Production and prebiotic activity of exopolysaccharides derived from some probiotics

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Received 15 April 2014
Accepted 15 December 2014

Egyptian Pharmaceutical Journal
2015, 14:1–9

Objective
The aim of this study was to focus on exopolysaccharides (EPSs) produced by *Lactobacillus delbrueckii bulgaricus*, *Lactobacillus helveticus* or *Lactobacillus casei* and their use as a prebiotic for *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* or *Bifidobacterium bifidum*.

Materials and methods
Optimization of culture conditions using different carbon and nitrogen sources and different temperature, pH and incubation periods for maximum EPS production was studied.

Results and conclusion
It was found that the best conditions were as follows: the use of sucrose (20%) instead of glucose in MRS medium, incubation at pH 7.0 and temperature 37°C for 72 h incubation under anaerobic conditions to give the highest EPS yield; a yield of 13.99 g/l was recorded in case of *L. helveticus* when grown on the aforementioned optimized conditions. It was found that *L. delbrueckii bulgaricus* EPS has the highest prebiotic indices (I), varying from 7.9 to 10.1. In contrast, *L. helveticus* and *L. casei* EPSs have the lowest prebiotic indices (I), varying from 1.4 to 2.4.

Keywords:
exopolysaccharides, *Lactobacillus casei*, *Lactobacillus delbrueckii bulgaricus*, *Lactobacillus helveticus*, prebiotic, probiotic activity

Egypt Pharm J 14:1–9

1687-4315

Introduction

Probiotics are feed and food supplements that beneficially affect the host's health. Strain identity is important to link a strain to a specific health effect and to enable accurate surveillance and epidemiological studies [1]. The term 'probiotic' includes a large range of microorganisms, mainly bacteria but also some yeasts. Because they can stay alive until they reach the intestine and contribute beneficial effects to the host's health, lactic acid bacteria (LAB), non-LAB and some yeasts can be considered as probiotics. LABs are the most important probiotics known to have beneficial effects on the human gastrointestinal tract.

These bacteria are Gram-positive bacilli and usually live in a nonaerobic environment, but they also can support aerobic conditions [2,3]. Probiotic bacteria have been postulated to play a positive role in maintaining health [4], alleviating the symptoms of traveller's diarrhoea [5] and irritable bowel syndrome [6] among other conditions [7]. 'Probiotic' is a broad term covering many strains of microbes, the majority of which belong to Gram-positive *Lactobacilli* or *Bifidobacteria*.

Prebiotics have been defined as a nondigestible food ingredient that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health [8].

There are three criteria that are required for an ingredient to be established as a prebiotic:

(1) A prebiotic must be resistant to hydrolysis by gastric acidity and should be nondigestible by host enzymes (hence not absorbed).
(2) It must be capable of being fermented by intestinal beneficial microflora.
(3) It should possess the property of selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being.

Among prebiotics, many exopolysaccharides (EPSs) were examined for their prebiotic activities. In a recent study, Hongpattarakere *et al.* [9] reported that the EPSs produced from *Lactobacillus plantarum*, *Weissella cibaria*, *Weissella confusa and Pediococcus pentosaceus* can be utilized (as carbon source) by *Bifidobacterium bifidum* DSM 20456.

Similarly, Russo *et al.* [10] reported that a β-glucan of nondairy bacterial origin has a prebiotic effect

on three probiotic strains. The glucan producer was *Pediococcus parvulus* 2.6, isolated for ropy cider. The same authors analyzed the potential prebiotic effect of β-glucan on the survival of three strains belonging to the *Lactobacillus* genus: *L. plantarum* WCFS 1, the isogenic recombinant *L. plantarum* WCFS 1β_gal and *Lactobacillus acidophilus* NCFM.

More recently, Grosu-Tudor *et al.* [11] studied the prebiotic potential of some EPSs produced by LAB. In this study, the authors investigated the digestibility and stability of six EPSs produced by LAB isolated from new milk and traditional dairy products to evaluate their prebiotic potential. Four homopolysaccharides (composed of glucose solely) and two heteropolysaccharides (composed of galactose : rhamnose in 5 : 2 ratio and galactose : glucose ratio 4 : 1) were tested. No degradation of the six EPSs was seen during in-vitro digestion. Two *Clostridium* strains, as well as *Anaerostipes caccae* and *Roseburia intestinalis*, could not metabolize any of the six EPSs tested; however, two bacteroides strains grow on all EPSs produced by *Lactobacillus lactis* 1.8 by *Bifidobacterium pseudocatenulatum* LMG 10505, indicating the prebiotic potential of this substrate.

Van Geel-Schutten *et al.* [12] stated that 'the ability to produce glucans appears to be widespread in genus *Lactobacillus*'. Thus, the presence of glucose as a structural unit of EPSs was recorded in the products of *Lactobacillus reuteri* [12–15], *Lactobacillus brevis* [16], *Lactobacillus fermentum* [17] and *Lactobacillus confusus* [15]. Similarly, fructose was reported as a structural unit of EPSs produced by many *Lactobacilli* spp. These included *L. reuteri*, *Lactobacillus sanfranciscensis*, *Lactobacillus frumenti*, *Lactobacillus pontis* and *L. confusus* [18]. In contrast, arabinose was defined as a minor component of EPSs produced by *Lactobacillus delbrueckii bulgaricus* [19] and *Lactobacillus casei* [20].

Materials and methods

Materials

Microorganisms (bacterial strains)

Seven bacterial strains, known as probiotics, were used for study of the production and prebiotic activity of EPS throughout the present work. These included the bifidogenic bacteria: *Lactobacillus delbrueckii bulgaricus, L. helveticus, L. casei, L. acidophilus and Bifidobacterium bifidum*. These were attained from Chr. Hasen's Lab. Inc., Danemark, in addition to *L. rhamnosus* NRRL B-442 and *L. reuteri* NRRL B-14171.

A pathogenic isolated bacterium, *Escherichia coli*, was isolated by El Nozha International Hospital Laboratory (Heliopolis, Cairo, Egypt) and used for comparison as a model for the bacteria incapable of utilizing EPSs and was maintained on nutrient agar medium.

Culture media

Unless stated, all of the following media were adjusted to pH 7.2 before subjecting to sterilization.

De Man–Rogosa–Sharp (MRS) medium: This medium was used for the growth and maintenance of the probiotics *L. delbrueckii bulgaricus*, L. casei, *L. reuteri*, *L. helveticus*, *L. rhamnosus*, *L. acidophilus* and *B. bifidum*. MRS broth medium by the studied bacterial exopolysaccharides and measure the cell intensity of the broth after inoculation and incubation. The prepared MRS broth tubes were inoculated and then incubated anaerobically at 37°C for 24 h and subcultured every month.

MRS base medium: This is a liquid medium comprising the inorganic components and nitrogen source as in the original MRS medium, with the carbon source replaced by an EPS at the same concentration.

High-performance liquid chromatography apparatus

(1) Liquid chromatograph: equipped with isocratic solvent delivery system, manual injector, refractive index detector and recording/computing integrator.
(2) Column: 300´4 mm (internal diameter) m-Bondapak/Carbohydrate (no. 84038; Waters Associates, 37 Gray St, Hamilton VIC 3300, (03) 5571 1777, Fax (03) 5571 9009).
(3) Test solution clarification kit: available in kit form from chromatography suppliers; 0.45 μm filters stable in organic solvents are suitable.
(4) Syringes: 10 μl point style no. 1, 2 × 0.020 inches outer diameter, 25-G needle (no. 701-N; Hamilton Co.).

Protons nuclear magnetic resonance apparatus

Elemental analysis data were performed by the Central Lab, the National Research Center.

Methods

Screening of some probiotics for their ability to produce exopolysaccharides

Seven probiotics were grown on a modified MRS broth medium (10 ml/test tube). This modified MRS medium comprised 20% glucose as carbon source and 1.0% peptone, 0.5% yeast extract and 0.1% beef extract as nitrogen sources, after incubation at 37°C for 72 h. These probiotics included *L. delbrueckii bulgaricus*, *L. helveticus*, *L. casei*, *L. acidophilus*, *B. bifidum*, *L. reuteri* NRRL B-14171 and *L. rhamnosus* NRRL B-442.

Productivity of exopolysaccharides by various probiotics

The bacterial strains were grown anaerobically in 100 ml flasks containing 90 ml of MRS medium (for each flask).

Probiotic strains were cultivated in a specific culture medium – that is, modified MRS broth medium [21] supplemented with 20% glucose as carbon source. As nitrogen sources, the media comprised 1% peptone, 0.5% yeast extract and 0.1% beef extract. It is worth clarifying that all other ingredients of the MRS medium remained unchanged, and fermentation was proceeded at 37°C, with initial pH 7 under anaerobic condition, for 72 h. The culture broth was centrifuged at 9000 rpm for 20 min at 4°C to remove bacterial cells. Addition of three volumes of 95% chilled ethanol to the supernatant was performed at 4°C, and the mixture was left overnight to precipitate EPSs, which were later collected by centrifugation at 9000 rpm for 20 min at 4°C and dried at 50°C until constant weight was obtained [22].

Optimization of physiological and cultivation conditions for higher productions of exopolysaccharides by chosen probiotics

The modified MRS medium was prepared using different sources of carbon and nitrogen, and fermentations were achieved under variable conditions of pH, temperature and incubation periods. These experiments were designed to investigate the effect of these factors on EPS production to reach the most optimal conditions for attaining the highest yields of EPSs.

Effect of different carbon sources: The probiotic strains were grown on modified MRS broth medium comprising 20% of each sugar. These included glucose (control), fructose, sucrose, lactose and cane sugar molasses. The other fermentation conditions and isolation steps of EPSs remained unchanged, as in previous experiments.

Effect of different nitrogen sources: The effect of different nitrogen source on the production of EPSs was studied using 100 ml flasks containing 90 ml MRS medium comprising 20% sucrose as carbon source. The nitrogen source of the MRS medium (1% peptone and 0.5% yeast extract) taken as control was replaced by substitutions of peptone, yeast extract, beef extract, casein, urea and soy bean according to their nitrogen contents so that they were equivalent to nitrogen sources used in the original MRS medium. The rest of the medium components, as well as the growing conditions, were kept unchanged.

Effect of different temperature: The effects of temperature were proceeded at 30, 37, 40 or 45°C. The rest of the growth conditions were kept unchanged using the optimal carbon and nitrogen sources.

Effect of different pH values of the culture medium: The effect of different initial pH values on the production of EPSs was studied. The medium pH was initially adjusted by using NaOH or HCl to cover a pH range from 5.5 to 8.0 (all adjustments were made before sterilization). The rest of the growth conditions were kept unchanged using the optimal carbon and nitrogen sources at optimized temperature.

Effect of different incubation periods: Different incubation periods (24, 48, 72 and 96 h) were tested at optimal conditions.

Preparation of the partially purified exopolysaccharides

A large number of cultures (90 ml × 10 flasks) of each of the three present probiotics were grown on the previously optimized media under the most convenient fermentation conditions. Growth was proceeded in static 100 ml flasks at 37°C for 3 days. The resultant bacterial cultures were centrifuged at 9000 rpm for 20 min. The crude EPSs (present in supernatants) were precipitated using three volumes of 96% ethanol. After separation, the precipitates were dissolved in least amounts of distilled water, and trichloroacetic acid (TCA) was added to give a final concentration of 10% to precipitate free protein. After centrifugation, the supernatants were shook three times with equal volumes of ether to remove excessive amounts of TCA. After dialysis against distilled water (48 h), the dialyzed solutions were concentrated under vacuum to half of their volumes, and the partially purified EPS preparations were separated by precipitation (with four volumes of ethanol) and centrifugation was carried out.

Analysis of the polysaccharide products

Determination of total carbohydrates: Total carbohydrates were determined as glucose, using the phenol–sulphuric method [23].

Determination of soluble protein: Protein assay was performed according to the method of Lowry *et al.* [24].

Determination of monosaccharide units comprising exopolysaccharides

Acid hydrolysis of EPS: Hydrolysis of the polysaccharides was achieved according to the method adopted by Perila and Bishop [25]. The quantitative paper chromatographic analysis using *n*-butanol–acetone–water in the ratio 4 : 5 : 1 v/v was applied according to the method of Wilson [26].

Quantitative paper chromatography of EPS hydrolysates: Chromatographic separation of the hydrolysed products was carried out on Whatman No. 1 filter paper

and *n*-butanol–(ethanol)EtOH–water in the ratio 40 : 11 : 19 v/v [27] and then quantitative determination of the separated sugars was carried out according to the method adopted by Wilson [26].

High-performance liquid chromatography (HPLC): The hydrolysates were examined for their monosaccharide contents with the HPLC technique according to the method of AOAC official method 1977 [28].

^{1}H NMR study

The partially purified polysaccharide samples were examined for features of their polysaccharide structure using the JOEL-ECA 500 Spectrometer: ^{1}H nuclear magnetic resonance (NMR) apparatus.

Evaluation of the biological activities of the obtained partially purified exopolysaccharides

Determination of prebiotic activities of the partially purified EPSs: The partially purified EPS products were used as carbon sources for growing four probiotics (previously found incapable to produce EPS) and (in parallel) a pathogenic strain of *E. coli*. The probiotics were grown on standard MRS broth medium, whereas *E. coli* was grown on nutrient broth medium. After incubation at 37°C for 24 h, the optical densities of the growths were determined at 600 nm, and the prebiotic index was calculated as follows:

$$\text{Prebiotic index} = \frac{\text{Optical denisty of the growth of probiotic culture}}{\text{Optical density of the growth of } E.coli}$$

Results and discussion

In the present work, studies were emphasized on the production and prebiotic evaluation of extracellular polysaccharides (EPSs) synthesized by *L. delbrueckii bulgaricus*, *L. helveticus* and *L. casei*. Chemical and physical characterization of EPS preparations (exhibited highest prebiotic activities) was also achieved.

Screening of some probiotics for their ability to produce exopolysaccharides

The above-mentioned seven probiotic bacteria were examined for their ability to produce EPSs. They were cultivated on a specific culture medium – that is, modified MRS medium [21] at 37°C for 72 h and initial pH 7. This modified MRS medium comprised 20% glucose as carbon source and 1% peptone, 0.5% yeast extract and 0.1% beef extract as nitrogen sources. It is worth clarifying that all the other ingredients of MRS medium remained unchanged, and fermentation was proceeded under anaerobic condition. These results indicated that the highest yield (1.76 g/l) of EPS was recorded in the culture of *L. delbrueckii bulgaricus*, whereas lower yields of 0.9 and 0.4 g/l were found in the cultures of *L. helveticus* and *L. casei*, respectively. In contrast, only trace amounts of EPS were observed in the cultures of the other four investigated probiotics. The results in Table 1 generally agree with those of Badel *et al*. [29], who reported the low yields of EPSs produced by the majority of *Lactobacillus* spp.

As previously described, the results indicated that three (out of seven) strains of the studied probiotics showed considerable yields of EPS. Accordingly, they were subjected to further investigations aiming to increase their EPS productions. This was achieved by optimization of the physiological and cultivation condition applied throughout the growth of these *Lactobacillus* spp.

Effect of carbon source

In this experiment, the selected probiotics were grown on an equal amount (20%) of each of glucose (control), fructose, lactose, sucrose or cane sugar molasses as sole carbon source. The results recorded in Table 2 indicate that the use of sucrose (at 20%) led to the highest production of EPSs by all of the chosen probiotics. The highest yields of EPSs exhibited by *L. helveticus*, *L. casei* and *L. delbrueckii bulgaricus* were 13.99, 7.06 and 4.54 g/l, respectively. In contrast, the use of lactose (at 20%) afforded lower yields of EPS: 0.60, 1.3 and 0.20 g/l by *L. delbrueckii bulgaricus*, *L. helveticus* and *L. casei*, respectively. However, the lower yield of EPS (0.4 g/l) attained from *L. casei* was recorded on using glucose (20%) as carbon source. The use of a higher concentration (20%) of glucose in the modified MRS medium based on unrecorded results showed that the utilization of lower concentrations (2–15%) of glucose led to the appearance of insignificant amounts of EPSs produced by the presently studied bacteria. This observation agrees with those of Badel *et al.* [29], who

Table 1 Polysaccharide productivities of some probiotic bacteria grown on a modified De Man–Rogosa–Sharp medium[a]

Probiotic strains	Polysaccharide yields (g/l)
Lactobacillus delbrueckii bulgaricus	1.76
Lactobacillus helveticus	0.9
Lactobacillus casei	0.4
Lactobacillus reuteri	Traces
Lactobacillus rhamnosus	Traces
Lactobacillus acidophilus	Traces
Bifidobacterium bifidum	Traces

[a]Carbon source: 20% glucose.

reviewed that the low yields (0.08–2.8 g/l) of EPS by the majority of LAB is the main reason of their noncommercial exploitation. Furthermore, the use of fructose or cane sugar molasses (as carbon source) led to the appearance of only trace amounts of EPSs.

As sucrose was the most favourable for the production of EPSs, it was necessary to study the effect of different sucrose concentration (10, 15 and 20%), whereas all of the other components remained unchanged. The results presented in Table 3 reveal that the highest yields of EPSs were attained by growing the chosen *Lactobacillus* strains on fermentation medium comprising 20% sucrose as carbon source. However, unrecorded data indicate that the use of sucrose concentration higher than 20% led to decline in the production of EPSs by all the studied *Lactobacilli* spp. These data are in agreement with those of Korakli *et al.* [30] and Seeuriyachan *et al.* [31]. In this respect, Korakli *et al.* [30] investigated the effect of sucrose concentration on the formation of EPSs by *L. sanfranciscensis* LTH 2590 in pH-controlled fermentations with sucrose concentrations ranging from 20 to 160 g/l. The EPS production increased and the relative sucrose hydrolysis decreased on increasing the sucrose concentration in the medium. The same authors reported that about 40 g of EPS/l was produced in the MRS medium containing 160 g of sucrose/l.

Table 2 Effect of carbon source on the polysaccharide productivities of the selected probiotics

Probiotic strains	Polysaccharide yields (g/l) using various carbon sources (20%)				
	Glucose	Sucrose	Lactose	Fructose	Cane sugar molasses
Lactobacillus delbrueckii bulgaricus	1.76	4.54	0.6	Traces	Traces
Lactobacillus helveticus	0.9	13.99	1.3	Traces	Traces
Lactobacillus casei	0.4	7.06	0.2	Traces	Traces

Table 3 Effect of using different sucrose concentrations on the polysaccharide productivities of selected probiotics

Probiotic strains	Polysaccharide yields (g/l) using various sucrose concentrations		
	20%	15%	10%
Lactobacillus delbrueckii bulgaricus	4.54	3.40	1.70
Lactobacillus helveticus	13.99	4.30	3.80
Lactobacillus casei	7.06	2.40	1.10

Effect of using different nitrogen sources

As peptone, yeast extract and beef extract are relatively expensive complex nitrogen sources, attempts were made to reduce the cost of the fermentation media comprising these sources [31,32]. The aim of this experiment was to study the effect of the use of different nitrogen sources on the EPS production by the chosen bacterial strains. The investigated nitrogen sources included peptone, yeast extract, beef extract, casein, urea and soy bean. The rest of the medium components, as well as the growing conditions, were kept unchanged. The results recorded in Table 4 show that the nature of nitrogen source had a profound effect on EPS biosynthesis. The control medium comprised peptone (1%), yeast extract (0.5%) and beef extract (0.1%) as standard nitrogen sources. Substitutions of these components with beef extract alone resulted in an increase in EPS yield (7.50 g/l) attained from the culture filtrate of *L. casei*. In contrast, none of the investigated bacterial strains exhibited increases in EPS yields as they were grown on individual nitrogen sources instead of mixed nitrogen sources, as in the control medium. On the basis of these data, the nitrogen sources used in the next experiment to grow the present *Lactobacilli* spp. were beef extract for *L. casei* and the mixed nitrogen sources, comprising the control medium, for *L. delbrueckii bulgaricus* and *L. helveticus*.

Effect of incubation temperature

This experiment was carried out to select the most suitable incubation temperature that supports the highest production of EPSs. In this experiment, the control medium with sucrose 20% was used to grow the strains *L. delbrueckii bulgaricus* and *L. helveticus*, whereas *L. casei* was grown on the medium comprising beef extract as nitrogen sources and sucrose 20% as carbon source for all the three chosen probiotics. The results in Table 5 indicate that the rise in incubation temperature up to 45°C led to sharp declines in EPS yields attained from all the investigated bacterial strains. However, the rise in incubation temperature up to 40°C resulted in similar decrease in EPS yields attained by both of *L. helveticus* and *L. casei*, whereas

Table 4 Effect of nitrogen source on the polysaccharide productivities of the selected probiotics

Probiotic strains	Polysaccharide yields (g/l) using various nitrogen sources[a]						
	Control[b]	Casein	Urea	Yeast extract	Beef extract	Peptone	Soy bean extract
Lactobacillus delbrueckii bulgaricus	4.54	3.00	0.4	4.00	0.30	4.00	2.00
Lactobacillus helveticus	13.99	3.30	2.00	2.00	0.30	0.30	3.00
Lactobacillus casei	7.06	4.00	0.80	3.00	7.50	0.40	4.00

[a]Equivalent amounts of nitrogen sources, [b]Control medium comprised mixed nitrogen sources.

that of *L. delbrueckii bulgaricus* remained closely related to the control value. Many research workers reported the same temperature (37°C) as optimum degree for the production of EPSs by some LAB. These included *L. delbrueckii bulgaricus* [33,34], *L. helveticus* [35] and *L. rhamnosus* [36–38].

Hence, the incubation temperature was adjusted at 37°C in the next experiments.

Effect of the initial pH values

The goal of the present experiment was to evaluate the effect of the initial pH values of the growth medium on the yields of EPSs produced by the chosen Lactobacilli. The bacteria were grown under the previously concluded optimum conditions at various initial pH values (pH 5.5, 6.0 and 8.0).

The results shown in Table 6 reveal that a change in initial pH values towards acidic (pH 5.5 and 6.0) or alkaline (pH 8) sides led to sharp decreases in the EPS yields afforded by each of *L. helveticus* and *L. casei* as compared with the control value (recorded at neutral conditions). In contrast, the same change in initial pH value resulted in slight variation in the EPS yields attained by *L. delbrueckii bulgaricus*. According to these data the initial pH value of the growth medium was adjusted at pH 7 in the next experiments. In agreement with these data, a closely related value (pH 6.5) was reported by Zhang *et al.* [17] as optimal fermentation condition for growth and EPS production by *L. fermentum* F6.

Effect of different incubation periods

The aim of this experiment was to define the optimum incubation period required for exhibition of the highest yields of EPSs produced by the chosen probiotics. The bacterial strains were grown on the previously optimized culture media adjusted at pH 7 (as initial value) and 37°C.

The data recorded in Table 7 indicate that EPS production of *L. delbrueckii bulgaricus* increased (up to 7.65 g/l) on the fourth day (96 h incubation) and the yield remained almost unchanged during a further 3 days of incubation. As regards the other two probiotic strains, *L. helveticus* and *L. casei*, the yield of EPSs was decreased by extending the incubation time after 72 h (control time).

In accordance with these results, the same incubation time (72 h) was reported as optimal for the production of EPSs by *L. rhamnosus* and *L. paracasei* [39]. In contrast, the optimal incubation period for production of EPSs by *L. delbrueckii bulgaricus* was found to be 96 h. As far as we are aware, there are no published data confirming this result, and this may be the first report on this observation.

Preparation of the partially purified exopolysaccharides

This was carried out to attain suitable amounts of partially purified EPSs to be used in the next part of this work. The analytical characters of both the crude and partially purified EPS samples are recorded in Table 8. The results indicated that the treatment with TCA eliminated portions of protein (present in free form) comprising crude EPSs. This appeared as increases

Table 5 Effect of incubation temperature on exopolysaccharide yields (g/l) attained by the chosen probiotics

Probiotic strains	EPS yields (g/l) using various incubation temperature			
	37°C (control)	30°C	40°C	45°C
Lactobacillus delbrueckii bulgaricus	4.54	3.13	4.50	2.49
Lactobacillus helveticus	13.99	5.41	7.02	6.21
Lactobacillus casei	7.06	5.70	5.79	4.19

EPS, exopolysaccharide.

Table 6 Effect of initial pH value on exopolysaccharides yields (g/l) attained by the chosen probiotics

Probiotic strains	EPS yields (g/l) using various initial pH values			
	pH 7 (control)	pH 5.5	pH 6	pH 8
Lactobacillus delbrueckii bulgaricus	4.54	4.60	4.4	4.3
Lactobacillus helveticus	13.99	3.00	4.8	4.8
Lactobacillus casei	7.06	4.00	4.4	4.8

EPS, exopolysaccharide.

Table 7 Effect of incubation period on exopolysaccharides yields (g/l) recorded for chosen probiotics

Probiotic strains	EPS yields (g/l) at various incubation time				
	24 h	48 h	72 h	96 h	120 h
Lactobacillus delbrueckii bulgaricus	4.02	4.24	4.54	7.65	6.91
Lactobacillus helveticus	5.53	5.95	13.99	13.44	11.44
Lactobacillus casei	5.50	6.80	7.06	6.17	5.25

EPS, exopolysaccharide.

Table 8 Carbohydrate/protein ratios in the crude and partially purified exopolysaccharides produced by the chosen probiotics

Bacterial sources of EPS	Carbohydrate : protein ratio	
	Crude EPS[a]	Partially purified EPS[b]
Lactobacillus delbrueckii bulgaricus	4.07 : 1.00	6.16 : 1.00
Lactobacillus helveticus	3.08 : 1.00	5.76 : 1.00
Lactobacillus Casei	3.08 : 1.00	4.80 : 1.00

EPS, exopolysaccharide, [a]Before treatment with trichloroacetic acid, [b]After treatment with trichloroacetic acid.

in the 'carbohydrate/protein' ratios in the treated samples of EPSs compared with those of the untreated (crude) ones. Accordingly, the partially purified EPSs were then subjected to further examination for their chemical, physical and biological characters.

Characterization of the partially purified exopolysaccharides produced by the presently studied probiotics

Chromatography of acids hydrolysates of the partially purified exopolysaccharides

This was undertaken to define the monosaccharide constituents comprising the chains of EPSs molecules synthesized by the chosen probiotic strains. Thus, complete acid hydrolysis of the investigated EPSs was carried out using concentration H_2SO_4 [40]. The resultant hydrolysates were chromatographed on Whatman No. 1 filter paper using butanol–acetone–water (4 : 5 : 1, v/v) as a solvent [27] and aniline phthalate as spraying reagent [41]. The paper chromatograms indicated the presence of two brown and one pink spots in the chromatographed hydrolysate of EPSs produced by *L. casei*. The brown spots were equidistant with the spots of authentic glucose and fructose, whereas the pink spot was equidistant with a spot of authentic arabinose. In contrast, the chromatographed hydrolysate of EPSs attained from *L. delbrueckii bulgaricus* and *L. helveticus* exhibited one brown and one pink spot equidistant with those of authentic glucose and arabinose, respectively. Quantitative determination of the aforementioned separated monosaccharides was achieved according to the method of Wilson [26]. The results in Table 9 reveal that EPSs synthesized by *L. casei* comprised glucose as a major component (10 mol) and fructose (1 mol) and arabinose (1 mol) as minor components. In contrast, EPSs attained from both *L. delbrueckii bulgaricus* and *L. helveticus* were devoid of fructose units and comprised glucose and arabinose units in molar ratios 3 : 1 and 2 : 1, respectively. The present data on paper chromatography were further confirmed by using another chromatographic technique, HPLC. On the basis of these chromatographic data, it can be concluded that the presently investigated bacterial EPSs are heteropolysaccharides comprising more than one type of monosaccharides or mixtures of homopolysaccharides each composed of one type of monosaccharides.

Table 9 Monosaccharide composition of the isolated bacterial polysaccharides

Bacterial sources of EPS	Monosaccharides constituent		
	Glucose	Fructose	Arabinose
Lactobacillus delbrueckii bulgaricus	3	–	1
Lactobacillus Helveticus	2	–	1
Lactobacillus Casei	10	1	1

EPS, exopolysaccharide.

Prebiotic activities of the partially purified exopolysaccharides produced by the chosen probiotics

It is well known that many polysaccharides (or their derivatives) from plant, animal and microbial sources possess biological activities such as anticoagulation, fibrinolytic [42], antitumor and prebiotic activities. This study focuses on prebiotic activities.

As previously noted, in Table 1, four probiotic strains seemed to be unable to produce EPSs; these included *L. reuteri*, *L. rhamnosus*, *L. acidophilus* and *B. bifidum*. Consequently, they were used to determine the prebiotic activities of the partially purified EPSs produced by the other three probiotics, *L. delbrueckii bulgaricus*, *L. helveticus* and *L. casei*. The prebiotic activity was expressed as the 'prebiotic index'. This index relates the growth intensities of the probiotics with that of the pathogen *E. coli*, as they were grown on the same EPS:

$$\text{Prebiotic index} = \frac{\text{Optical density of probiotic culture at 600 nm}}{\text{Optical density of } \textit{E.coli} \text{ culture at 600 nm}}$$

The results recorded in Table 10 indicate that the highest prebiotic indices (7.9–10.1) were exhibited by each of *L. acidophilus*, *L. rhamnosus*, *B. bifidum* and *L. reuteri* as they were grown on the partially purified EPSs produced by *L. delbrueckii bulgaricus*. In contrast, the lowest prebiotic indices (1.4–2.4) were shown by the aforementioned four probiotics, as they utilized the partially purified EPSs produced by *L. helveticus* or *L. casei*.

The present data collectively refer to EPSs produced by *L. delbrueckii bulgaricus* to be more susceptible to attack by the enzyme system of each of *L. acidophilus*,

Table 10 Prebiotic activities of the partially purified exopolysaccharides using various probiotics

Sources of EPS	Prebiotic indices using various probiotics			
	Bifidobacterium bifidum	*Lactobacillus reuteri*	*Lactobacillus rhamnosus*	*Lactobacillus acidophilus*
Lactobacillus delbrueckii bulgaricus	92	101	91	79
Lactobacillus helveticus	16	14	17	14
Lactobacillus casei	20	18	24	18

EPS, exopolysaccharide.

L. rhamnosus, *B. bifidum* and *L. reuteri* than that caused by *E. coli* attack. Thus, it can be concluded that EPSs produced by *L. delbrueckii bulgaricus* is a better prebiotic than those produced by *L. helveticus* or *L. casei*.

In a recent study, Hongpattarakere *et al.* [9] reported on in-vitro prebiotic evaluation of EPSs produced by marine isolated LAB. These included *L. plantarum*, *W. cibaria*, *W. confusa and P. pentosaceus*. EPSs produced by these strains were found to be utilized as carbon source. More recently Grosu-Tudor *et al.* [11] studied the prebiotic potential of some EPSs produced by LAB.

Conclusion

As a final comment on the present work, EPS models (produced under optimized conditions by three probiotics) exhibit variable levels of prebiotic activities. This offers valuable bases for future studies aiming to utilization of such native products as more safe and more effective food and drugs.

Acknowledgements

The authors thank National Research Centre for supporting this work.

Conflicts of interest

There are no conflicts of interest.

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