pathogenic bacteria colonization (Allen *et al.* 1997; Fernandez *et al.* 2000), and increased beneficial bacterial population (Fernandez *et al.* 2002) as well as serving as a defense against viral-caused disease in broilers (Zulkifli *et al.* 2003), suggesting the potential use of PKE as source of prebiotic.

The benefits of oligosaccharides to modulate intestinal microflora of animals have been demonstrated *in vitro* (Ofek and Beachey 1978; Kaplan and Hutkins 2003; Saminathan *et al*. 2011) and in animal trials (White *et al*. 2002; Sang and Fotedar 2010; Corrigan *et al*. 2012). Theoretically, these oligosaccharides can withstand the digestive enzymes and flow to the lower intestines unaltered to be used by the beneficial bacteria, such as *Lactobacillus sp.* and *Bifidobacterium* sp. (Mitsuoka *et al*. 1987). This has been demonstrated in the animal study of Corrigan *et al*. (2012), who reported that, in turkeys, the *Lactobacillus sp.* population increased when they were fed MOS-supplemented rations (1 g/kg diet). Similarly, Baurhoo *et al*. (2007) demonstrated that dietary MOS increased *Lactobacillus* and *Bifidobacterium* contents. However, inconsistencies exist on the effect of oligosaccharides on growth performance and bacterial population of host animals, presumably due to differences in sources (Asano *et al.* 2004; Yalcinkaya *et al.* 2008), types (Swanson *et al*. 2002a; Cieslik *et al.* 2005), and inclusion levels (Mourao *et al*. 2006; Kim *et al*. 2011) among the studies.

Therefore, the purpose of this study was to assess whether enzyme treatment on PKE can increase the availability of oligosaccharides and the influence of the latter to support the growth of beneficial bacteria. This study consisted of two experiments: an *in vitro* assessment of PKE-extracts to support the growth of *Lactobacillus* sp., followed by an *in vivo* trial using experimental rats as a study model to evaluate the efficacy of the PKE-extracts to modify the gut bacterial population of the host animals.

EXPERIMENTAL

Materials

Samples of PKE were collected from a commercial kernel oil extraction mill in Kuantan, Malaysia. The crude enzyme used in this study was obtained through solid state fermentation of PKE using *Aspergillus terreus* K1 as inoculum. Briefly, distilled water (pH 5.8) was added to ground PKE to achieve a moisture content of approximately 627 g/kg, inoculated with 6.0×10^5 *A. terreus* spore per gram of PKE, and incubated at 30.5 °C for 7 days (Chen *et al.* 2013). After incubation, the crude enzyme produced was extracted by shaking the PKE in distilled water at 4 °C for 24 h, centrifuging at 10,000 × *g* for 10 min, and filtering through Whatman No. 1 filter paper. The extracted crude enzyme was concentrated by freeze drying and stored at 4 °C for subsequent use. The above crude enzyme was reported to consist of 19.97, 44.12, and 262.01 U/g DM of endogluconase, mannanase, and xylanase, respectively (Chen *et al*. 2013).

Preparation of PKE-Extract

In this study, three PKE-extracts: raw PKE-extract (PKE_{RAW}), enzyme-treated PKE-extract (PKE_{ENZ}), and steam + enzyme-treated PKE-extract ($SPKE_{ENZ}$) were prepared using solid-state fermentation, respectively. The PKE_{RAW} was prepared by shaking PKE in distilled water at 4 °C for 24 h, followed by centrifugation at 3,000 × *g* for 10 min, and filtered through Whatman No. 1 filter paper. The PKE_{ENZ} and $SPKE_{ENZ}$ were prepared by following the procedure of Saenphoom *et al*. (2011), with minor modifications. Briefly, PKE was moistened with distilled water to achieve a moisture content of approximately 600 g/kg, and it was fermented with 1 mL of crude enzyme per 100 g of PKE at 55 °C for 24 h. After incubation, PKE filtrate was obtained by shaking the PKE in distilled water at 4 °C for 24 h, centrifuging at 3,000 × *g* for 10 min, and filtering through Whatman No. 1 filter paper. All three types of PKE filtrate were lyophilized by freeze drying to obtain solid PKE-extracts. For the $SPKE_{ENZ}$ preparation, moist PKE (60% moisture) was first autoclaved at 121 °C for 15 min, and the autoclaved PKE was then used as the substrate in SSF, which was carried out using the procedure described above.

In vitro Assessment of PKE-extract on Growth of *Lactobacillus sp.*

The PKE-extracts obtained through the different pre-treatment methods were assessed for their prebiotic ability to support the growth of three *Lactobacillus* strains, according to the method described by Kneifel *et al.* (2000). This method reports the growth of each *Lactobacillus* strain relative to its growth in a modified MRS broth containing glucose, which is the favourable growth media for *Lactobacillus* strains. This allows the comparison of growth between different *Lactobacillus* strains since each *Lactobacillus* strain has its own growth kinetics. The three strains of *Lactobacillus* (*L. gallinarum* I 26, *L. salivarius* I 24, and *L. brevis* I 218) were originally isolated from the ileum of local chickens in Malaysia (Jin *et al.* 1996) and re-classified by sequencing the gene and 16S-23S rRNA gene intergenic spacer region (Lee *et al.* 2008).

Each PKE-extract (30 g/L) was prepared in de-ionized water and sterilized by filtering each solution through 0.2 µm filters (Minisart, Sartorius AG, Germany). Modified MRS medium containing 10 g/L peptone (Oxoid Ltd), 5 g/L yeast extract (Oxoid Ltd), 5 g/L sodium acetate (Sigma-Aldrich, St Louis, MO, USA), 2 g/L $K_2HPO_4 \cdot 3H_2O$ (Sigma-Aldrich), 2 g/L $(NH_4)_3C_6H_5O_7 \cdot 2H_2O$ (Sigma-Aldrich), 0.2 g/L $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich), 0.05 g/L $MnSO_4 \cdot 4H_2O$ (Sigma-Aldrich), and 1 ml/L Tween 80 (Merck, Darmstadt, Germany), was adjusted to a final pH of 6.2, and sterilized by autoclaving at 121°C for 15 min. Prior to the test, 10 g/L of filter-sterilized glucose was added to the modified MRS broth. PKE-extract, modified MRS broth with glucose (control), and modified MRS broth without glucose (control-blank), was then inoculated with an overnight inoculum (18 h old) and incubated anaerobically in an anaerobic jar with gas generating kits (Oxoid Ltd.) at 37 °C for 24 h. Experiments were conducted in triplicate for each *Lactobacillus* strain and for each PKE-extract. After incubation, each culture was vortex for 30 s, and absorbance was read at 620 nm. The growth of *Lactobacillus* in the PKE-extracts is reported as a relative comparison to its growth in the modified MRS broth containing glucose, calculated using the method of Kneifel *et al.* (2008). Relative growth of *Lactobacillus* strain on PKE-extract was calculated for each of the three replicate from each treatment as follows,

$$\text{Relative growth} = (A/B) \times 100\% \quad (1)$$

where *A* is the mean absorbance of each replicate of PKE-extract and *B* is the mean absorbance of the same strain on control.

Results of the relative growth of *Lactobacillus* on different PKE-extracts show that only *L. brevis* I 218 had a relatively moderate growth on enzyme treated PKE. Thus, the monosaccharides and oligosaccharides concentrations in each extract (before and after incubation) were determined by HPLC. This was to determine whether the differences in growth observed were due to the different levels of oligosaccharides present in each PKE-extract, or due to the excess glucose in the enzyme treated PKE.

Monosaccharide and oligosaccharide concentrations were determined by HPLC (Waters, USA, 2690), using a COSMOSIL Sugar-D column (4.6 mm I.D. × 250 mm) (Nacalai, San Diego, USA). The monosaccharide concentration was assayed by using an acetonitrile/water mixture (80/20; v/v) with a flow rate of 1.0 mL/min at 30 °C, while the oligosaccharides concentration was determined by using a mobile phase consisting of an acetonitrile/water mixture (65/35; v/v) with a flow rate of 0.7 mL/min. Different concentrations of monosaccharides (fructose, xylose, mannose, and glucose) (Sigma-Aldrich) and Mannan oligosaccharides [degree of polymerization (DP) of 2 to 6] (Megazyme, Co., Wicklow, Ireland) were used as standards.

In vivo Assessment of PKE-Extract on Feacal Bacterial Population

Thirty-two Sprague-Dawley rats (A-Sapphire Enterprise, Selangor, Malaysia), weighing 180 to 200 g, were individually housed in wire-topped plastic cages (47 cm length × 35 cm width × 20 cm height) with appropriate space, free access to clean drinking water, and a standard rodent diet (Specialty Feeds, Glen Forest, Australia). The cages were kept in a room with a mean temperature of 24 ± 3 °C and a 12 h light–dark cycle. The rats were randomly divided into four groups: i) Control, ii) PKE, iii) PKE_{ENZ}, and iv) $SPKE_{ENZ}$, in a completely randomized design with 8 rats (replicate) per group. After one week of acclimatization, the rats were fed 3 mL each with one of the following four treatments: i) distilled water (Control), ii) PKE-extract, iii) PKE_{ENZ}-extract, or iv) $SPKE_{ENZ}$-extract through oral administration daily, for a period of 10 days. The PKE-extracts used for the feeding trial were prepared by diluting 50 g of the respective PKE-extract in 100 mL of distilled water. At the end of the experiment period, feacal samples (collected between 2 and 5 h post administration of the extracts) were collected using Falcon tubes, weighed, and then immediately frozen at -20 °C. Animals were cared for according to the guidelines of the Animal Care and Ethics Committee of the University Putra Malaysia.

Enumeration of Feacal Bacterial Populations

The feacal samples collected at the end of experimental period were used for microbial quantification by real time PCR. A QIAamp DNA Stool Minikit (Qiagen) was used to extract DNA from 0.2 g of the frozen stool sample. The extracted DNA was used as a template for real-time PCR assay. The amplification reactions were carried out using iQ^{TM}SYBR Green Supermix (BioRad, USA) in a total volume of 25 µL containing 12.5 µL SYBR Green Supermix, 1 µL of each Primer, 1 µL of DNA samples and 9.5 µL H_2O. The primers used to detect the different population of microorganisms are shown in Table

1. Amplification (5 min at 94 °C, followed by 40 cycles of 20 s at 94 °C, 30 s min at annealing temperature as listed in Table 1, 20 s at 72 °C), and detection was performed using BioRad CFX96 Touch (BioRad, USA). Each assay was performed in duplicate in the same run. The cycle threshold (C_T) was calculated as the cycle number at which the reaction became exponential. The cycle threshold of each sample was then compared to a standard curve made by serial dilution of plasmid DNA of each microbial group.

Statistical Analysis

Data were analyzed using the SPSS statistical package program, version 16.0 (SPSS Inc., Chicago, IL, USA). The effect of different PKE-extracts, *Lactobacillus* strains and feacal bacteria population was tested using one-way analysis of variance (ANOVA). Significant differences between means were analyzed using Duncan's multiple range test at $P < 0.05$.

Table 1. Names, Sequences, Application, Product Size, Annealing Temperature, and References of the Primers Used

Target organism	Primer set (5'→3')	Annealing Temperature	Reference
Total Bacteria	F: CGGCAACGAGCGCAACCC	55°C	1
	R: CCATTGTAGCACGTGTGTAGCC		
Enterococcus spp.	F: CCCTTATTGTTAGTTGCCATCATT	50°C	1
	R: ACTCGTTGTACTTCCCATTGT		
Enterobacter spp.	F: CATTGACGTTACCCGCAGAAGAAGC	50°C	1
	R: CTCTACGAGACTCAAGCTTGC		
Lactobacilli	F: CATCCAGTGCAAACCTAAGAG	58°C	2
	R: GATCCGCTTGCCTTCGCA		
E. coli	F: GTGTGATATCTACCCGCTTCGC	50°C	3
	R: GAACGCTTTGTGGTTAATCAGGA		
Bifidobacteria	F: GGGTGGTAATGCCGGATG	60°C	4
	R: TAAGCCATGGACTTTCACACC		

[1]Navidshad *et al.* (2012); [2]Wang *et al.* (1996); [3]Frahm and Obst (2003); [4]Bartosch *et al.* (2005)

RESULTS

In vitro Assessment of PKE-extract on Growth of *Lactobacillus*

Growth responses of the three *Lactobacillus* strains relative to their respective growth on MRS are presented in Table 2. The results show that all three strains could grow on the PKE-extracts, but their growth varied among strains and PKE-extracts ($P < 0.05$). All three *Lactobacillus* strains demonstrated the best growth ($P < 0.05$) on

$SPKE_{ENZ}$ followed by $PKE_{ENZ.}$ Despite the differences, the growth of *L. salivarius* I 24 and *L. gallinarum* I 16 on PKE-extract was low, with relative growth rate of ranging between 0.22 and 0.37. Growth of *L. brevis* I 218 was poor on raw PKE extract, but moderate ($P < 0.05$) on enzyme-treated PKE (PKE_{ENZ} and SPE_{ENZ}) with relative growth of between 0.42 and 0.61.

The mono- and oligosaccharide contents before and after incubation with *L. brevis* I 218 were analyzed using HPLC to explain the differences in its growth rates among the control and the enzyme treatment groups. The results are presented in Figs. 1 and 2. The monosaccharides of PKE-extract was comprised of fructose, glucose, and a small amount of mannose (in the ratio of 5:4:1) with no xylose detected. With enzyme treatment, the total monosaccharides content of PKE-extract increased 3 and 4 folds, respectively, for the PKE_{ENZ}-extract and $SPKE_{ENZ}$-extract. After incubation with *L. brevis* I 218, total monosaccharides decreased by 32%, 51% and 49% in the non-treated PKE, PKE_{ENZ} and $SPKE_{ENZ}$-extract, respectively.

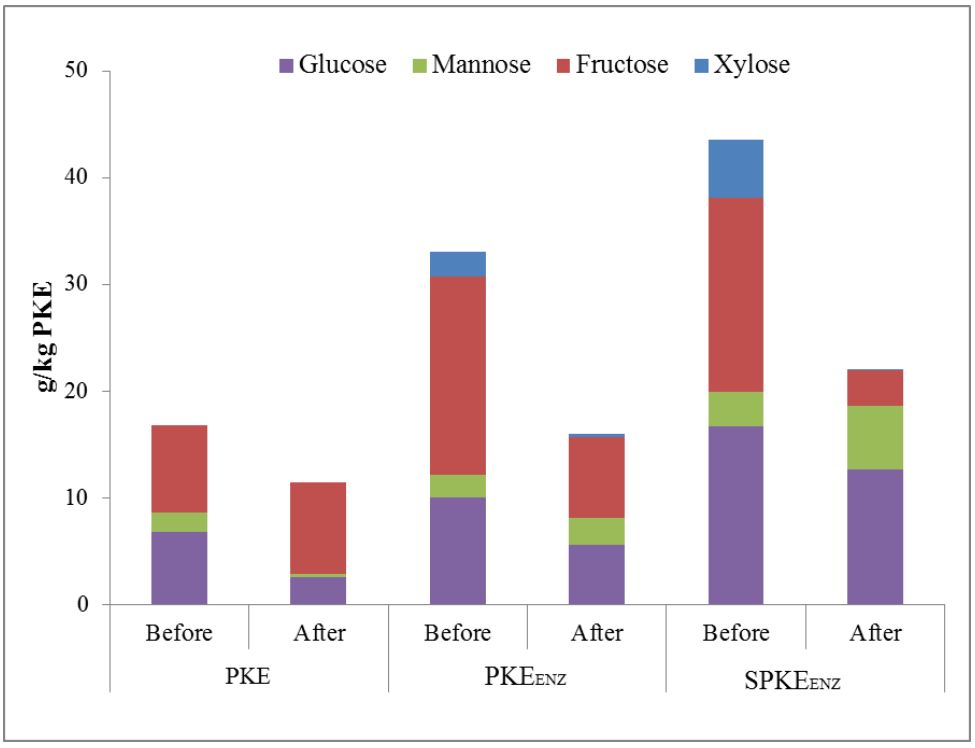

Fig. 1. Monosaccharides concentration before and after incubation with *L. brevis* I 218 in PKE-extract, PKE_{ENZ} extract and $SPKE_{ENZ}$ extract

In terms of glucose, the reduction was in the range of 4 to 4.5 g/kg for the different PKE-extracts. Fructose content decreased in enzyme-treated PKE after incubation, but increased in untreated PKE-extract after incubation. Xylose content in both PKE_{ENZ}-extract and $SPKE_{ENZ}$-extract was utilized almost completely (87 and 97 % reduction, respectively) after incubation, whereas mannose was utilized almost completely in the non-treated PKE-extract (85% reduction). In contrast, there was an increase in mannose content in the two enzyme-treated PKE-extracts, 19 and 83 %, respectively, for PKE_{ENZ} and $SPKE_{ENZ}$.

Despite knowing that the PKE-extracts used in this study were not purified and may consist of a mixture of oligosaccharides [such as MOS, FOS, galactooligosaccharides (GOS) or xylooligosaccharides (XOS)], it is assumed that MOS is the main component because the majority of the NSPs in PKE is in the form of mannan (Dusterhoft *et al.* 1991). Pure MOS (DP of 2 to 6) fractions were used as standards to estimate the concentrations of the different oligosaccharides in each treatment using HPLC.

Results of the HPLC analysis showed that PKE_{ENZ} and $SPKE_{ENZ}$ also contained more mannan oligosaccharides (28.91 and 59.71 g/kg PKE, respectively) compared to the PKE-extract (20.93 g/kg PKE). In addition, untreated PKE typically consisted of higher DP oligosaccharides (mannohexose and mannopentose), and with enzyme treatment, these oligosaccharides were broken down into smaller DP oligosaccharides (mannobiose, mannotriose, and mannotetrose) (Fig. 2).

Nevertheless, the total oligosaccharides content of the untreated PKE (PKE-extract) decreased by 15.30% after incubation with *L. brevis* I 218, while that of both enzyme-treated PKE (PKE_{ENZ} and $SPKE_{ENZ}$) increased upon incubation with the same *Lactobacillus* (15.67 and 9.16 %, respectively).

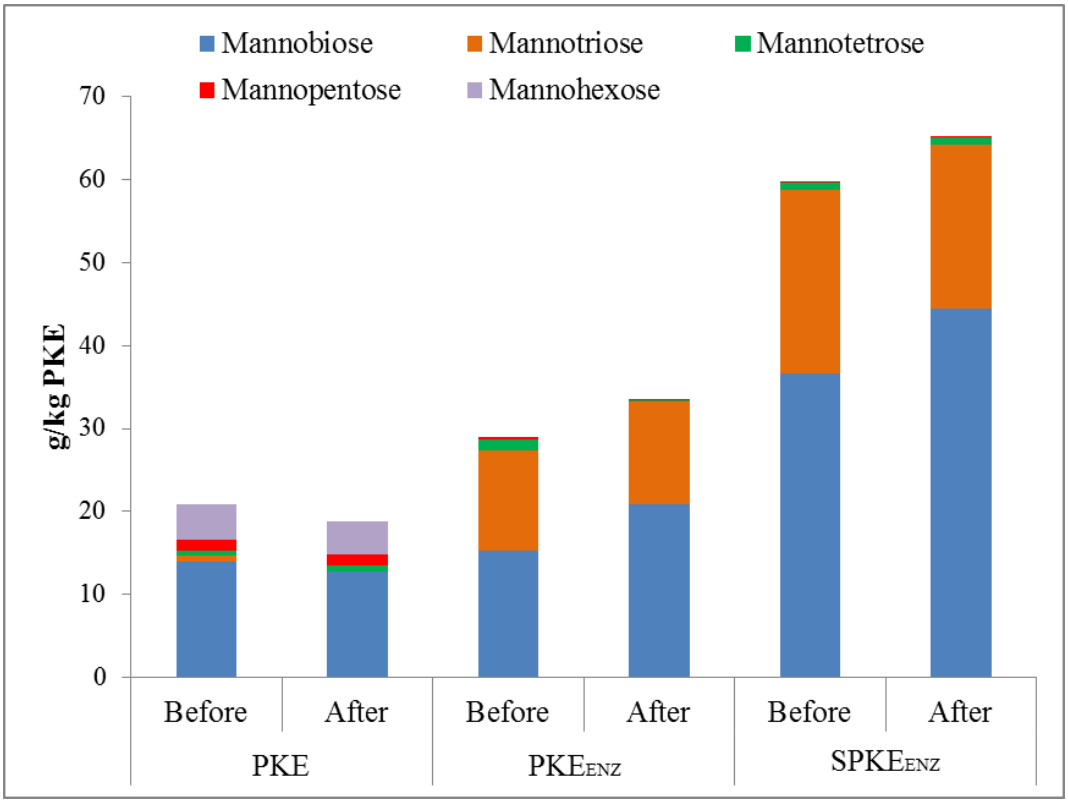

Fig. 2. Oligosaccharides concentration before and after incubation with *L. brevis* I 218 in PKE, PKE_{ENZ}, and $SPKE_{ENZ}$ extracts by using MOS of DP 2-6 as standard

Table 2. Relative Growth of *Lactobacillus* Strains on Different PKE-extracts[1]

Strain	PKE	PKE_{ENZ}	$SPKE_{ENZ}$
L. salivarius I 24	0.27 ± 0.024 ^{Aa}	0.31 ± 0.018 ^{Aa}	0.37 ± 0.056 ^{Ab}
L. gallinarum I 16	0.22 ± 0.021 ^{Ba}	0.28 ± 0.034 ^{Ab}	0.30 ± 0.005 ^{Bb}
L. brevis I 218	0.23 ± 0.005 ^{Ba}	0.42 ± 0.005 ^{Bb}	0.61 ± 0.017 ^{Cc}

[1] Results are mean values for 3 replicates ± standard deviation; $^{A-C}$ means in the same column with different uppercase letter are significantly different ($P < 0.05$); $^{a-c}$ means in the same row with different lowercase letter are significantly different ($P < 0.05$).

Enumeration of Feacal Bacteria Population

This study was performed to examine whether supplementing rats with PKE-extracts can modify the feacal bacteria population of the experimental rats. Overall, the data show that supplementation of PKE-extract increased the beneficial bacterial population, while the pathogenic bacteria population was suppressed (Table 3). The feacal microbial population of rats fed PKE-extract did not differ from those in the control. PKE_{ENZ} supplementation significantly increased the feacal *Lactobacillus* number but not that of *Bifidobacterium* and *E. coli* ($P < 0.05$), as compared to control and PKE supplementation. In contrast, $SPKE_{ENZ}$ significantly increased ($P<0.05$) total bacteria, *Lactobacillus,* and *Bifidobacterium*, and decreased ($P< 0.05$) *Enterococcus*, *Enterobacter,* and *E. coli* populations compared to the control.

Table 3. Effect of PKE-extracts on Feacal Bacterial Population (cells/g feacal DM)

Microorganism	Control	PKE	PKE_{ENZ}	$SPKE_{ENZ}$	SEM
Lactobacillus ($\times 10^6$)	0.62^a	1.51^a	3.56^b	4.46^b	0.41
Bifidobacterium($\times 10^5$)	0.52^a	1.16^{ab}	1.20^{ab}	1.87^b	0.09
Enterococcus ($\times 10^6$)	7.09^a	3.76^b	3.70^b	4.02^b	0.47
Enterobacter($\times 10^5$)	5.61^a	2.33^b	3.56^{ab}	1.20^b	0.51
E. coli ($\times 10^5$)	2.29^a	0.49^b	0.84^{ab}	0.27^b	0.20
Total bacteria ($\times 10^{12}$)	0.72^a	1.00^a	1.10^a	2.29^b	0.13

DISCUSSION

The PKE is generally regarded as high in fiber (16 to 18 g/100 g) with NSPs making up about 410 g/kg of the total carbohydrate (excluding lignin). Similar to the seed of most Arecaceae, the NSP of PKE exists mainly in the form of linear mannan with low galactose substitution (580 to 780 g/kg), while the remainder portions of its NSP are

cellulose and xylans (120 and 30 g/kg, respectively) (Dusterhoft *et al*. 1991). These hemicellulosic constituents are comprised of a variety of polysaccharides with linear or branched polymers derived from monosaccharides such as D-mannose, D-galactose, D-xylose, D-glucose, and L-arabinose (Alang *et al*. 1988; Cervero *et al.* 2010). Thus, it seems possible that complete hydrolysis of the hemicellulose would give rise to a mixture of monosaccharides, with mannose being the major constituent, and glucose and others being present in smaller quantities. For the purpose of this study, the hemicellulose of PKE was partially hydrolysed in order to obtain oligosaccharides (DP<6) from PKE. Based on the present results, the untreated PKE contains 20.93 g/kg oligosaccharides, which, after treatment with enzyme, the oligosaccharides content increased to 28.91 and 59.71 g/kg for PKE_{ENZ} and $SPKE_{ENZ,}$ respectively. Although mannan made up most of the PKE-NSP, results of this study show that fructose and xylose contents were increased through enzymatic treatment. Thus, it can be hypothesized that the oligosaccharides produced through hydrolysis of NSP of PKE are a mixture of MOS, XOS, and FOS.

The benefits of oligosaccharides, particularly MOS and FOS, have been demonstrated *in vitro* to promote growth of beneficial bacteria (Ofek and Beachey 1988; Kaplan and Hutkins 2003; Saminathan *et al*. 2011). However, the growth of bacteria differed even between strains of the same species (Kneifel *et al*. 2000). In agreement with the above finding, the present results show that growth of the three strains of *Lactobacillus* on MRS (Control) was varied, with *L. salivarius* I 24 exhibiting the highest growth potential, followed by *L. gallinarum* I 16 and *L. brevis* I 218. However, when PKE-extract was used as a substrate, *L. salivarius* I 24 and *L. gallinarum* I 16 exhibited inferior growth compared to *L. brevis*. This observation could be due to the presence of growth inhibitors such as organic acids, furan derivatives, and phenolic compounds, which are common by-products of lignocellulose biomass fermentation (Chandel *et al*. 2011). Unlike *L. salivarius* and *L. gallinarum*, *L. brevis* has been shown to possess strong resistance to the potential growth inhibitors (Guo *et al.* 2010; Kim *et al*. 2010).

Despite the poor growth, all the three *Lactobacillus* strains had slightly better growth on the enzyme-treated PKE, probably due to the higher monosaccharides and low DP oligosaccharides contents in the enzyme-treated PKE, as shown by the HPLC analysis. It has been documented that *Lactobacillus* is only able to utilize oligosaccharides with DP less than 5 (Kaplan and Hutkins 2000), and those of higher DP are utilized at a lower efficiency (Chung and Day 2004). As the PKE extracts used in this study were in their crude forms, and it is beyond the scope of this study to identify the types and proportions of oligosaccharides present in the crude extracts (except assuming MOS is the main oligosaccharides). The exact components that support the growth of *Lactobacillus* cannot be confirmed.

The higher growth rates of *L. brevis* I 218 in the PKE extracts compared to the other two *Lactobacillus* strains observed in this study is interesting and deserved some comments. The crude enzyme used in this study was similar to that of Chen *et al.* (2013), which has previously reported to have high xylanase activity (262.01 U/g). The presence of xylose in both the enzyme-treated PKE, but its absence in the non-enzyme treated PKE samples, seems to suggest that the high xylanase activity is efficient in producing xylose and XOS, which could be a prebiotic source to support, specifically the growth of *L. brevis* I 218 observed in this study. The present finding is in agreement with the observation by Saminathan *et al.* (2011), who reported that *L. brevis* I 218 grew best on

XOS, whereas *L. salivarius* I 24 grew poorly on XOS, and *L. gallinarum*I 16 could not grow on XOS at all. In addition, the concurrent decrease in the different types of monosaccharides observed in this study seems to suggest that *L. brevis* was able to simultaneously consume different monosaccharides in the presence of glucose, which is also in agreement with Kim *et al.* (2009), who reported that *L. brevis* lacks normal hierarchical control in carbohydrate utilization. Furthermore, enzyme treated PKE-extracts had higher oligosaccharides contents after incubation, which may also indicate further hydrolysis of polysaccharides (DP >6), into oligosaccharides (DP < 6), continued during incubation, either through fermentation by the *L. brevis* itself, or by the residual enzyme still present in the PKE-extracts. The above observation suggests an important characteristic for the PKE-extract to be considered as prebiotics, since most of the available monosaccharides would have been absorbed in the upper intestine before it reached the lower intestinal tract to be used as prebiotics. The hydrolysis of polysaccharides into oligosaccharides and other simple sugars in the lower intestine will provide the necessary growth substrates to promote the growth of beneficial gut microbes.

The enhancement of beneficial bacteria by oligosaccharides could be due to the inhibition of pathogenic bacteria colonization caused by decreased intestinal and feacal pH (Djouzi and Andrieux 1997; Bruggencate *et al.* 2004; van Meer *et al*. 2008) and/or that adherence of numerous grams negative bacteria to animal cells is specifically inhibited by mannose or its derivatives through the competitive absorption to the mannose specific type 1 fimbriae, thereby limiting their colonization in intestinal epithelium (Ofek and Beachey 1978). White *et al.* (2002), using yeast as a source of MOS in swine diet, showed no significant effect on the microbial population in the gut. In contrast, Strikling *et al.* (2000) and Swanson *et al*. (2002a) demonstrated that MOS significantly increased feacal *Lactobacillus* population, but not *Bifidobacterium* population, in dogs. However, both bacterial populations were conversely significantly increased when MOS was used in combination with FOS in dogs (Swanson *et al*. 2002b). Similarly, MOS supplementation in poultry diet has been shown to increase both *Lactobacillus* and *Bifidobacterium* populations (Baurhoo *et al*. 2007). The above findings show that the prebiotic effect of oligosaccharides generally differed between different organisms. The efficacy of oligosaccharides from PKE to modulate intestinal microflora was demonstrated by results of the present *in vivo* study. In agreement with previous research findings, these results show that only supplementation of $SPKE_{ENZ}$ extract resulted in a significant increase in both *Lactobacillus* and *Bifidobacterium* populations in rats, whereas only supplementing with PKE_{ENZ} extract increased the population of *Lactobacillus* significantly when compared to the control. In addition, only $SPKE_{ENZ}$ extract significantly reduced *E. coli* population. This may be due to higher oligosaccharides content in the $SPKE_{ENZ}$ extract, which underwent steaming before enzyme treatment. Steam treatment may have helped to expose the mannan polymers and allowed cellulolytic and hemicellulolytic enzymes to further break down the fiber of PKE, thus producing more short chain oligosaccharides in the $SPKE_{ENZ}$ extract. These results, whilst appearing to be useful, need further investigations, since other studies (Sang and Fotedar 2010; Geraylou *et al.* 2013) have shown opposite effect when excess oligosaccharides were included.

CONCLUSIONS

1. It was demonstrated that enzymatic hydrolysis of PKE, particularly when coupling with steam treatment, can increase the release of mono-oligosaccharides and lower DP oligosaccharides of higher prebiotic efficiency.
2. The present study, using rats as an animal model to represent monogastric species, shows that these oligosaccharides can be used as a dietary supplement to enhance the growth of beneficial gut bacteria for improvement of the wellbeing of the host. However, the efficiency of utilization of the different PKE oligosaccharides as prebiotic needs further investigations.

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