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Enhancement of Short Chain Fatty Acid Production by Co-cultures of Probiotics Fermentation with Pearl Millet (*Pennisetum glaucum*) Fibre Fractions

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Pearl millet dietary fibre fermentation using four co-culture of three probiotic strains *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Bifidobacterium bifidus* was studied for production of short chain fatty acids (SCFA). All co-cultures were grown on specific medium containing pearl millet fibre fractions; IDF, SDF, TDF as a main carbon source. SCFA production of the probiotics co-cultures was measured at 0, 6, 24, and 48 h using gas liquid chromatography. SCFA formation among the fibre fractions followed the pattern of TDF>SDF>IDF irrespective of co-cultures, indicated that TDF is the best possible dietary fibre for SCFA production. During all co-cultures fermentation with pearl millet fibre fractions, quantity of different SCFA's produced was acetate>propionate >butyrate. The SCFA quantity was observed low with co-culture of probiotic comprised of same genus ($p < 0.05$). During 48 h fermentation, co-cultures (BL+BB, BB+LR and BL+LR) digested 81-88 % of the millet fibre fractions. *Bifidobacterium bifidum* + *Bifidobacterium longum* + *Lactobacillus rhamnosus* co-culture was found most efficient during pearl millet fermentation which digested the 97% fibre fractions and produced more SCFA than other combinations in the study. These findings conferred pearl millet dietary fibre fractions as new potential prebiotic for tested probiotic co-cultures.

Keywords: Probiotic fermentation, Short chain fatty acid, Co-cultures, Gas liquid chromatography.

Complex carbohydrates and plant polysaccharides cannot be hydrolyzed by human enzymes. However colon microflora can ferment these non-digestible carbohydrates, including cellulose, xylans, resistant starch and inulin, to yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs). The major SCFA produced during dietary fibre fermentation (acetate, propionate, butyrate) are absorbed from the colonic lumen and used by body tissues to modulate various important activities. Butyrate is usually metabolized by colonocytes thus; butyrate levels in portal blood is abate relative to acetate and propionate. Propionate regulates hepatic carbohydrate and lipid metabolism. Acetate predominately escapes colonic and hepatic metabolism and act as a fuel for peripheral tissues. [1, 2, 3]Today gut microflora is openly being accepted as an environmental factor that regulates host metabolism and in future may become a target for treating metabolic comorbidities.[4,5] Recent studies indicated that diet can be a factor to determine the composition of human gut microflora. Long-term dietary habits have a significant influence on the gut microflora. Changes in daily carbohydrate intake

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may regulate specific groups of colonic bacteria over a short period of time. The types and amount of SCFAs produced, also co-relate with type of carbohydrate consumed and the composition of the gut microflora. For instance, fermentation of dietary fructans is higher when gnotobiotic mice were co-colonized with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii* than the mice colonized with only *B. thetaiotaomicron*. *B. thetaiotaomicron* yielded more acetate and formate, and *M. smithii* utilize formate for methanogenesis.[6, 7]

The influence of dietary ûbres from millet as main carbon sources for fermentation using pure probiotic cultures has been reviewed to understand microûora and short chain fatty acid (SCFA) formation relation pattern.[8] However, the role of combination of organisms on the digestion of millet dietary ûbres and SCFA formation not been reported. Since human gut contains 300 to 400 different species of bacteria then co-cultures should provide more scope in dietary ûbres fermentation to SCFA production. This study was aimed to understand the fermentation of pearl millet ûbre and the formation of SFCA (acetate, propionate and butyrate) using co-cultures of three probiotic strains. Therefore, current study used fermentation to determine the effects of pearl millet fibre fractions on probiotics co-cultures during in vitro conditions.

The main objective was to understand the influence of the different combinations of *Lactobacillus rhamnosus* (LR), *Bifidobacterium longum* (BL) and *Bifidobacterium bifidum* (BB) on the rate of SCFA production in the media containing millet fibre fractions as the main carbon source and to determine the extent of fermentation of dietary fibre by combinations of probiotics. The results from this study will help to understand the fibre utilization and the interactions among the probiotics in the human gut.

MATERIALS AND METHODS

Chemicals and bacterial strains

The present study used microbiological media MRS (Oxoid, UK) for *Lactobacillus rhamnosus* and Reinforced Clostridial Medium (RCM) (Oxoid) for *Bifidobacterium bifidum* and *Biûdobacterium longum* as the cultivation media. The basal medium, peptone D yeast extract D fildes (PYF) solution, was taken as the carbohydrate-free medium. PYF enrichment solution was used as the growth medium for in vitro fermentation process. Probiotic bacterial strains used in this study, were obtained from Bioresource collection and research centre, food industry research and development institute Taiwan. Pure cultures of probiotic strains *Lactobacillus rhamnosus* (LR), *Bifidobacterium bifidum* (BB), *Biûdobacterium longum* (BL) were combined in a 1:1(*v*/*v*) ratio to prepare four co-cultures in equal proportions. The co-cultures were termed as LR+BB, LR+BL, BB+BL and LR+BB+BL.

Pearl Millet

Pearl millet (*pennisetum glaucum*) was selected for this study based on its popularity and in order of worldwide production.

Estimation of soluble, insoluble and total dietary ûbre

Pearl millet samples were analyzed for soluble, insoluble and total dietary ûbre fractions using total dietary fibre assay kit from Megazyme international (Wicklow, Irland). [8,9]

Fermentation substrate

Pearl millet dietary ûbre fractions (TDF, IDF and SDF) were used as the main substrates for the fermentation process.

Cell suspension preparation

Freeze dried cultures of *L. rhamnosus* and *B. bifidum* and *B. longum* were rehydrated by sub culturing in MRS and reinforced clostridial medium respectively under strict anaerobic condition. Anaerobic jars were used to apply anaerobic conditions. *Lactobacillus* and *Bifidobacterium* species were incubated at 37 ºC for 24 h and 72 h respectively. Before in vitro fermentation, again the bacteria were pre- cultured twice in 10 mL of the respective medium containing $10gL^{-1}$ glucose as the carbon source. After incubation, the bacteria cells were harvested, washed twice with saline (0.85 % NaCl solution), and resuspended in the basal medium (PYF solution) to remove excess carbon. The suspension was then diluted to 1:10 with the basal medium.[10]

In Vitro fermentation using extracted pearl millet ûbre fractions

Duplicate in vitro fermentation of pearl millet fibre fractions using co-cultures of *L. rhamnosus, B. bifidum* and *B. longum* was carried

out in sterile 100 mL bottles. Each bottle contained culture medium, substrate and tested probiotic culture. Culture medium (60mL) and 1% (vD v, 0.6mg) substrate (extracted TDF, SDF and IDF from pearl millet) was added to each bottle, and sealed for 24 h for complete hydration of ûbre before adding the inoculum. The bottles were incubated at 37°C for 2 h prior to inoculation. Then 1h before inoculation, the bottles were placed in a 37°C shaking water bath. A reducing solution (1.6 mL) was added, and sealed with rubber stoppers. Inoculum 6 mL (10 %(v D v) [9] of the bacterial suspension (10^7 cfu $mL^{-1}$of all the cultures) was added to each bottle and fermentation was conducted under strict anaerobic conditions at 37°C.Strict anaerobic conditions were maintained using anaerobic jars (Anearobic plus system; Oxoid, Dioxo, Prague). Anaerobic jars were equipped with palladium catalysts (Oxoid) and ûlled with CO_2D H_2 (10 D 90%) by gas packs (Merck, Darmstad, Germany).A volume (2 mL) and (5mL) of aliquots was removed at 0, 6, 24 and 48 h for SCFA and fibre analysis respectively. Microbial growth was stopped by adding 1 mL of 10 g L^{-1} copper sulphate. Gas packs were replaced after each removal of aliquots.

Determination of SCFA

Analysis of SCFA was conducted using a modiûed method.[11] Duplicate aliquot samples were thawed for 30 min, and centrifuged at 5000 $_g$ at room temperature. The supernatant (0.75 mL) was transferred to a sterile vial and vortex mixed with meta-phosphoric acid (20%, 0.3 mL). Vials were incubated with meta-phosphoric acid at room temperature for 30 min. After which samples were centrifuged for 20 min at 20 000 $_g$ for 10 min. The supernatant was analyzed for SCFA by GLC. Conditioned were maintained as described.[6] Packard model 5890 series 11 gas chromatography and a HP–innowax, 30m×25mm×25 µm column at an initial temperature of 60°C and ûnal temperature of 250°C.Flow rates of nitrogen, hydrogen and air were 20, 30 and275 mL min^{-1}respectively.

Calculation to determine the SCFA concentration (mM 100 mL^{-1})

$$\frac{\text{Peak area of the SCFA}^{*}\times\text{internal standard concentration per 100 mL}}{\text{(RRF of SCFA)}\times\text{peak area of the internal standard}}$$

Where RRF = relative response factor = peak area of 1 mM of SCFAD peak area of 1.0 mM of internal standard; SCFA*= acetic acid/propionic acid/butyric acid

Estimation of the indigestible percentage of total dietary fibre (after fermentation of TDF)

The indigestible quantity of SDF and IDF was measured from 5mL of the aliquots of the fermentation broth at each time point. The sum of both SDF and IDF was considered as the indigestible amount of TDF.

Calculation to estimate the percentage of dry matter disappearance

This calculation is a derived from previous formula [12].

$$\frac{\text{Substrate dry matter-Residual dry matter* –blank weight}}{\text{Substrate dry matter}}\times 100$$

Residual dry matter* = sum of IDF and SDF

Statistical analysis

The analyses were performed in duplicates and results were expressed as mean values and standard deviation. Data were analysed using the statistical analysis package of Microsoft Excel 2007.Treatment was factorially arranged with three substrates (IDF, SDF and TDF) and four lengths of fermentation (0, 6, 24 and 48h). The diûerence between the experimental groups and the control containing glucose were evaluated using *t*-student test and anova.

RESULTS AND DISCUSSION

This study focused primarily on SCFA production potential of probiotic co-cultures on different fibre fractions from pearl millet. Extracted and purified fibre fractions (IDF,SDF,TDF) of pearl millet were used as a main substrate during fermentation with four co-cultures of three probiotics strains (*L. rhamnosus*, *B. bifidum*, *B.longum*) .Similar to previous findings, the current study also used in vitro approach to understand metabolism of co-cultures and interspecies interactions with fibre due to complexity of human colon.[13,14]

Fermentation results of IDF, SDF, and TDF fraction of pearl millet with four co-cultures (BB+BL, BB+LR, BL+LR and BB+BL+LR) of three tested probiotics bacteria, showed a significant increase in SCFA production at different length of fermentation (Table 1(a, b, c, d). Data

Table 1(a). Fermentation of Pearl millet fibre with probiotics co-cultures(BB+BL) leading to the formation of SCFA, (m Moles/100mL) at 0 , 6, 24 and 48th h.

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA		Acetate				Propionate				Butyrate		
IDF	0.26±0.02	1.35±0.01	1.6±0.01	1.83±0.03	0.03	0.04	0.06	0.06	0.02	0.02	0.03	0.04
SDF	0.29±0.01	1.43±0.01	1.76±0.01	1.98±0.02	0.04	0.05	0.07	0.07	0.02	0.03	0.04	0.05
TDF	0.30±0.03	1.50±0.01	1.84±0.01	2.02±0.02	0.04	0.06	0.09	0.10	0.02	0.04	0.05	0.05
Glucose	0.25	1.57±0.03	1.90±0.02	1.99±0.01	0.04	0.06	0.10	0.10	0.02	0.03	0.04	0.04
Control	0.24±0.02	0.21±0.01	1.22±0.02	1.22±0.02	0.04	0.04	0.05	0.05	0.02	0.03	0.03	0.03

Results are expressed as the mean value of two trials±standard error. Standard error less than 0.01 was not included

Table 1(b). Fermentation of Pearl millet fibre with probiotics co-cultures (BB+LR) leading to the formation of SCFA, (m Moles/100mL) at 0, 6, 24 and 48th h.

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA		Acetate				Propionate				Butyrate		
IDF	0.28±0.01	1.41±0.03	3.16±0.02	3.20±0.02	0.03	0.04	0.05	0.06	0.02	0.02	0.03	0.04
SDF	0.29±0.01	1.53±0.02	3.28±0.01	3.34±0.02	0.03	0.04	0.06	0.06	0.02	0.02	0.03	0.04
TDF	0.30±0.02	2.15±0.01	3.74±0.02	4.18±0.02	0.03	0.05	0.08	0.08	0.02	0.03	0.05	0.05
Glucose	0.31±0.02	1.58±0.01	2.90±0.02	3.54±0.02	0.03	0.05	0.05	0.05	0.02	0.03	0.04	0.04
Control	0.22±0.01	1.26±0.01	1.27±0.02	1.27±0.02	0.03	0.04	0.05	0.05	0.02	0.03	0.03	0.03

Results are expressed as the mean value of two trials±standard error. Standard error less than 0.01 was not included

Table 1(c). Fermentation of Pearl millet fibre with probiotics co-cultures (BL+LR) leading to the formation of SCFA, (m Moles/100mL) at 0 ,6,24and 48th h

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA		Acetate				Propionate				Butyrate		
IDF	0.25±0.01	1.40±0.01	3.63±0.02	4.39±0.03	0.03	0.04	0.05	0.05	0.02	0.03	0.04	0.04
SDF	0.28±0.01	1.61±0.02	4.17±0.14	4.65±0.02	0.03	0.04	0.06	0.06	0.02	0.04	0.04	0.04
TDF	0.30±0.02	2.12±0.02	4.41±0.02	4.75±0.02	0.03	0.05	0.07	0.07	0.02	0.04	0.05	0.05
Glucose	0.31±0.02	1.93±0.02	3.33±0.02	3.72±0.01	0.03	0.05	0.05	0.05	0.02	0.04	0.05	0.05
Control	0.22±0.03	1.10±0.01	1.16±0.01	1.22±0.02	0.03	0.04	0.05	0.05	0.02	0.03	0.03	0.03

Results are expressed as the mean value of two trials±standard error. Standard error less than 0.01 was not included

Table 1(d). Fermentation of Pearl millet fibre with probiotics co-cultures(BB+BL+LR) leading to the formation of SCFA, (m Moles/100mL) at 0 ,6, 24 and 48th h

H	0	6	24	48	0	6	24	48	0	6	24	48
SCFA		Acetate				Propionate				Butyrate		
IDF	0.27±0.01	3.72±0.02	4.07±0.01	5.02±0.03	0.03	0.04	0.05	0.05	0.02	0.03	0.03	0.03
SDF	0.31±0.01	3.91±0.09	4.46±0.03	4.88±0.04	0.03	0.04	0.06	0.06	0.02	0.03	0.04	0.04
TDF	0.36±0.01	4.43±0.01	5.75±0.04	5.88±0.03	0.043	0.06	0.07	0.07	0.02	0.04	0.05	0.05
Glucose	0.30±0.01	5.32±0.06	5.49±0.12	5.13±0.03	0.03	0.05	0.06	0.05	0.02	0.04	0.04	0.04
Control	0.22±0.01	1.23±0.01	1.24±0.01	1.26±0.02	0.03	0.04	0.05	0.05	0.02	0.03	0.03	0.03

Results are expressed as the mean value of two trials±standard error. Standard error less than 0.01 was not included

shown here in the table is only for 0, 6, 24 and 48h. Each probiotic co-culture after fermentation with pearl millet ûbre fractions produced SCFA, predominantly acetate, at more than twice the level compared with the negative control, which consisted of only the growth media (without ûbre). However, no significant difference ($p<0.5$) was observed in propionate and butyrate production by different co-cultures. This suggested that the quantity of propionate and butyrate did not alter significantly with or without fibre using the co-cultures in this experiment. Co-cultures (BB+BL) comprised of same genus yielded less quantity of SCFA ($p<0.05$) than BB+LR, BL+LR and BB+BL+LR (Table 1(a, b, c, d). Interestingly, co-cultures of BB+BL+LR produced a significantly higher amount of SCFA as compared to all other combinations at the end of fermentation (Table 1). This result indicated that fermentation of dietary fibre might happen at faster rate if there was a compatible combination of bacteria. Lowest ratio for acetate to propionate was also noted in BB+BL than BB+LR, BL+LR and BB+BL+LR. The TDF yielded higher concentration of SCFA as compared with other ûbre fractions SDF and IDF of pearl millet. Among millet fibre fraction, amount of SCFA were formed in following pattern; TDF > SDF > IDF, irrespective of the co-cultures involved during fermentation.

Fermentation for each co-culture was measured by determining the percentage of substrate remained at each sample time during course of fermentation (Table 2). Co-cultures BB+BL+LR digested 100% SDF, 97% TDF and almost 94% IDF whereas BB+LR and BL+LR digested 85-88% of all pearl millet fibre fractions during fermentation. However, co-culture comprised of same genus BL+BB showed 81-83% digestion for all fibre fractions. Data shown here in the table 2 is only for 6, 24 and 48h.

Table 2. Percentage of substrate remained after 6, 24 and 48th h time point.

Co-cultures	H	IDF	SDF	TDF
BB+BL	6h	64.9	59.1	88.0
	24h	32.3	26.2	49.5
	48h	19.0	13.5	18.4
BL+LR	6h	60.6	55.5	87.2
	24h	23.7	22.9	39.8
	48h	12.6	7.7	11.7
BB+LR	6h	54.9	65.8	85.0
	24h	26.2	23.9	42.7
	48h	14.2	12.0	10.9
BB+BL+LR	6h	41.5	39.5	70.6
	24h	10.0	6.8	33.2
	48h	5.4	-	3.0

Result are expressed as the mean values of two trials± standard error

Co-cultures of different genus produced significantly higher SCFA than the other bacterial combinations comprising of same genus. It might be due to co-cultures of the same genus may have a similar requirement of dietary fibre fraction which makes them prone to competition. It supported our earlier assumption that mixed bacterial co-cultures might carried out pearl millet fibre fermentation more efficiently than same genus. Similar findings were observed with cellulitic bacteria which exhibited two different microbial interactions - competition and synergism.[15,16] It might be due to bacteria belonged to the same genus more likely competed for the same substrate while microflora from different genus, were more likely involved synergistically.[17,18]The low SCFA proûle from the control fermentation flask indicated components of the medium did not contribute to SCFA. This finding was in agreement with other results obtained from various types of ûbre sources using pure cultures and co-culture.[19]

Acetate production in all co-cultures increased during length of fermentation. However, this trend was not found with propionate and butyrate production. Less propionate and butyrate, and almost unchanged amount of propionate and butyrate was observed from the start of the fermentation to end, indicated that tested bacteria might have used propionate and butyrate as an energy source for their survival in the co-culture. The ratio of propionate and butyrate was significantly less ($p<0.05$) than acetate (Table 1(a, b, c, d).The SCFA production pattern was observed, acetate >propionate >butyrate for all the combinations. Ability of SDF to synthesize more SCFA than IDF and less than TDF was also in agreement with previous studies on diûerent ûbre fractions.[20] Differences in the individual SCFA formation amongst various co-cultures might be due to the physiological properties of the strains, potential to ferment the substrate, or medium

compositions.[21] Previous findings have reported that butyrate formation during fermentation mainly occurred from an inter conversion reaction of acetate. Butyrate might have resulted from acetate in the present study and its formation can be stimulated by enhancing acetate formation in the medium. It is significant to note how butyrate is produced from these co cultures since butyrate is the most important SCFA for human health.[22, 23] The lower ratios between acetate to propionate is important to reduce the human serum cholesterol level.[24] SDF was found a more fermentable fibre and formed higher SCFA as compared to IDF but less than TDF. It indicated that substrate had more fermentable material than the fractions. SCFA production in batch fermentation was dependent on the availability of the substrate for the digestion by microbes. [21] This study showed that co-cultures prepared from two species hydrolyzed less millet fibre fractions than BL+BB+LR, which were comprised of three species irrespective of genus.

It was evident that fermentation length and co-culture growth during course of study was enough for maximum digestion of millet fibre fractions. BB+BL+LR co-culture comprised of *Bifidobacterium bifidum*, *Bifidobacterium longum* and *Lactobacillus rhamnosus*, was found the most efficient for all pearl millet dietary fibre fraction fermentation in terms of bye product formation and fermentation time. It even yielded a slight higher SCFA than glucose, which was used as the positive control. This was the first study which documented pearl millet fibre fraction fermentation with human faecal probiotic bacteria co-cultures, produced SCFA and involved low oxygen and highly reducing experimental conditions, were similar to human gut distal colon. That aspect helped us to assume experimental co-cultures fermentation of millet fibre occurred in gut, which strongly indicated that a millet rich diet will modulate the production of acetate in human gut. These findings suggested that all pearl millet fibre fractions produced SCFA during fermentation with all bacterial co-cultures. However, more research is needed to understand the difference in the fermentation of complex and simple structures by bacteria.

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