

## COMPARATIVE ASSESSMENT OF THE PREBIOTIC ACTIVITY OF SOME PECTIN POLYSACCHARIDES

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The isolation of pectinic polysaccharides from the aerial part of *Ferula kuhistanica* growing wild in Uzbekistan, *Malus sieversii* rosemary variety apples cultivated under local conditions (apple pomace after juice production was used), and tangerine peels (*Citrus reticulata*, tangerine variety) is reported. Their prebiotic activities for several probiotic cultures of *Bifidobacterium longum* 17x and *Propionibacterium avidum* 1 in addition to monocultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 906 and *Lactobacillus rhamnosus* 925ak were determined. According to the results, pectinic polysaccharide from *F. kuhistanica* was most promising in this respect.

**Keywords:** pectinic polysaccharides, *Ferula kuhistanica*, apple pomace, tangerine peels, prebiotic activity.

Prebiotics have recently been used for prevention and correction of disrupted microbiocenosis in the gastrointestinal tract and also for complex therapy of nonspecific ulcerative colitis [1, 2], irritable bowel syndrome [3], prevention of necrotizing enterocolitis [4], allergic reactions in children [5-7], and treatment of acute diarrhea [8]. They reach the large intestine intact and metabolize selectively its microflora by stimulating active growth of useful microorganisms such as lactobacilli and bifidobacteria. Edible plant fiber (polysaccharides, oligosaccharides, etc.) are used most often as prebiotics. Research and development on new effective prebiotics are constantly performed because these preparations are in high demand and indications for their use are expanding. Herein, the prebiotic potentials of pectinic polysaccharides isolated from the aerial part of *Ferula kuhistanica*, a broadly distributed wild plant of Uzbekistan; pomace of locally cultivated apples (*Malus sieversii*, Rosemary variety); and tangerine peels (*Citrus reticulata*, tangerine variety) were compared. The studies were carried out using local strains of probiotic cultures by estimating their vitality as

compared with the widely used prebiotic lactulose (on which the preparation Duphalac<sup>®</sup> is based).

### EXPERIMENTAL CHEMICAL PART

**Isolation of pectinic polysaccharides from leaves of *F. kuhistanica*.** Pectinic substances (PS) were extracted (2×) by a mixture of oxalic acid and ammonium oxalate solutions (0.5%, 1:1) at 70°C for 3 h [9] from leaves of *F. kuhistanica* (100 g) that were collected in Kashkadarya Oblast in Takhta-Karacha pass (Uzbekistan) and from which water-soluble polysaccharides had been extracted. The extracts were combined, dialyzed against distilled H<sub>2</sub>O, evaporated to a thick residue, and precipitated by EtOH in a 1:3 ratio. The precipitate was separated by centrifugation, dehydrated by Me<sub>2</sub>CO, and dried. The yield of PS from *F. kuhistanica* (PS-F) was 8.6 g.

**Isolation of pectinic polysaccharides from apple pomace.** Pectinic polysaccharides were extracted by the known method [10] that included hydrolysis and extraction (2×) of apple pomace (100 g) by HCl at raw-material – extractant ratios of 1:8 and 1:4 at 65 – 70°C and pH 2.0 for 1.5 – 2 h. The extracts were combined, dialyzed against tap water until neutral, evaporated, and precipitated by EtOH in

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**TABLE 1.** Physicochemical Characteristics of Pectinic Polysaccharides

Plant, organ	Yield, %	MM, Da	$\eta_{rel}$ (c 1%, H <sub>2</sub> O)	Titrimetric parameters, %		
				Kf	Ke	$\lambda$
<i>Ferula kuhistanica</i> (leaves)	8.6	25500	8.4	13.5	44.6	76.7
<i>Malus sieversii</i> (pomace)	5.47	45000	3.06	2.7	16.8	85.9
<i>Nitrus reticulata</i> (peels)	4.58	30000	14.0	3.96	10.44	72.5

**Note.** Kf and Ke are the amounts of free and esterified carboxylic acids;  $\lambda$ , degree of esterification.

a 1:3 ratio. The precipitate was separated by centrifugation, rinsed with EtOH of increasing concentration (80–96%), and dried. Yield of PS from apples (PS-A), 5.47 g.

**Isolation of pectinic polysaccharides from tangerine peels.** Ground tangerine peels (50 g) were extracted (2 $\times$ ) with HCl (600 mL, pH 2) for 1.5–2 h at 80–85°C [11]. The extracts were combined, evaporated, and precipitated by two volumes of EtOH. The precipitate of PS from tangerine peels (PS-T) was worked up by the aforementioned method. Yield of PS-T, 2.29 g.

All isolated pectinic polysaccharides were light-cream-colored amorphous powders that dissolved completely in H<sub>2</sub>O to form viscous solutions.

The molecular masses of the pectinic polysaccharides were determined by sedimentation on a MOM-3170 ultracentrifuge. Viscosity was measured on an Ostwald viscometer with capillary diameter 0.73 mm at 22°C. Table 1 shows that PS from tangerine peels had the greatest viscosity; from apple pomace, a high molecular mass.

The monosaccharide compositions of the pectinic polysaccharides (Table 2) were determined after complete acid

**TABLE 3.** Effect of Lactulose and the Studied Polysaccharides on Vitality of *Bifidobacterium longum* 17x + *Propionibacterium avidum* 1

No.	Experimental conditions	log CFU/mL	<i>p</i>	Effect, %	Total titratable acidity, °T
1	MRS broth with glucose (control)	8.89 ± 0.23	-	100	150
2	MRS broth + lactulose	10.68 ± 0.37	< 0.001	120.1	180
3	MRS broth + PS-F	10.56 ± 0.34	< 0.001	118.8	175
4	MRS broth + PS-A	9.86 ± 0.28	< 0.001	110.9	162
5	MRS broth + PS-T	10.64 ± 0.42	< 0.001	119.76	170

**TABLE 2.** Monosaccharide Composition of Pectinic Polysaccharides

Pectinic polysaccharide	Ratio of neutral monosaccharides, GC					Amount of GalA, %
	Rha	Xyl	Ara	Glc	Gal	
<i>Ferula kuhistanica</i> (leaves)	-	1.0	3.0	-	1.4	64.5
<i>Malus sieversii</i> (pomace)	1.0	1.1	3.5	2.3	1.2	70.3
Citrus reticulata (peels)	1.3	1.2	4.2	1.0	2.5	78.2

**Note:** Rha = rhamnose; Xyl, xylose; Ara, arabinose; Glc, glucose; Gal, galactose; GalA, galacturonic acid.

hydrolysis. Thus, samples (100 mg) were hydrolyzed by H<sub>2</sub>SO<sub>4</sub> (3 mL, 2 N) at 100°C for 24 h. The hydrolysates were worked up and analyzed using paper chromatography (PC, Filtrak FN 18) and BuOH–Py–H<sub>2</sub>O (6:4:3). Monosaccharides were detected by anilinium biphthalate.

GC analysis of aldonitrile acetates used a Chrom-5 chromatograph with a flame-ionization detector, stainless-steel column (200  $\times$  0.3 cm), Silicone XE-60 (5%) on Chromaton NAW-0.200-0.250 mm, 210°C, and N<sub>2</sub> carrier gas at flow rate 60 mL/min.

Pectinic polysaccharides from *F. kuhistanica* were dominated by the monosaccharide arabinose. Galactose and xylose were found in smaller quantities (2–3 times). An analogous ratio was typical of pectinic polysaccharides from apple pomace. However, the large amount of glucose was noteworthy. The monosaccharide ratios for PS-T, where arabinose and galactose were dominant, differed slightly. The degree of esterification was an important parameter of the pectinic polysaccharides. It was determined by titration [12] and reached 72.5–85.9%. The content of galacturonic acid was determined by the carbazole method [13] and varied in the range 64.5–78.2%. Therefore, the principal

**TABLE 4.** Effect of Lactulose and the Studied Polysaccharides on Vitality of *Lactobacillus delbrueckii* subsp. *bulgaricus* 906

No.	Experimental conditions	log CFU/mL	<i>p</i>	Effect, %	Total titratable acidity, °T
1.	MRS broth with glucose (control)	10.45 ± 0.48	-	100	160
2.	MRS broth + lactulose	12.07 ± 0.54	< 0.05	116	180
3.	MRS broth + PS-F	12.40 ± 0.62	< 0.05	118.7	170
4.	MRS broth + PS-A	12.03 ± 0.50	< 0.05	115.2	165
5.	MRS broth + PS-M	12.66 ± 0.70	< 0.05	121.2	190

**TABLE 5.** Effect of Lactulose and the Studied Polysaccharides on Vitality of *Lactobacillus rhamnosus* 925

No.	Experimental conditions	log CFU/mL	<i>p</i>	Effect, %	Total titratable acidity, °T
1	MRS broth with glucose (control)	10.33 ± 0.38	–	100	130
2	MRS broth + lactulose	11.7 ± 0.48	< 0.05	113.25	149
3	MRS broth + PS-F	11.77 ± 0.52	< 0.05	113.9	135
4	MRS broth + PS-A	10.95 ± 0.42	< 0.05	106.1	134
5	MRS broth + PS-T	11.45 ± 0.32	< 0.05	110.8	138

monosaccharides of the studied pectinic polysaccharides were galacturonic acid and the pentoside arabinose.

IR spectra were taken from pressed KBr pellets on a Perkin-Elmer Model 2000 FTIR spectrometer (100 scans). Absorption bands at  $832 \pm 2 \text{ cm}^{-1}$  were characteristic of pectins with  $\alpha$ -glycosidic bonds between D-galacturonic acid residues. Absorption bands at  $889 \pm 2 \text{ cm}^{-1}$  were characteristic of 1,4-glycoside bonds;  $950 \pm 1$ , methyl bending vibrations;  $1150 \pm 2$ , esterified carboxylic acids. An absorption band at  $1370 \pm 3 \text{ cm}^{-1}$  was characteristic of a methyl ester;  $1630 \pm 3$  and  $1740 \pm 2$ , stretching vibrations of carboxylic acid carbonyl, i.e., an ester carbonyl band. All aforementioned absorption bands had different intensities [14].

The IR spectroscopic data agreed with the titration results and indicated that the studied pectinic polysaccharides were esterified at the carboxylic acid by methyls.

Thus, highly esterified pectinic polysaccharides that had different molecular masses and gave aqueous solutions of high relative viscosity were isolated. The main chain of the pectinic polysaccharides consisted of  $\alpha$ -1,4-bonded D-galacturonic acid residues with neutral monosaccharides forming side branches.

## EXPERIMENTAL BIOLOGICAL PART

The research used associations of local strains of bifidobacteria, i.e., *Bifidobacterium longum* 17x and *Propionibacterium avidum* 1 in addition to monocultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* 906 and *Lactobacillus rhamnosus* 925ak.

Prebiotic lactulose and the studied PS-F, PS-A, or PS-T were added to a final concentration of 0.25% to sterile MRS broth (Hi-Media).

The inoculate (1 mL) obtained from growth of *B. longum* 17x and *P. avidum* 1; *L. delbrueckii* subsp. *bulgaricus* 906; and *L. rhamnosus* 925ak in MRS broth for 24 h at 38°C was placed into growth medium (9.0 mL) in a tube. Bifidobacteria and *L. rhamnosus* were grown at  $38 \pm 1^\circ\text{C}$ ; *L. delbrueckii* subsp. *bulgaricus*, at 43°C in an anaerobic  $\text{N}_2$  atmosphere for 24 h. The number of living cells per mL of growth medium was calculated (log CFU/mL). Experiments were carried out with five replications. Results were processed statistically using the Student *t*-criterion.

## RESULTS AND DISCUSSION

The results showed that potential prebiotics were actively utilized when placed into growth medium and that the number of living cells increased.

A comparison of culture growth on balanced standard MRS medium and in media with prebiotic additives found that the last provided a high titer of living cells, i.e., from 10.64 to 12.66 log CFU/mL (Tables 3 – 5). The stimulating effect of all studied compounds depended on the genus, species, and strain of microorganism. The results indicated that lactulose at a concentration of 0.25% increased the growth of bifido- and propionibacteria by 20%. Growth was stimulated about the same by adding pectins from *F. kuhistanica* and tangerine peels (18-19%) and much less by adding pectin from apple pomace. PS were the most effective for *L. del-*

**TABLE 6.** Effect of Lactose and the Studied Polysaccharides on the Number of Living Immobilized Cells

Preparation	Initial cell titer before immobilization, log CFU/mL	Titer of immobilized cells in recovered preparation (log CFU/mL) as a function of sorbent concentration, %		
		0.5	1.0	1.5
			Apple pectin	
Bifidumbacterin PL	10.6 ± 0.56	8.65 ± 0.50*	9.8 ± 0.20	9.8 ± 0.20
Lactobacterin Orom	10.12 ± 0.61	8.25 ± 0.40*	8.95 ± 0.20	8.65 ± 0.15*
			Tangerine pectin	
Bifidumbacterin PL	10.6 ± 0.56	10.6 ± 0.79	10.8 ± 0.28	9.2 ± 0.70
Lactobacterin Orom	10.12 ± 0.61	9.9 ± 0.44	9.98 ± 0.80	9.5 ± 0.52

\* Statistically significant vs. initial titer ( $p < 0.05$ ).

*brueckii* subsp. *bulgaricus* 906 of all studied cultures (Table 4).

In this instance, the living cell titers after adding lactulose, PS-F, PS-A, and PS-T were 12.03-12.66 log CFU/mL. Addition of PS-T had the greatest stimulating effect (growth increased by 21.24%). The high number of living cells corresponded to high acid formation, i.e., 190°T.

The increase in the number of living cells in the growth medium was slightly less for *L. rhamnosus* 925ak with these same substances. Nevertheless, the titer of living cells increased by 13% after adding lactulose and PS-F. This culture typically developed little acid consisting from 130 to 149°T (Table 5).

Active utilization of lactulose and the studied PS by the microorganisms was closely related to increased acid formation in the growth medium. Thus, the total titratable acidity for growth on standard MRS broth was 130 – 160°T. It was significantly greater at 134 – 190°T with the additives.

Exceedingly significant results were also obtained in experiments on pectins as sorbents for preparing probiotic preparations (using apple and tangerine pectins as examples). The immobilization methods were described before [15]. Table 6 presents results from studies of the influence of various concentrations of these pectins on the number of living microorganisms in lyophilized preparations manufactured by OROM – BIOPREPARAT GAK of the pharmaceutical industry of the Republic of Uzbekistan, i.e., Bifidumbacterin PL and Lactobacterin Orom, the microbiological compositions of which were reported [16, 17]. Both tested pectins exhibited under these conditions a certain absorption capacity for probiotic cultures. However, it is noteworthy that PS-T had a higher content of living immobilized cells.

PS-A absorbed significantly fewer probiotic-culture living cells than PS-T. This could possibly be explained by the physicochemical features of these pectins.

PS-A absorbed significantly fewer living probiotic cells than PS-T. This was possibly explained by the physicochemical properties of these pectins.

Electron-microscopy studies of immobilized biomass showed that *B. longum* cells formed a thick coating on carrier particles and were coccoid cells that tended to aggregate.

Immobilization did not affect the metabolic activity and stability of manufactured batches of Bifidumbacterin PL and Lactobacterin Orom during prolonged storage for >3 yr.

Thus, pectinic polysaccharides isolated from leaves of *F. kuhistanica*, pomace of *M. sieversii*, and peels of *Citrus reticulata* were highly esterified polysaccharides that gave

aqueous solutions of relatively high viscosity and molecular mass (25,500 – 45,000 Da). The main chain of the studied pectins consisted of  $\alpha$ -1,4-bonded D-galacturonic acid residues with side branches of neutral monosaccharides. They all exhibited a certain degree of prebiotic activity that was comparable with that of lactulose (Duphalac® preparation). PS from leaves of *F. kuhistanica* had the most pronounced effect on the vitality of *B. longum* and *P. avidum* 1 culture and monocultures of *L. delbrueckii* subsp. *bulgaricus* and *L. rhamnosus*. The results as a whole indicated that PS isolated from leaves of *F. kuhistanica* were of greatest interest for formulating a new effective prebiotic agent.

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