Antioxidant property of synbiotic combination of *Lactobacillus* sp. and wheat bran xylo-oligosaccharides

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Abstract Wheat bran water unextractable portion (WB-WUP) was subjected to xylanase treatment to obtain a mixture of xylo-oligosaccharides (XOS). XOS mixture was purified on charcoal-celite column and the individual oligosaccharides were separated on a Bio-Gel P-2 column. The sugar composition of the purified oligosaccharides was determined by GLC and their structure was deduced by ESI-MS and ^1H and ^{13}C NMR. The major oligosaccharides identified were xylobiose and xylotriose (consisting of arabinose). Five strains of lactobacilli (probiotics), XOS (prebiotics) and a combination of both (synbiotics) in milk (as medium) were monitored for antioxidant activity. DPPH radical scavenging activity (~70 %) as well as ferric reducing power (~80 mg/100 ml FeSO_4eq) were significantly higher ($p < 0.05$) in all the synbiotic preparations compared to that of control. The present study indicated that the synbiotic preparations consisting of XOS and lactobacilli can be effectively used as dietary supplement.

Keywords Xylo-oligosaccharides · Probiotic · Synbiotic · Antioxidant

Introduction

Reactive oxygen species (ROS) which include free radicals from various metabolic and biochemical reactions may react rapidly with cellular components causing significant damage to DNA, enzymes and cell membranes. Oxidative stress occurs when the body's natural antioxidants like catalase,

superoxide dismutase and a combination of peroxidase enzymes are insufficient to neutralize the free radicals (Halliwell and Gutteridge 1989). Increasing antioxidant defenses through food is considered crucial in maintaining human health and in disease prevention (Serafini and Del Rio 2004). A pioneering approach in this direction is the development of functional foods containing probiotic, prebiotics or their combination (synbiotics) capable of exerting antioxidant activity and nullifying the oxidative stress in the host.

Gibson and Roberfroid (1995) defined prebiotics as 'nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon'. Among the various non digestible oligosaccharides that have prebiotic potency, xylo-oligosaccharides (XOS) are now being explored for their physiological roles. XOS are oligomers of 2–10 xylose residues linked together by β (1–4) linkage which are produced by the hydrolysis of xylan, the major component of plant hemicelluloses either by autohydrolysis or by chemical treatment/enzymatic hydrolysis. Corncobs, hardwoods (birchwood and larchwood), straws, bagasse, rice hulls, malt cakes and bran serve as starting materials for XOS production (Vázquez et al. 2000). XOS have been enzymatically produced from wheat bran alkali extractable hemicelluloses (Reddy and Krishnan 2010) and wheat bran water soluble polysaccharides (Madhukumar and Muralikrishna 2010). Wang and Lu (2013) reported the extraction of XOS from wheat bran by microwave assisted enzymatic hydrolysis. XOS are reported to possess prebiotic, immunomodulatory, anticancerous, antimicrobial, growth regulatory and antioxidant properties (Aachary and Prapulla 2011).

Probiotics are 'living microbial supplements that beneficially affect the host animals by improving its intestinal microbial balances' (Fuller 1989). Probiotics, most notably lactobacilli and bifidobacteria are being extensively studied for their ability to positively influence the host gut by

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producing metabolites with bioactive properties and have been reported to possess an array of health promoting properties (Nagpal et al. 2012).

Studies have shown that milk is an excellent vehicle for delivery of probiotics and prebiotics to the gastrointestinal tract, the site of action. In the recent years there has been focus on preparing fermented milk with the combination of prebiotics and probiotics with the aim of promoting health. There are reports on the use of probiotic strains, commonly, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Bifidobacterium breve* and prebiotics such as fructooligosaccharides, inulin and lactulose to prepare synbiotic fermented milk (Casiraghi et al. 2007; Amiri et al. 2010). To the best of our knowledge the antioxidant activity of synbiotic combination of *Lactobacillus* sp. and XOS in the delivery system of fermented milk has not been reported till date.

The antioxidant potential of synbiotic combination of XOS and *Lactobacillus* sp. needs to be explored for the production of new dairy functional foods with improved quality. The present study focuses on (a) the extraction, purification and characterization of XOS from wheat bran water unextractable portion (WB-WUP), (b) its use in combination with selected *Lactobacillus* sp. in preparation of synbiotic fermented milk and (c) evaluation of antioxidant functionality of the preparation.

Materials and methods

Materials

Wheat bran was procured from the local market. Termamyl (EC 3.2.1.1) from *Bacillus licheniformis*, glucoamylase (EC 3.2.1.3) from *Aspergillus niger*, xylanase from *Thermomyces lanuginosus*, 2,2-Diphenyl-1-picrylhydrazyl, 2,4,6-Tris(2-pyridyl)-s-triazine and other chemicals were purchased from Sigma Chemicals (St Louis, USA). *Lactobacillus* strains *Lactobacillus brevis* NCDC01 (Lb), *Lactobacillus acidophilus* NCDC011 (La), *Lactobacillus plantarum* NCDC020 (Lp), *Lactobacillus casei* NCDC017 (Lc) and *Lactobacillus fermentum* NCDC156 (Lf) were procured from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal. The strains were subcultured thrice before use in sterile de Mann, Rogosa, Sharpe (MRS) broth using 1 % inoculum and 24 h incubation at 37 °C. Microbiological culture media and media ingredients were obtained from HiMedia, Mumbai, India. All other chemicals and solvents were of analytical grade.

Preparation of xylo-oligosaccharides

XOS was prepared from WB-WUP according to the method described earlier (Lasrado and Muralikrishna 2013). Briefly,

wheat bran was destarched by termamyl and glucoamylase treatments, water soluble polysaccharides were removed and the residue was dried by solvent exchange and designated as WB-WUP. WB-WUP was subjected to xylanase treatment and the crude XOS obtained was passed through charcoal-celite column to remove monosaccharides. The oligosaccharide mixtures obtained were separated on Bio-Gel P-2 column (0.9×100 cm) by using triple distilled water as the eluant. The appropriate fractions were pooled, quantified and concentrated for further characterisation.

Determination of sugar composition by gas liquid chromatography (GLC)

The oligosaccharides (5 mg) were hydrolysed with sulphuric acid (2 N) for 8 h, followed by neutralization with barium carbonate and deionization with Amberlite IR-120(H+) resin. Sodium borohydride was added to reduce the monosaccharides into alditols. The excess borohydride was decomposed by adding acetic acid (2 N) and co-distilled with methanol to remove the boric acid formed. The alditols were acetylated using acetic anhydride and pyridine (1:1). After acetylation excess reagents were removed by co-distilling with water and toluene (2 ml×3 each). The alditol acetates were dissolved in chloroform, filtered through glass wool and dried by flushing with nitrogen. The derivatives were taken in known amount of chloroform (Sawardeker et al. 1965) and analysed on RTX 2330 column by GLC (Shimadzu GLC system, 2010 Plus).

ESI-MS of purified oligosaccharides

Mass spectra of the oligosaccharides were analysed using Alliance, Waters 2695 mass spectrometer instrument by positive mode electrospray ionisation with the following operational conditions, i.e. core voltage 100 V, capillary voltage 3.5 kV, desolvation temperature 150 °C, source temperature 80 °C, desolvation gas (nitrogen) 500 l/h and coregas (Argon) 35 l/h (Reis et al. 2002).

NMR spectra of oligosaccharides

¹³C and ¹H NMR of purified oligosaccharides samples (2–5 mg) were carried out by taking samples in D₂O. The shifts were referenced to external TMS. The spectra were recorded in a Bruker AQS 500 MHz NMR spectrometer (5 mm BBO probe).

Probiotic and synbiotic preparations

Pasteurized fresh skim milk was used for the preparation of samples. To prepare probiotic samples, sterile milk was inoculated with different strains of *Lactobacillus* individually corresponding to 10⁸ cfu/ml. To prepare synbiotic samples, XOS

mixture was added to sterile milk at 1 % concentration and then inoculated with different strains of *Lactobacillus*. Uninoculated sterile milk with and without XOS served as control (Table 1). The preparations were incubated at 37°C for 24 h to prepare fermented probiotic and synbiotic milk.

The pH of the preparations both before and after fermentation was measured using the digital pH meter. Following fermentation, the pH of the preparations was adjusted to 4.6 using 1 N HCl, the samples were centrifuged at 5,000×g for 10 min, and the supernatant was collected.

Antioxidant activity

DPPH (2, 2-Di Phenyl – 1 Picryl- Hydrazyl) assay

DPPH free radical scavenging activity was estimated using a slight modification to the procedure reported earlier (Rao and Muralikrishna 2006). In brief, an aliquot of sample (0.5 ml) was added to 0.5 ml of a solution of DPPH, prepared fresh, at a concentration of 80 mg/l in methanol and incubated for 30 min at room temperature (~25 °C). The samples were centrifuged at 3,000×g for 2 min and the absorbance was measured spectrophotometrically at 517 nm. Methanol was used as blank and DPPH solution in methanol (1:1) served as control. The radical scavenging activity of the samples was expressed in terms of percentage inhibition of DPPH absorbance

$$\text{DPPH scavenging activity (\%)} = \left[\frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100$$

Ferric reducing anti- oxidant power (FRAP) assay

Reducing power of the samples was estimated according to Benzie and Strain (1999). Briefly, sample (0.1 ml) was mixed with 0.9 ml of freshly prepared FRAP reagent [(a) 300 mM

acetate buffer, pH 3.6 (b) 10 mM TPTZ in 40mM HCl (c) 20 mM FeCl₃·6H₂O. The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1]. The mixture was incubated for 30 min at room temperature (~25 °C) and absorbance was read against a suitable blank at 593 nm. The increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing TPTZ and FeCl₃·6H₂O is used to estimate the antioxidative activity. FRAP values were calculated with reference to a standard curve [ferrous sulphate (FeSO₄·7H₂O)] solutions and results were expressed as mg Fe²⁺/100 ml.

Statistical analysis

All the experiments were performed in triplicates and the values were represented as mean values±standard deviation (SD). One way analysis of variance was performed by ANNOVA procedure. Results were considered statistically significant at $p < 0.05$.

Results and discussion

Preparation of XOS from WUP of wheat bran

Wheat bran water unextractable portion was obtained in the yield of 54 %. In studies published earlier, WB-WUP was isolated in the yield ranging from 40 to 65 % and was reported to be composed of mainly arabinoxylans and cellulose (Gibson and Roberfroid 1995). To obtain XOS, WB-WUP was subjected to xylanase treatment. Xylanase from *Thermomyces lanuginosus* is an endo-β-(1→4)-xylanase which cleaves the xylan backbone randomly at places with two or more successive unsubstituted xylan residues resulting in XOS which vary in degree of polymerization. The crude XOS mixture obtained by enzymatic hydrolysis WB-WUP consisted significant amount of monosaccharides in addition to XOS and was thus further purified.

Purification of XOS

In order to obtain XOS mixture devoid of monosaccharides, charcoal-celite chromatography was used. This method uses different ethanol concentrations to selectively extract carbohydrate adsorbed to charcoal column (Whistler and Durso 1950). Further, the oligosaccharide mixture was separated into individual oligosaccharides by gel permeation chromatography using Bio-Gel P-2. The elution of sugars from the column was based on molecular weight. Three minor fractions containing larger oligosaccharides eluted first in decreasing order of their molecular weight and were designated as WO-I, WO-II and WO-III. These were followed by two major fractions

Table 1 Probiotic and synbiotic preparations used for antioxidant activity experiments

Preparation	Combination	
Probiotic	C	Milk (Control)
	PLa	Milk + <i>Lactobacillus acidophilus</i> NCDC011
	PLf	Milk + <i>Lactobacillus fermentum</i> NCDC156
	PLb	Milk + <i>Lactobacillus brevis</i> NCDC01
	PLp	Milk + <i>Lactobacillus plantarum</i> NCDC020
	PLc	Milk + <i>Lactobacillus casei</i> NCDC017
Synbiotic	CX	Milk + XOS (Control)
	SLf	Milk + <i>Lactobacillus fermentum</i> NCDC156+XOS
	SLb	Milk + <i>Lactobacillus brevis</i> NCDC01+XOS
	SLp	Milk + <i>Lactobacillus plantarum</i> NCDC020+XOS
	SLc	Milk + <i>Lactobacillus casei</i> NCDC017+XOS

(designated WO-IV and WO-V). The major fractions were used for further structural characterization.

Structural analysis of oligosaccharides

Sugar composition and ESI-MS of oligosaccharides

The neutral sugar composition of the oligosaccharides analysed by GLC indicated the presence of arabinose and xylose in the relative ratios of ~1:2 and 0:1 in WO-IV and WO-V respectively (Table 2). In ESI-MS analysis, XOS were detected as cationised species, $[M+Na]^+$ (Reis et al. 2002). The ions identified in the ESI-MS spectra are summarized in Table 2. WO-IV gave molecular ion signals at m/z 437 indicating it to be a trisaccharide. The spectra of WO-V gave the molecular ion signal at m/z 305 indicating that the fraction consists of neutral low molecular weight XOS i.e. xylobiose. Madhukumar and Muralikrishna (2010) purified XOS from wheat bran water soluble polysaccharides (WSP) and reported that it predominately consists of neutral XOS varying in degree of polymerization of 3–7.

NMR spectra

The signals obtained from ^{13}C and ^1H NMR are assigned by comparing with previously reported data (Hoffmann et al. 1991; Gruppen et al. 1992, 1993)

WO-IV: In ^{13}C NMR the anomeric carbons of β -xylopyranoside gave signals at δ 96.2 ppm and δ 101.5 ppm which are assigned to reducing β -xylopyranoside and non-reducing β -xylopyranoside respectively and the resonance in the region of δ 107.3 is assigned to the anomeric carbon atoms of α -L-arabinofuranoside. The α -L-arabinofuranoside linked to O-3 of the second β -xylopyranoside from the reducing end position were characterized by the signals δ 107.3 ppm (C1), δ 80.4 ppm (C2), δ 76.9 ppm (C3), δ 84.5 ppm (C4) and δ 61.0 ppm (C5) respectively (Table 3). The ^1H NMR spectrum signal around δ 5.30 ppm (H1), δ 4.15 ppm (H2), δ 3.92 ppm (H3), δ 4.30 ppm (H4) and δ 3.80 ppm (H5) indicated that arabinofuranoside residue is linked to the O-3 position of the second β -xylopyranose residue (Gruppen et al. 1992).

WO-V: In ^{13}C NMR, the anomeric carbons gave resonance signals at δ 96.2 ppm and δ 101.5 ppm, corresponding to reducing β -xylopyranoside and non-reducing β -

Table 2 ESI-MS fragmentation pattern of XOS

Bio-Gel P-2 major fractions	m/z	A:X ratio	Probable product
WO-IV	437	1:2	Xyl_2Ara
WO-V	305	0:1	Xyl_2

A:X ratio-Arabinose to Xylose ratio as determined by gas chromatography

Table 3 ^{13}C NMR chemical shifts of purified XOS

Oligosaccharide	Residue	C-1	C-2	C-3	C-4	C-5
WO-IV	Xylp-1	96.26	73.66	73.75	76.31	62.72
	β -Xylp-2 ^{II}	101.59	72.51	75.35	76.14	64.95
	α -Araf-A ^{3X2}	107.33	80.45	76.90	84.52	61.06
WO-V	Xylp-1	96.22	73.63	73.72	76.28	62.70
	β -Xylp-2 ^{II}	101.56	72.49	75.33	76.11	64.93

Xyl p-1 means xylopyranose residue at reducing end position

α -Araf-A^{3X2} means arabinofuranose linked to O-3 of Xyl p-2

xylopyranoside respectively. C-2 to C-4 signals are observed between δ 71–84 ppm. Signals at δ 64.9 ppm and δ 62.7 ppm correspond to C5 of β -Xylp-1 and β -Xylp-2 respectively (Table 3). The ^1H NMR spectrum showed signals at δ 4.47 ppm (H1), δ 3.28 ppm (H2), δ 3.56 ppm (H3), δ 3.78 ppm (H4) and δ 3.41 ppm (H-5) corresponding to the non-reducing second β -D-xylopyranose residue.

Based on the structural analysis [GC, ESI-MS, and NMR] WO-IV and WO-V were identified as arabinose containing triose and xylobiose, respectively. The probable structure can be assigned as follows

Change in the pH of probiotic and synbiotic preparations upon fermentation

The pH changes of the preparations are shown in Table 4. It can be observed that addition of XOS to milk lowers the pH of milk by ~0.3 units. After fermentation, pH of all the preparations, except of probiotic milk containing *L.brevis*

Table 4 Change in the pH of probiotic and synbiotic preparations upon fermentation

Prebiotic			Synbiotic		
Preparation	0 h	24 h	Preparation	0 h	24 h
C	6.52±0.01 ^a	6.27±0.02 ^a	CX	6.38±0.03 ^a	6.28±0.02 ^a
PLa	6.52±0.01 ^a	5.02±0.07 ^b	SLa	6.40±0.02 ^a	4.95±0.19 ^b
PLf	6.51±0.02 ^a	5.02±0.07 ^b	SLf	6.35±0.03 ^a	4.91±0.05 ^b
PLb	6.52±0.01 ^a	5.88±0.14 ^a	SLb	6.39±0.01 ^a	5.07±0.06 ^b
PLp	6.50±0.01 ^a	4.96±0.06 ^b	SLp	6.36±0.02 ^a	4.94±0.04 ^b
PLc	6.53±0.01 ^a	5.03±0.14 ^b	SLc	6.38±0.02 ^a	4.91±0.05 ^b

Values are Mean±SD, $n=3$. Values not sharing small letter superscripts within the row are significantly different ($p<0.05$)

Refer to Table 1 for expansion of the abbreviations used

(PLb), decreased to about 4.9. The absence of decrease in pH in PLb, suggests poor utilization of lactose by *L. brevis* which may be due to slow transport of lactose into the microorganism (Honda et al. 2012). However in synbiotic fermented milk SLb, *L. brevis* utilizes XOS as carbon source hence there is decrease in pH. The reduction of milk pH improves its nutritional value. Acid production during fermentation promotes finer coagulation of casein and improves the bioavailability of calcium and other minerals (Bronner and Pansu 1999).

Antioxidant activity of fermented milk

The DPPH radical scavenging activity of the samples is shown in Fig 1. Upon fermentation, there was a significant increase ($p<0.05$) in the scavenging activity of probiotic samples fermented by four of the five selected strains i.e. *Lactobacillus acidophilus* (PLa), *Lactobacillus plantarum* (PLp), *Lactobacillus casei* (PLc) and *Lactobacillus fermentum* (PLf) compared to the control. There was no increase in the scavenging activity of milk inoculated with *L. brevis* (PLb) due to lack of fermentation.

Synbiotic preparations and control (CX), which contain XOS showed significantly high ($p<0.05$) radical scavenging activity even before fermentation compared to probiotic preparations and control sample (C) which are devoid of XOS. This is because, XOS, owing to the presence of phenolic acids have potent antioxidant activity (Veenashri and Muralikrishna 2011). However, upon fermentation there was increase in the radical scavenging ability of all synbiotic preparations (SLa, SLf, SLb, SLp and SLc), suggesting the role of fermentation in increasing the antioxidant potential of milk. In sample SLb, *L. brevis* which is a poor lactose metabolizer utilized XOS as substrate to ferment milk and showed an increase in the radical scavenging activity.

Table 5 Antioxidant activity of probiotic and synbiotic preparations milk by FRAP assay

Preparations	FRAP value equiv. FeSO ₄ (mg/100 ml)	
	0 h	24 h
C	17.76±3.62 ^{aA}	18.51±2.57 ^{aA}
CX	71.44±2.35 ^{aB}	70.28±1.29 ^{aB}
PLa	19.23±0.60 ^{aA}	25.89±1.85 ^{bA}
PLf	19.46±0.96 ^{aA}	32.42±2.50 ^{bA}
PLb	19.74±2.90 ^{aA}	18.24±2.29 ^{aA}
PLp	18.75±1.92 ^{aA}	27.83±0.62 ^{bA}
PLc	17.63±2.00 ^{aA}	30.89±1.50 ^{bA}
SLa	70.31±1.70 ^{aB}	78.92±0.88 ^{bB}
SLf	68.51±3.29 ^{aB}	80.79±2.28 ^{bB}
SLb	68.55±3.06 ^{aB}	76.13±2.02 ^{bB}
SLp	69.70±2.89 ^{aB}	76.00±1.80 ^{bB}
SLc	70.70±1.94 ^{aB}	76.54±1.5 ^{bB}

Values are Mean±SD, $n=3$. Values not sharing same small letter superscripts within a row are significantly different ($p<0.05$). Values not sharing same capital letter superscripts within a column are significantly different ($p<0.05$). Refer to Table 1 for expansion of the abbreviations used

The results of the FRAP assay are presented in Table 5. The pattern of ferric reducing ability of the probiotic and synbiotic preparations was similar to that of the radical scavenging activity. Both these assays illustrate that the fermentation of milk by selected strains (with the exception of *L. brevis*) increased the antioxidant capacity of milk. Also, addition of XOS to milk and its subsequent fermentation by *Lactobacillus* strains significantly ($p<0.05$) increased the antioxidant activity of milk. Hence, synbiotic fermented milk has significantly higher antioxidant potential compared to that of probiotic fermented milk and control. The increase in antioxidant

Fig. 1 Antioxidant activity of probiotic and synbiotic preparations by DPPH assay. Values are Mean±SD, $n=3$. ($p<0.05$, between 0 and 24 h, # $p<0.05$, between probiotic and synbiotic preparations). Refer to Table 1 for expansion of the abbreviations used

activity in the synbiotic preparation is contributed both by XOS and the fermentation products. Increase in antioxidant activity upon fermentation can be ascribed to the formation of metabolic endproducts due to utilization of XOS along with lactose by the *Lactobacillus* strains. Previous studies have reported increased antioxidant activity of yogurt enriched with fructooligosaccharides (Madhu et al. 2012) and date palm syrup (Gad et al. 2010). Pihlanto (2006) reported that the hydrolysates from milk proteins resulting from fermentation of milk have antioxidant ability. There are a few reports on the antioxidant properties of different *Lactobacillus* strains and the ability of the *Lactobacillus* strains to improve the antioxidant potential of milk on fermentation (Parrella et al. 2012; Ramesh et al. 2012). The results obtained from this study indicate that supplementation of milk with XOS and *Lactobacillus* strains significantly increases the antioxidant potential of milk.

Conclusions

This study focused on the preparation of XOS from wheat bran water unextractable portion (WB-WUP), its use in the preparation of synbiotic fermented milk and the evaluation of the antioxidant activity of this preparation. XOS from WB-WUP predominately consisted of disaccharide (Xyl-(1→4) β -Xyl) and trisaccharide (β -Xyl-(3→1- α -Ara)-1→4- β -Xyl). The antioxidant potential as measured by DPPH and FRAP assay's showed that synbiotic combination of XOS and *Lactobacillus* strains significantly increased the antioxidant potential of milk. Thus, synbiotic combination of XOS and *Lactobacillus* strains has the potential to be a good supplement for addition to milk formulations in order to prevent oxidative stress and associated diseases.

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