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Pearl Millet (*Pennisetum glaucum*) Fibre Fractions; A New Substrate for the Growth of Co-cultures of Probiotics

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This is the first study to report the growth kinetics of co-cultures of probiotics on pearl millet dietary fibre fractions. In vitro, fermentation of pearl millet fibre fraction (SDF, IDF, TDF) with four co-cultures of three probiotic stains was determined by measuring cell viable count, pH and specific growth rates at 0, 6, 24 and 48h. All co-cultures utilized millet fibre fraction, predominantly TDF, as a fermentation substrate for their growth but showed more preference for glucose. Co-culture comprised of different species produced more cell yields than combination of same species ($p<0.05$). pH 4.19-4.40 was found limited factor for bacterial growth. Co-culture prepared from *Lactobacillus rhamnosus*, *Bifidobacterium bifidum* and *Bifidobacterium longum* was found the most viable and effective during this study. It was clear evidence of synergistic activity which influenced the degree of fermentation of dietary fibre. These results suggest that millet dietary fibre fractions could stimulated the growth of the *bifidobacterium* species and *lactobacillus* rhamnosus but *bifidobacterium* species indicated longer, slow and constant growth compared to *lactobacillus rhamnosus*. Pearl millet dietary fibre has showed potential of developing into a new prebiotic for these tested probiotics.

Key words: Pearl millet, Probiotic, *Lactobacillus rhamnosus*, *Bifidobacteria*, Fermentation, Dietary fibre.

Recent years, have witnessed many studies, which are primarily focused on characterizing the physiological effects resulting from human consumption of different varieties of dietary fiber sources.[1] Dietary fibres play an important role in human gut functions, provide a ready source of available carbohydrates to the human colon microflora, resulting an increase in stool output, dilution of colonic contents, and an accelerated rate of passage through the gut.[2] Fibre enriched diet can direct the expansion of specific microflora to enhance metabolic regulation. Changes in daily carbohydrate intake may influence specific groups of gut microflora over a short span of time. It has been reported that diets enriched with resistant starch resulted an increased faecal levels of *Ruminococcus bromii* and *Eubacterium rectale*, which associate with fibre fermentation.[3]

Fibre contribution towards stimulation of microbial growth is the more usual one in man[4]. *Bifidobacterium* species and *lactobacillus* species have traditionally been known as potential health promoting microbes in the human gut. Last decades have seen increased use of these species as probiotics in pharmaceuticals and in foods. It can also be clearly reflected by probiotic supplements in the market.[5, 6]Many studies have been conducted to identify the substances that aid in survival and growth of these bacteria.[7, 8] People usually prefer natural food for their health promoting properties. Feeding studies with human volunteers have confirmed that *Bifidobacterium* and *Lactobacillus* species viable count and growth

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can be stimulated by cereal dietary fibre fractions.[9]Growth rate and amount of substrate used in fermentation often determine inherent ability of potential probiotic strains. Both factors also influence the ability to compete with other bacteria in the gut. The amount of substrate converted into bacterial mass or cell number can be used as an indicator to understand the efficiency of prebiotic or probiotic activity using dietary fibre fractions.

Pearl millet is the staple food in many developing countries of Asia and Africa. The role of millet fibre in digestion is not well understood. Therefore, current study used fermentation to measure the effects of fibre from pearl millet on human gut microbiota during in vitro conditions. In the large intestines, fibre is typically fermented by *bifidobacterium* and *lactobacilus* species but nutrient requirements are strain specific.[10]This arise the importance of substrate source and composition for overall function of the microbial combination. In order to analyze, pearl millet status as a potential prebiotic for tested bacterium which can support their growth, many microbiological questions have to be addressed to get advance in the field. The ability of co-cultures to obtain high viable cell yield during millet fibre fermentation is one of them.[9]

The present study was aimed to measure, the effect of pearl millet dietary fibre fraction (TDF, SDF, and IDF) fermentation with different co-cultures of probiotic and understand the growth kinetics of four co-cultures of three probiotic strains bifidobacterium bifidum, bifidobacterium longum and lactobacillus rhamnosus during course of fermentation. Following the inoculation of millet fibre with co-cultures of probiotics, fermentation process was studied for viable cell counts, acidification and the specific growth rates.

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).[11]This method involved following steps.

Digestion of original sample with enzymes

PM grains were powdered to 300-400 µm. Samples were weighed in duplicate to 1g into 400 mL beakers. 40mL of MES-TRIS buffer solution (pH 8.2) was added to each beaker with a magnetic stirring bar. 50 ìL of heat stable á-amylase solution was added to the samples while stirring at lower speed. Then incubated for 35 min in a shaking water bath at 95-100°C with continuous agitation. All sample beakers were removed from the water bath and cooled to 62°C. Then 100 ìL of protease solution was added to each sample and incubated in a shaking water bath at 62°C, with continuous agitation for 25 min. After incubation for 30 min, all sample beakers were removed from the water bath. While stirring 5 mL of 0.561 M HCl solution was dispensed into the samples. The pH was measured and adjusted to 4.1-4.8 with 5 % NaOH or 5 % HCl solution. 200 ìL of amylo glucosidase solution was added to the samples while stirring on a magnetic stirrer. Again beakers were incubated in a shaking water bath at 62°C for 25 min, with constant agitation. Blank without millet sample was used to find any involvement of the reagents to residue throughout enzymatic digestion.

Soluble/insoluble dietary fibre determination

Insoluble dietary fibre (IDF) was filtered,

and then residue washed with warm distilled water. Combined solution of filtrate and water washings were precipitated with 4 volumes of 95% ethanol (EtOH) for soluble dietary fibre (SDF) determination. Precipitate was then filtered and dried. Both SDF and IDF residues were corrected for protein, ash and final calculation of SDF and IDF values.

Total dietary fibre determination

SDF was precipitated with EtOH, and residue then filtered, dried and weighed. Total dietary fibre (TDF) value was corrected for protein and ash content.

Filtration

Dietary fibre fraction (residues) filtration was completed according to AOAC method to estimate the percentage of ûbre fractions in the millet.[11] However ûltration was modiûed by using ûlter paper 541 (Whatman International Ltd, Maidstone, UK) instead of celite. The purpose of this step was to purify the ûbre fractions for fermentation, since celite proved diûcult to remove from the isolated ûbre fractions.

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.[12]

In vitro fermentation

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 to make 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 at 37°C in shaking water bath. A reducing solution (1.6 mL) was added, and flasks were sealed with rubber stoppers. Inoculum 6 mL (10 %(v D v)[13]of the bacterial suspension (10^7cfu $mL^{-1}$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). Gas packs were replaced after each removal of aliquots. A volume (5 mL) of aliquots was removed at 0, 6, 24 and 48 h. One mL was used to prepare a 10-fold dilution series to enumerate viable count where as 1.5 mL was taken for optical density (540 nm) to measure specific growth and 2-3 mL was taken for pH measurement.

Determination of pH, viable count and specific growth rate

Two ml aliquot was used to measure the pH, viable count and specific growth of fermented samples. Bacterial growth was determined by measuring the optical density of samples (2 mL) at 540 nm. Samples were subjected to optical density and viable count measurements immediately following the incubation periods. The viable counts were enumerated using the pour plate technique, by inoculating 1 mL of ten-fold serial dilutions into PYF medium comprising 10 g/L glucose as the carbon source and 1.5 % agar. The plates were incubated anaerobically at 37 ºC for 24 h for *Lactobacillus* species and 72 h for *Bifidobacterium* species respectively.[13, 14]

Statistical Analysis

The results were expressed as mean values with standard error. Data were analysed using the statistical analysis package of Microsoft Excel 2003. Differences between bacterial groups and counts /Cell yield /pH at 0, 6, 24 and 48h fermentation were measured for significance using paired *t*-tests. Differences were assumed significant if $p \leq 0.05$.

RESULTS AND DISCUSSION

The viable count, specific growth rate and acidification rates were main parameters used to determine growth kinetics of co-culture during fermentation with pearl millet dietary fibre. 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*) to understand the growth kinetics of co-cultures on fibre fractions. All co-cultures of our tested probiotics utilized pearl millet as their main substrates during fermentation. The current study used high initial levels of cell counts in the fermentation medium 10^7 cfu/mL. Pearl millet fibre used as substrate in the fermentation medium, showed an observable ($p<0.05$) influence on the culture growth and the viability, for all co-cultures at 24 h fermentation. Co-cultures prepared comprised of all three probiotic strains (BB+BL+LR) showed more viable counts than the other three combinations (BB+BL, BB+LR, LR+BL). This high cell count may be due to the different substrate and/or different fermentation potential of the strains used in current study (Table 1). That result was similar to previous studies involving more than two species of gut bacteria, showed potential to ferment various carbohydrates.[15, 16, 17]

Time was found a limiting factor for cell counts during fermentation. Maximum number of co-cultures viable cells obtained after 24 h fermentation, indicating optimum time for the fermentation of millet dietary fibre fractions. Viable count of co-cultures prepared from different genus (BB+LR and BL+LR) exhibited significant difference ($p<0.05$) to co-culture of same genus (BB+BL). Viable counts of counterpart organisms showed a rapid decline after the 24 hours. (Table 1)This study also used optical density to determine the growth of co-culture on pearl millet fibre fractions. It was a simple parameter to understand bacterial growth but in total cell content (Table 2 and 3). We used the same quantity of pearl millet dietary fibre fractions (IDF, SDF and TDF) for all co-cultures. These fractions were 99 % pure. However, co-cultures showed differences in the growth during the fermentation of the different fibre fractions; IDF, SDF, and for TDF. Specific growth rates were measured to found the potential of the co-cultures to utilize pearl millet fibre fractions as a main carbon source (Table 2 and 3).

Our all probiotic co-cultures exhibited varied specific growth rates. *L.rhamnosus* yielded maximum biomass up to 24h after initial lag phase of fermentation, indicated that the maximum metabolite formation was also occurred at that time while bifidobacterium species indicated longer slow and constant growth(Table 2 and 3). It suggested metabolite formation continued for longer time but there were a number of non viable cells at this later stage of fermentation, but microbes were in the death phase.[18,19]Co-cultures showed significantly ($p<0.05$) higher growth on glucose compared with the fibre (Table 2 and 3). Therefore, the potential prebiotic supplement which enhanced the viable count of most of the co-cultures was glucose, and to some extent, TDF. Among the co-cultures evaluated, those comprising species from same genus demonstrated less biomass formation than the other combinations during the fermentation ($p<0.05$). There was no significant difference in the biomass yield from the different pearl millet fibre fractions ($p>0.05$).The highest pH drop after 24th h fermentation indicated the formation of acids rather than biomass. The results of specific growth indicated synergistic relationships between the different microbial combinations during fermentation. This relationship is considered as synergistic since higher numbers of viable cells were formed during the growth using co-cultures, when compared with the individual organisms. The variations in cell numbers between the different co-cultures were statistically significant ($p<0.05$). Previous work has also found evidence of synergism when culture grown in combination in milk.[20, 21]However, this study did not observe any higher growth of *Lactobacillus rhamnosus* compared to Bifidobacterium species (Table 1 and 2). It might be due to the absence of specific nutrients in the medium.[10]The variations in chemical structure and the quantity of sugars of fibre fractions might contribute to difference in the specific growth rates. Specific growth rates become low, when the substrate concentration decreased with the fermentation time. Metabolic waste that accumulates in the medium might hindered the cells and influenced specific growth rate.[3, 4]

pH measurement was used to understand

Table 1. Viable count of (Log_{10} (CFU)/mL) *L. rhamnosus, B. bifidum and B. longum* in co-culture combinations on pearl millet

Co-culture combinations	Strains	Pearl Millet fibre fractions												Glucose			
		IDF				SDF				TDF							
		0h	6h	24h	48h	0h	6h	24h	48h	0h	6h	24h	48h	0h	6h	24h	48h
BB+BL	*B. bifidum*	9.2±0.01	10.3±0.01	10.5±0.02	10.4±0.01	9.5±0.01	10.4±0.02	10.43±0.03	10.5±0.02	9.13±0.03	10.5±0.02	10.4±0.02	10.3±0.03	9.2±0.02	10.2±0.03	10.3±0.02	10.5±0.02
	B. longum	9.1±0.02	10.4±0.03	10.5±0.02	10.3±0.02	9.4±0.01	10.4±0.02	10.41±0.03	10.52±0.02	9.12±0.03	10.5±0.01	10.3±0.02	10.4±0.01	9.1±0.02	10.4±0.03	10.4±0.02	10.4±0.02
BL+LR	*B. longum*	9.1±0.02	9.4±0.03	12.4±0.03	10.2±0.02	9.4±0.02	10.4±0.02	12.4±0.03	10.2±0.03	9.5±0.02	10.4±0.01	12.4±0.02	10.2±0.02	9.4±0.02	9.5±0.02	12.5±0.02	10.4±0.03
	L. rhamnosus	9.4±0.02	10.4±0.02	11.4±0.02	8.3±0.02	9.4±0.02	10.4±0.02	11.4±0.02	9.2±0.02	9.4±0.02	10.4±0.03	11.4±0.01	10.3±0.02	9.4±0.02	10.5±0.02	11.5±0.03	9.5±0.03
BB+LR	*B. bifidum*	9.1±0.02	9.4±0.02	12.4±0.03	10.4±0.02	9.2±0.03	9.4±0.02	12.4±0.02	10.4±0.03	9.5±0.01	9.4±0.01	12.4±0.02	10.4±0.02	9.2±0.03	9.4±0.02	11.4±0.02	10.4±0.02
	L. rhamnosus	9.3±0.02	10.4±0.02	10.3±0.02	8.4±0.01	9.4±0.02	10.5±0.02	10.4±0.01	9.3±0.01	9.4±0.02	10.4±0.01	10.4±0.02	8.2±0.0	9.4±0.02	10.5±0.03	11.4±0.01	9.5±0.02
BB+ BL+LR	*B. bifidum*	10.4±0.01	12.4±0.01	14.1±0.01	11.4±0.01	10.4±0.01	12.4±0.02	14.4±0.01	11.4±0.01	10.4±0.01	12.4±0.0	14.2±0.01	11.4±0.01	10.4±0.02	12.5±0.03	14.5±0.01	11.4±0.01
	B. longum	10.3±0.01	12.2±0.03	13.9±0.02	11.3±0.02	10.5±0.01	12.5±0.02	14.43±0.03	11.5±0.02	10.2±0.03	12.5±0.02	14.4±0.02	11.3±0.03	10.2±0.02	12.2±0.03	14.2±0.02	11.3±0.02
	L. rhamnosus	10.2±0.01	12.4±0.02	14.1±0.01	8.3±0.01	10.1±0.01	12.2±0.01	13.3±0.01	8.6±0.01	10.1±0.02	12.2±0.02	14.2±0.02	8.1±0.01	10.2±0.02	12.5±0.03	14.3±0.01	9.2±0.01

Results are expressed as the mean value of two independent trials±SE.

the growth of co-cultures at different length of fermentation (Table 4). During fermentation, pH varied with time. The lowest pH was noted after 24 h fermentation, signaling maximum metabolite production. Tested bacterial co-culture belonging to a different genus, exhibited slow growth when the pH was 4.19- 4.40 after 24 h fermentation (Table 4). Higher cell population of Bifidobacterium species compared with the Lactobacillus rhamnosus in co cultures might be due to the higher growth of Bifidobacterium species in the fermentation medium before the pH value decreased to 4.19-4.40. Previously, it was found that the pH values which regulate the growth of Lactobacillus species was dependent on the cereal used in the fermentation.[5, 22] Less acidification in the medium resulted an increase of the cells of the bacterial combinations, and allowed the production of more metabolites in the fermentation medium. In current study co-culture of BB+BL+LR exhibited the higher stimulation of growth in the presence of the TDF, SDF, IDF and Glucose in terms of viable count, specific growth rates and pH with an average count of 13.4 log CFU/mL for this combination. This presented an excellent synergistic relationship between these tested probiotic strains, capable of using the millet fibre fractions.

Table 2. Specific growth rates of different co-culture of *L. rhamnosus, B. bifidum* and *B. longum* between 0 and 6 h of fermentation

Co culture	Strains	Pearl millet fibre fractions			Glucose
		TDF	IDF	SDF	
BB+BL	*B. bifidum*	0.50±0.01	0.43±0.01	0.48±0.01	0.48
	B. longum	0.47±0.01	0.44	0.45±0.01	0.46
BL+LR	*B. longum*	0.07±0.01	0.05	0.07±0.01	0.08
	L. rhamnosus	0.42	0.40	0.41	0.47
BB+LR	*B. bifidum*	0.09	0.07	0.07	0.10
	L. rhamnosus	0.45	0.37	0.39±0.01	0.47
BB+BL+LR	*B. bifidum*	0.78	0.75	0.75	0.89
	B. longum	0.78±0.01	0.75	0.76±0.01	0.86
	L. rhamnosus	0.85±0.01	0.84	0.84±0.01	0.85

Results are expressed as the mean value of two trials ± SE.
Standared error less than 0.01was not indicated.

Table 3. Specific growth rates of different co-culture of *L. rhamnosus, B. bifidum* and *B. longum* between 6 and 48 h of fermentation

Co culture	Strains	Pearl millet fibre fractions			Glucose
		TDF	IDF	SDF	
BB+BL	*B. bifidum*	0.29	0.26	0.27	0.46
	B. longum	0.29	0.26	0.27±0.01	0.46
BL+LR	*B. longum*	0.26	0.26	0.25	0.40
	L. rhamnosus	0.15	0.14	0.14	0.19
BB+LR	*B. bifidum*	0.14	0.12	0.13	0.39
	L. rhamnosus	0.41	0.39±0.01	0.40±0.01	0.41
BB+BL+LR	*B. bifidum*	0.16	0.15	0.14	0.39
	B. longum	0.12	0.15	0.15	0.39
	L. rhamnosus	0.11	0.11	0.11	0.39

Results are expressed as the mean value of two trials ± SE.
Standared error less than 0.01was not indicated.

Table 4. pH value of *L. rhamnosus, B. bifidum and B. longum* in co-culture during pearl millet fibre fermentation at 0,6,24 and 48 h

0H	IDF1	SDF1	TDF1	Glucose	Control
BB+BL	5.67±0.01	5.57±0.02	5.61±0.04	5.60±0.01	5.66±0.03
BL+LR	5.58±0.15	5.66±0.03	5.64±0.01	5.62±0.01	5.74±0.02
BB+LR	5.67±0.01	5.64±0.03	5.61±0.02	5.69±0.01	5.76±0.01
BB+BL+LR	5.65±0.04	5.56±0.03	5.54±0.01	5.65±0.04	5.68±0.05
6th H	IDF1	SDF1	TDF1	Glucose	Control
BB+BL	4.67±0.01	4.65±0.01	4.62±0.01	4.66±0.01	5.62±0.01
BL+LR	4.52±0.01	4.55±0.03	4.55±0.04	4.56±0.01	5.66±0.02
BB+LR	4.46±0.02	4.43±0.03	4.42±0.01	4.41±0.01	5.66±0.02
BB+BL+LR	4.24±0.01	4.25±0.01	4.27±0.01	4.375±0.01	5.73±0.01
24th H	IDF1	SDF1	TDF1	Glucose	Control
BB+BL	4.53±0.01	4.52±0.01	4.54±0.03	4.44±0.02	5.65±0.04
BL+LR	4.40	4.46±0.01	4.43±0.01	4.39±0.01	435±0.01
BB+LR	4.38	4.4±0.01	4.35±0.04	4.42±0.01	5.73±0.01
BB+BL+LR	4.23±0.01	4.22±0.03	4.19±0.02	4.27±0.01	5.74±0.03
48th H	IDF1	SDF1	TDF1	Glucose	Control
BB+BL	4.66±0.02	4.64±0.01	4.65±0.02	4.52±0.01	5.63±0.01
BL+LR	4.57±0.01	4.55±0.01	4.53±0.01	4.44±0.02	5.73±0.02
BB+LR	4.47±0.01	4.52±0.02	4.44±0.02	4.4±0.01	5.73 ±0.01
BB+BL+LR	4.40±0.02	4.32±0.02	4.36±0.01	4.40±0.01	5.64±0.04

Results are expressed as the mean value of two trials ± SE.

CONCLUSION

It was evident in the present study that co-cultures grew with pearl millet fibre fractions obtained higher cell yield. Interestingly, co-cultures comprised of different genus showed more cell count than combinations from same genus. It also indicated synergism between these bacterial co-cultures. Co-cultures prepared of more than one genus reached a lower pH than those made up of bacteria from the same genus, providing more evidence for the synergistic association in these co-cultures. The specific growth rate varied among the co-cultures for all the fibre substrates indicated the differences in the potential to digest millet fibre. Co-culture BB+BL+LR showed growth on all substrates tested (TDF, SDF, IDF and Glucose). Synergism between tested bacteria within the co-cultures regulates the degree of fermentation of millet dietary fibre fractions. The present study also found, TDF was the most accessible fibre fraction for all probiotics co-culture. It was clearly indicated by the highest viable count, pH decrease, and growth rates for the fermentation of TDF by all tested co-cultures. Pearl millet dietary fibre has showed potential of developing into a new prebiotic for these tested probiotics but its efficacy should be analyzed with other gut microbes.

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