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Original Research Article

Enhancement of Short Chain Fatty Acid Production from Millet Fibres by Pure Cultures of Probiotic Fermentation

Umar Farooq[1], Muhammad Mohsin[2], Xiaoming Liu[1] and Hao Zhang[1*]
[1]State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Lihu Road 1800, Wuxi 214122, Jiangsu, China, [2]University of Engineering and Technology Lahore, Faisalabad Campus, Pakistan.

*For correspondence: *E-mail: welcometoall19@hotmail.com, zhanghao@jiangnan.edu.cn;* ***Tel:*** *+86-15161501530; +86-510-85912087*

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Abstract

Purpose: *To enhance the in vitro synthesis of short chain fatty acids through millet dietary fibre fermentation by human faecal probiotic bacteria.*
Methods: *The effect of millet dietary fibre fermentation on production of short chain fatty acids (SCFA) by four probiotics was studied. Dietary fibre was extracted from two millet varieties viz Pearl millet, Pennisetum glaucum (PM) and Foxtail millet (FxM, Setaria italica), and separated into total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). Four probiotic bacteria (Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium longum and Bifidobacterium bifidus) were grown on specific medium containing IDF, SDF and TDF. SCFA production by the probiotics was measured at 0, 6, 24, and 48 h using gas liquid chromatography.*
Results: *SCFA production in the fibre fractions followed the rank order, TDF > SDF > IDF, irrespective of millet variety, indicating that TDF is the best possible dietary fibre for SCFA production. Lactobacillus and Bifidobacteria spp. digested 60 – 80 and 75 – 85 % of the millet fibre fractions from both millet samples, respectively. The quantity of different SCFAs produced was in the rank order: acetate > propionate > butyrate.*
Conclusion: *The results from this study suggest that millet dietary fibre has a potential for conversion into new nutraceuticals.*

Keywords: *Probiotic, Millet, Short chain fatty acid, Prebiotic, Probiotic, Dietary fibre*

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INTRODUCTION

A synbiotic is a combination of prebiotics and probiotics in which the prebiotic substance favor growth of probiotic microbes or other beneficial bacteria in the host and sustain microbial balance in the gut which promote gastrointestinal functions [1,2]. During fermentation of prebiotic by probiotic microorganisms in the human gut, short chain fatty acids (SCFA), H_2, CO_2, CH_4 and organic acids are the main metabolites formed as end products [3].

Previous studies have documented a large number of dietary fibre fractions (inulin, oligofructose, lactulose, galacto oligosaccharides (GOS), trans galacto-oligosaccharides (TOS) and iso malto oligosaccharides (IMO) which act as prebiotics for probiotics and the influence of organisms on SCFA formation [4].

The influence of dietary fibre from different cereals as main carbon sources for fermentation has been reviewed using faecal cultures in order to analyze the relationship between microflora

and short chain fatty acid (SCFA) formation [5]. However, there is no significant information on the fermentation of millet fibre fractions using human faecal microorganisms. Faecal microfloras contain variety of microbes but little is known about the role of individual organisms on the digestion of dietary fibre and SCFA formation [3]. It is our assumption that pure cultures may provide more scope on the contribution of specific microbes and dietary fibre to SCFA production. To understand the capability of probiotics to produce SCFAs on IDF, SDF and TDF from two millet varieties, the digestion of millet fibre and the formation of SFCA (acetate, propionate and butyrate) using pure cultures of *Bifidobacterium spp*.and *Lactobacillus spp*. were carried out in this study.

EXPERIMENTAL

Chemicals and Bacterial strains

Microbiological media MRS and Reinforced Clostridial Medium (RCM) (Oxoid, UK) were used as the cultivation media for pure cultures, and fildes PYF enrichment solution was used as the growth medium for in vitro fermentation process. The basal medium, peptone/yeast extract/fildes (PYF) solution, was used as the carbohydrate-free medium. Probiotic bacterial strains *Lactobacillus rhamnosus*, *Lactobacillus acido-philllus*, *Bifidobacterium bifidum*, *Bifidobacterium longum* used in this study were obtained from Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Taiwan.

Millet varieties

Two Millet varieties, Pearl millet, PM (*Pennisetum glaucum*) and Foxtail millet FxM (*Setaria italica*) were selected for the study based on their availability and worldwide production.

Estimation of soluble, insoluble and total dietary fibre

Millet samples were analyzed for soluble insoluble and total dietary fibre using total dietary fibre assay kit from Megazyme International (Wicklow, Ireland) [6]. This method involved the following steps.

Digesting the original sample with enzymes

Millet grains were powdered to 300-400 µm. Samples were weighed in duplicate to 1gram into 400 mL beakers. 40 ml of MES-TRIS buffer solution (pH 8.2) was added to each beaker with a magnetic stirring bar. Fifty micro liters (50 µL) of heat-stable α-amylase solution was added to the samples while stirring at lower speed. The samples were 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 60 °C. Then 100 µL 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. 5 ml of 0.561 M HCl solution was dispensed into the sample while stirring. The pH was measured and adjusted to 4.1-4.8 with 5 % NaOH or 5 % HCl solution. Amylo glucosidase solution (200 µL) was added to the sample while stirring on a magnetic stirrer. Again the beakers were incubated in a shaking water bath at 62 °C for 25 min, with constant agitation. A blank containing no millet along with the test samples to find any involvement of the reagents throughout enzymatic digestion was used.

Soluble/insoluble dietary fibre determination

Insoluble dietary fibre (IDF) was filtered, and the residue was 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. The precipitate was then filtered and dried. Residues were analyzed for protein and ash contents to determine the final contents of SDF and IDF.

Total dietary fibre determination

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

Filtration

Dietary fibre fraction (residues) filtrations were carried out according to AOAC method [6] to estimate the percentage of fibre fractions in the millet. However the filtration procedure was modified by using filter paper 541 (Whatman International Ltd, Maidstone, UK) instead of celite. This purpose of this step was to purify the fibre fractions for fermentation, since removal of celite from the isolated fibre fractions was difficult.

Fermentation substrate

The millet-isolated dietary fibre fractions (TDF, IDF and SDF) were used as the substrates for the fermentation process.

Preparation of cell suspension

Freeze-dried culture of *Lactobacillus spp.* and *Bifidobacterium spp.* were rehydrated by sub-culturing in MRS and reinforced clostridial medium respectively under strict anaerobic conditions. Then cell suspension was prepared according to method [7]. *Lactobacillus* and *Bifidobacterium* species were incubated at 37 ºC for 24 and 72 h, respectively. After incubation, the bacterial 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.

***In vitro* fermentation using extracted millet fibre fractions**

Duplicate fermentations were done in sterile 100 mL bottles. Each bottle contained culture medium, substrate and pure culture. 60 ml of culture medium and 0.6 mg substrate, viz, TDF, SDF and IDF from each of the millet varieties was separately added to each bottle, and sealed for 24 h for complete hydration of fibre before adding the inoculum. The bottles were incubated at 37 °C for 2 h prior to inoculation. At 1h before inoculation, the bottles were placed in a 37 °C shaking- water bath. A reducing solution (1.6 mL) was added, and the flasks were sealed with rubber stoppers. 6 ml of inoculum (10 %v/v) 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 in anaerobic jars (Anearobic plus system; Oxoid, Dioxo, Prague). Anaerobic jars were equipped with palladium catalysts (Oxoid) and filled with CO_2 and H_2 (10: 90 v: v) by gas packs (Merck, Darmstad, Germany). Aliquots (2 and 5 mL) were 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 modified method [8]. Duplicate aliquot samples were thawed for 30 min, and centrifuged at 5000g at room temperature value 25°C. The supernatant (0.75 mL) was transferred to a sterile vial and vortex-mixed with 0.3 ml of 20% meta-phosphoric acid. Vials were incubated with meta-phosphoric acid at room temperature for 30 min after which samples were centrifuged at 20 000g for 20 min. The supernatant was analyzed for SCFA by GLC. Conditions were maintained as described [9].

Calculation of SCFA concentration (mmoles /100ml**)**

The concentration of SCFA (mmoles/100 ml) was determined as in Eq 1.

SCFA = {(Pa x Cs)/(RRF x Ps)} …… (1)

where Pa is the peak area of SCFA, Ps is the peak area of internal standard, Cs is the concentration of internal standard/100 mL, RRF = relative response factor of SCFA, i.e., peak area of 1 mM of SCFA/ peak area of 1.0 mM of internal standard, and SCFA* = combined acetic acid/propionic acid/butyric acid

Determination of % indigestible 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.

Evaluation of % dry matter disappearance

Dry matter disappearance (DMD, %) was determined as in Eq 2 [10].

DMD (%) = {SDM – RDM – BW)/(SDM)}100 …. (2)

where SDM is substrate dry matter, RDM is residual dry matter, and BW is blank weight; RDM is the sum of IDF and SDF.

Statistical analysis

The experiment was conducted as a randomized complete block with four bacteria species serving as blocks. Treatment was factorially arranged with three substrates (IDF, SDF and TDF) and four lengths of fermentation (0, 6, 24 and 48 h).The analyses were performed in duplicate and results were expressed as Mean ± mean deviation (MD). Data were statistically analysed using Microsoft Excel 2003. The difference between experimental groups and the control containing glucose were evaluated using Student's t- test and one-way analysis of variance (ANOVA).

RESULTS

The fermentation of IDF, SDF, and TDF fractions of the two millet varieties by pure cultures of probiotics microbe, produced significant increase in SCFA (Table 1) at different durations of fermentation ($p < 0.05$). Each probiotic strain (after fermentation with millet fibre) produced SCFA, mainly acetate, at more than twice the level from the negative control which consisted of only the growth media (without fibre and probiotics). TDF yielded higher concentration of SCFA than any other fibre fractions. Lactobacillus spp. and Bifidobacterium spp. exhibited a very unique pattern of production, yielding the highest SCFAs at 24 h and 48 h respectively. It was also found that SDF was a more fermentable fibre and formed higher SCFA than IDF but less than TDF. Among millet fibre fractions, amount of SCFA varied in following pattern; TDF > SDF > IDF, irrespective of millet variety. In this study, glucose was used as the positive control, because it is completely available for bacterial fermentation. We also noticed that Lactobacillus acidophilus became more active in the first 24 h of incubation and had less ability to use PM.

Percent data for indigestible material are shown in Table 2. Lactobacillus spp digested 60 – 80 % and Bifidobaceria spp. 75 – 85 % of millet fibre during incubation periods. Data shown in Table 2 is only for 6, 24 and 48 h. Both millet varieties (PM and FxM) contributed to acetate formation but the difference between them was not significant ($p < 0.05$).

Similarly, formation of acetate was most favoured by the probiotics bacteria while butyrate and propionate were comparatively ($p < 0.05$) less. Among the microbes tested, Bifidobacterium spp. generally exhibited a lower ratio of acetate to propionate than Lactobacillus spp. at 24 and 48 h (Table 1). Pure cultures used fibres at different rates (Table 2). Fibre was significantly fermented

Table 1: Concentration of SCFAs from fermentation of millet fibre (mmoles/100ml)

Millet type	Fibre type	*L. acidophilus*			*L .rhamnosus*			*B. longum*			*B.bifidus*		
		Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate
0 hr													
	IDF1	0.35	0.03	0.02	.29	0.03	0.02	.32	0.03	0.02	.31	0.03	0.03
PM	SDF1	0.31	0.03	0.02	.31	0.03	0.02	.31	0.03	0.02	.28	0.03	0.02
	TDF1	0.32	0.03	0.02	.29	0.03	0.02	.29	0.03	0.02	.29	0.03	0.02
	IDF2	0.33	0.03	0.02	.28	0.03	0.02	.30	0.03	0.02	.32	0.03	0.02
FxM	SDF2	0.30	0.03	0.02	.32	0.03	0.02	.28	0.03	0.02	.31	0.03	0.02
	TDF2	0.30	0.03	0.02	.30	0.03	0.02	.29	0.03	0.02	.29	0.03	0.02
	Glucose	0.31	0.04	0.03	.31	0.05	0.04	.31	0.04	0.02	.30	0.03	0.02
	Control	0.29	0.03	0.02	.33	0.04	0.03	.29	0.03	0.02	.29	0.03	0.02
6th h													
PM	IDF1	.44	0.04	.03	0.71	0.04	.03	.56	0.13	0.08	.48	.10	0.04
	SDF1	.54	0.04	.03	.74	0.04	.03	.53	0.14	0.08	.55	.09	0.05
	TDF1	.66	0.04	.03	.60	0.04	.03	.54	0.13	0.09	.54	.08	0.04
FxM	IDF2	.44	0.04	.04	.76	0.04	.04	.57	0.15	0.08	.57	.10	0.04
	SDF2	.45	0.04	.03	.72	0.04	.03	.59	0.13	0.10	.59	.09	0.04
	TDF2	.58	0.04	.04	.64	0.04	.02	.51	0.16	0.09	51	.07	0.06
	Glucose	.40	0.06	.03	.65	0.05	.03	.55	0.13	0.08	.48	.11	0.04
	Control	.32	0.04	.03	.32	0.04	.02	.31	.04	0.03	.31	.04	0.03
24th h													
PM	IDF1	.52	.15	.05	.44	.22	.08	.49	.19	.12	.58	.17	.12
	SDF1	.63	.15	.06	.78	.21	.06	.62	.18	.13	.68	.18	.12
	TDF1	.72	.16	.05	.85	.27	.09	.71	.22	.21	.71	.21	.11
FxM	IDF2	.50	.13	.05	.71	.20	.06	.56	.13	.13	.54	.18	.12
	SDF2	53	.14	.06	.83	.21	.07	.71	.16	.15	.61	.17	.13
	TDF2	.62	.15	.05	.90	.24	.08	.59	.21	.17	.63	.19	.11
	Glucose	.75	.22	.09	1.10	.33	.12	.56	.24	.21	.53	.21	.07
	Control	.31	.04	.03	.32	.04	.03	.30	.04	.03	.32	.01	.02
48th h													
PM	IDF1	.55	.14	.06	.49	.15	.06	.63	.21	.23	.60	.21	.12
	SDF1	.60	.14	.07	.52	.15	.06	.84	.22	.25	.65	.22	.15
	TDF1	.71	.15	.08	.53	.18	.07	1.06	.23	.31	.91	.27	.17
FxM	IDF2	.53	.16	.06	.50	.19	.05	1.10	.31	.30	.47	.19	.13
	SDF2	.57	.17	.07	.57	.14	.07	1.01	.30	.29	.52	.20	.14
	TDF2	.66	.18	.08	.58	.16	.08	1.05	.29	.28	.53	.18	.16
	Glucose	.68	.19	.09	.47	.21	.10	1.41	.31	.32	.54	.17	.15
	Control	.31	.04	.03	.28	.04	.02	.33	.04	.03	.31	.01	.02

Table 2: Substrate (%) left after 6, 24 and 48 h

Probiotics	Time (h)	IDF1	SDF1	TDF1	IDF2	SDF2	TDF2
	6	82.71±2.1	78.6±3.1	72.4±.9	85.91±1.1	76.41±1	86.3±1.2
Lactobacillus	24	41.7±1.8	43.7±1.2	57.7±.8	44.6±0.90	44.9±.90	59.3±.72
acidophilus	48	30.1±.7	40.6±.6	21.20±.20	32.10±1.1	37.70±.80	38.23±2.1
	6	66.18±.90	70.6±.70	85.40±.30	77.60±.10	78.50±.70	83.10±.30
Lactobacillus	24	28.4±.30	33.4±.70	49.8±.70	40.7±.20	37.20±.60	45.20±.10
rhamnosus	48	15.8±.20	27.70±.20	24.12±.50	30.90±.30	26.50±45	25.20±.30
	6	83.57±2.10	77.91±.90	92.4±1.3	83.18±2.70	78.70±1.3	81.10±1.20
Bifidobacterium	24	46.47±1.20	54.6±1.1	64.18±2.1	62.1±1.8	60.60±1.4	46.10±.45
bifidum	48	18.70±.20	13.8±2.1	25.10±1.60	22.12±1.50	19.6±1.5	25.51±.10
Bifidobacterium	6	79.67±23	82±1.3	91.32±.54	88±.45	81.65±2.3	81.12±.78
longum	24	66.23±.27	57.32±.78	71.43±.43	65.34±.29	58.43±1.3	61.43±.90
	48	28.57±.69	22.65±.56	22.15±.57	28.32±.75	23.97±.78	13.47±.20

Results are expressed as mean ± MD.

by Lactobacillus spp. in the first 24 h and for Bifidobacterium spp. after 24 h.

DISCUSSION

This is probably the first study on the fermentation of millet fibre fractions with human faecal probiotic bacteria to yield SCFA. These results are in agreement with previous findings involving fermentation with other cereal crops [11]. The low SCFA profile from the control fermentation flask indicates that components of the medium and the substrate did not contribute to SCFA. This finding is contrary to results from previous study on the fermentation of dietary fibre with faecal inocula [12] but in agreement with other results obtained from various types of fibre sources using pure cultures and co-culture [11, 13]. The ability of SDF to synthesize more SCFA than IDF and less than TDF is in agreement with previous studies on different fibre fractions reporting SDF has more fermentable fibre than IDF [14]. Several studies had reported that the production of total SCFA from the mixed culture of microbial fermentation of complex dietary fibre was linearly substrate dependant [15]. It has been reported that the difference in SCFA production may be due to the chemical and structural differences between fibres [12]. However effects of chemical and structural difference on SCFA production were minimal for microorganisms grown on these millet fibres. Studies on cereal fibre fermentation by Bifidobacterium and Lactobacillus spp. had produced higher acetate formation than propionate and butyrate [16]. Potential of both millet varieties towards acetate production indicated their similar amounts of fermentable carbohydrate.

The lower ratio of acetate to propionate in *Bifidobacterium spp.* is considered an important finding because the formation of propionate and butyrate from *Bifidobacterium spp*. has not been reported previously. It could be the best choice in a functional food containing millet fibre. A higher proportion of butyrate was yielded by *Bifidobacterium spp*. than *Lactobacillus spp*. *Bidfidobacterium spp*. thus, produced a lower ratio of acetate to propionate and a higher proportion of butyrate than *Lactobacillus spp*. This property is important for human health [17]. Butyric acid is important in prevention and treatment of diseases of the colonic mucosa, such as distal ulcerative colitis and cancer [18]. Previous studies had shown that *L. rhamnosus* has the ability to improve serum propionate level in the human body [10]. Present study also observed a low propionate compared to acetate by *L. rhamnosus* and a lower ratio of acetate to propionate than *L. acidophilus*, more or less similar to *Bifidobacterium spp*. analyzed. The observation of a higher viable count of *Bifidobacterium* and *Lactobacillus* spp. during the final stages of fermentation may suggest the influence of propionic acid on the growth of the probiotics but not clear [19].

In the gut, the synthesis of SCFA from fibre depends on the length of time the substrate remains in system. SCFA production in batch fermentation is dependent on the availability of the substrate for the digestion by microbes. The fate of substrate is directly proportional to the hydration of substrate prior to the fermentation and the rate of substrate disappearance.

CONCLUSION

Millet dietary fibre fractions enhanced the formation of acetate, propionate and butyrate by *in vitro* fermentation with probiotic *L. acidophilus*, *L. rhamnosus*, *B. bifidus* and *B. longum* strains

originated from human faecal microflora. This study demonstrateded the use of millet fibre as a prebiotic for the microorganisms tested. However, in the human gut, microbes do not ferment the fibre as individual organisms. An *in vivo* study where the organisms work synergistically would be more revealing.

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