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Original Article

Evaluation of the prebiotic effects of citrus pectin hydrolysate

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ABSTRACT

Citrus pectin enzyme hydrolysate (PEH) of different hydrolysis time intervals (6 hours, PEH-6; 12 hours, PEH-12; 24 hours, PEH-24; or 48 hours, PEH-48) or concentrations (1%, 2%, and 4%) was tested for its growth stimulation effect on two probiotics, *Bifidobacterium bifidum* and *Lactobacillus acidophilus*. Higher monosaccharide concentrations and smaller molecular weights of PEHs were obtained by prolonging the hydrolysis time. In addition, higher PEH concentrations resulted in significantly higher ($p < 0.05$) probiotic populations, pH reduction, and increase in total titratable acidity than the glucose-free MRS negative control. Furthermore, significantly higher populations in the low pH environment and longer survival time in nonfat milk ($p < 0.05$) were observed when the two probiotics were incubated in media supplemented with 2% PEH-24, than in glucose and the negative control. In comparison with other prebiotics, addition of 2% PEH-24 resulted in a more significant increase in the probiotic population ($p < 0.05$) than in the commercial prebiotics. This study demonstrated that PEH derived from citrus pectin could be an effective prebiotic to enhance the growth, fermentation, acid tolerance, and survival in nonfat milk for the tested probiotics.

1. Introduction

Since the 1990s, there has been an increase in the demand for healthy foods. Among them, both probiotics and prebiotics are popular items. Prebiotic is defined as: "the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbes that confer(s) health benefits to the host" [1]. The effects of prebiotics can be evaluated on the basis of the growth of probiotic bacteria such as lactobacilli and bifidobacteria, the decrease in intestinal pathogens, and the changes in production of health-related bacterial metabolites. Several studies have shown that probiotics and prebiotics are effective in reducing some

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specific pathogens and physiologic dysfunctions, such as enhancing the resistance to infection [2], enhancement of immune status [3], antitumorigenic effects [4], prevention of diarrhea [5], cholesterol reduction [6], protection against allergic diseases [7], and reducing lactose intolerance [8].

Pectin is considered a soluble dietary fiber and exerts physiological effects on the gastrointestinal tract, such as reducing glucose absorption [9], enhancing hypocholesterolemia effect [10], and delaying gastric emptying [11]. In addition, the oligosaccharides obtained from pectin have been proposed as an excellent candidate for new-generation prebiotics [12]. Hotchkiss et al [12] reported that the fermentation products of Valencia orange peel demonstrated bifidogenic effects, and the concentrations of some short-chain organic acids such as acetate, butyrate, and propionate, increased upon fermentation. The prebiotic potential of oligosaccharides obtained by enzymatic hydrolysis of bergamot peel was also demonstrated [13]. In addition, the oligosaccharide was found to offer protection against pathogenic *Escherichia coli* [14] and to have the ability to inhibit the invasion of Caco-2 cells from *Campylobacter* [15]. Furthermore, the protection of colonocytes against *E. coli* verotoxins and the stimulation of apoptosis in human colonic adenocarcinoma cells has been reported [16].

Huang et al [17] reported that the pectin enzyme hydrolysate (PEH) prepared from citrus pectin which was hydrolyzed by a commercial enzyme showed antitumor activity and enhanced membrane permeability of human cancer cells. The PEH contained a certain amount of oligosaccharides which were assumed to be the main cause for those biological functions. However, the use of PEH as a prebiotic was not determined. Thus, the objectives of this research were to hydrolyze citrus pectin at different time intervals and determine the prebiotic effects of the PEHs, including the stimulation of growth and fermentation of probiotics, and enhancement of survival abilities in low pH environments and a food system. These prebiotic effects were evaluated by two commonly used probiotics, *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. In addition, the prebiotic function of PEH was compared with commonly used commercial prebiotics.

2. Methods

2.1. Tested probiotic bacteria

B. bifidum DMS20082 and *L. acidophilus* DMS20079 were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). All media and ingredients were purchased from Difco Laboratories (Detroit, MI, USA). These cultures were maintained at −80°C and stored in GermBank (Creative Microbiologicals, Taipei, Taiwan). Bacteria were subcultured in MRS broth at 37°C anaerobically for 48 h twice before the experiments. L-cysteine HCl (0.05%) was added into MRS medium for the incubation of *B. bifidum*.

2.2. Preparation of the citrus PEH

Citrus pectin with a 60% degree of esterification (Nacalai Tesque, Kyoto, Japan) was treated with a commercial pectin enzyme, Peclyve CP (CPE), according to the modified method described by Huang et al [18]. This CPE (Lallemand Australia Pty. Ltd., North Adelaide, Australia) was produced by *Aspergillus niger* and contained 51.2 U/mL pectin methyl esterase and 22.4 U/mL polygalacturonase. The citrus pectin (1% w/v) was hydrolyzed by CPE (0.1% w/v) at pH 4 and 45°C, then heated in a boiling water for 10 minutes to stop the reaction. The hydrolysis time intervals were 6 hours, 12 hours, 24 hours, or 48 hours, respectively. After cooling down to room temperature, the reaction mixture was centrifuged at 5000*g* for 15 minutes to precipitate the nondigested pectin and the supernatant was collected. The pH value of the supernatant was adjusted to 6.5 by 0.01N NaOH, then sterile filtrated (0.22 μm), freeze-dried, and stored at −20°C. PEHs was collected and marked as PEH-6, PEH-12, PEH-24, or PEH-48 based on their hydrolysis intervals and sterile deionized water was added before testing.

2.3. Determination of the molecular weight of PEH

The molecular weight of pectin or PEH was determined by a high performance size-exclusion chromatography method. The rehydrated solution was filtered through a 0.22 μm membrane and a PD-10 desalting column (GE Healthcare, Piscataway, NJ, USA). A sample solution 20 μL was injected into a TSK-Gel G5000 PWXL column (Tosoh, Tokyo, Japan). Mobile phase was water, flow rate was 0.6 mL/min, and column temperature was 40°C. A refractive index detector (Hitachi L-2490, Hitachi, Tokyo, Japan) was used. This system was calibrated with six dextran standards with particular molecular weights at 1 kDa, 50 kDa, 150 kDa, 270 kDa, 410 kDa, or 750 kDa. The molecular weights of PEHs were determined by comparing the sample retention time with the standard curve of molecular weight and retention of the six standards.

2.4. Determination of sugar content

The monosaccharide profiles of PEH samples were determined according to the methods described by Englyst et al [19] and allose was used as an internal standard. The PEH sample (pH = 6.5) was filtered through a 0.45 μm membrane, acetylated, then analyzed with a gas chromatograph (Hitachi G-5000, Tokyo, Japan). A flame ionization detector was used and the analysis parameters were as follows: capillary column, Quadrex 007–225 (15 m × 0.53 mm i.d.; Quadrex Corporation, Woodbridge, CT, USA); oven temperature, initially held at 100°C for 3 minutes and then raised to 220°C at a rate of 3°C/min; injector and detector temperatures, 270°C; gas flow rates, 2.1 mL/min (carrier gas: nitrogen) and 500 mL/min (air and hydrogen).

2.5. The growth effect of PEH on the probiotic bacteria

After incubation, the culture of tested probiotics were centrifuged and resuspended in phosphate buffered saline (PBS, pH 7.4) to 1.0 optical density at 600 nm (OD_{600}) that equaled to 10^9 CFU/mL based on the preliminary study. Two bacterial populations, 10^4 CFU/mL and 10^6 CFU/mL, were tested and the population change was more significant in the media inoculated at 10^4 CFU/mL than at 10^6 CFU/mL. Thus, later

experiments were all conducted in the inoculating population of 10^4 CFU/mL. All ingredients of the glucose-free MRS broth were prepared in the laboratory and used as the base medium. The glucose-free MRS broth supplemented with 1%, 2%, or 4% (w/v) of PEH-6, PEH-12, PEH-24, or PEH-48, was then inoculated at 10^4 CFU/mL with *L. acidophilus* or *B. bifidum*. The glucose-free MRS broth with or without the addition of 2% glucose was used as the positive and negative controls, respectively. After incubation at 37°C in a 20-mL glass tube for 24 hours or 48 hours inside an anaerobic jar which contained Anaero Pack (MGC, Tokyo, Japan), the media were decimally serial diluted and spread on commercial MRS agar plates. The probiotic populations were enumerated based on the colony number on the commercial MRS agar plates after being incubated at 37°C for 48 hours anaerobically. L-cysteine-HCl was added (0.05%) to the MRS broth and agar for *B. bifidum*, but not for *L. acidophilus*.

2.6. Total titrate acidity and pH values

Total titrate acidity (TTA) and the pH values were also measured as indicators of the fermentation of the inoculated probiotics. TTA and pH values were determined according to the Association of Official Analytical Chemists' methods [20]. TTA was determined by titration with 0.01N NaOH solution and expressed as percent lactic acid. The pH values were measured using a pH meter (SP-2100, Suntex Co. Ltd., Taipei, Taiwan).

2.7. Acidity tolerance of prebiotics

Testing of acidity tolerance was modified based on the study by Lin [21]. A 100-μL of bacterial culture at 10^7 CFU/mL was inoculated into 20 mL of the glucose-free MRS broth containing 2% PEH, the negative or positive control medium. After incubation for 24 hours, 1 mL of the probiotic culture was added into 9 mL of PBS, adjusted to pH 2.0, pH 2.5, or pH 3.2 with 0.1N HCl. PBS with the adjusted pH value of 7.0 was used as the control. The mixture of bacterial culture and PBS was incubated at 37°C with 80 rpm shaking. After 3 hours, 1 mL of the mixture was decimally diluted and spread onto commercial MRS agar plates. Bacterial population was enumerated after anaerobic incubation at 37°C for 48 hours.

2.8. Survivability of prebiotics in nonfat milk

A 100-μL of bacterial culture at 10^8 CFU/mL was inoculated in 20 mL of nonfat milk (negative control), or nonfat milk containing 2% PEH, or 2% glucose (positive control). The milk samples were stored at 4°C for 2 days, 4 days, 6 days, 8 days, 10 days, and 15 days. On the day of sampling, 1 mL of the nonfat milk was decimally diluted and spread onto commercial MRS agar plates. Bacterial population was enumerated after anaerobic incubation at 37°C for 48 hours.

2.9. Comparison of PEH and commercial prebiotics for the growth of probiotic bacteria

Although the glucose-free MRS broth containing 4% (w/v) PEH-24 showed the greatest growth of probiotic bacteria, the growth of *B. bifidum* showed no significant difference between 2% and 4% PEH. In addition, 2% of commercial prebiotics were used in a previous study [22]. Thus, the glucose-free MRS broth was supplemented with 2% (w/v) PEH-24 or commercial prebiotics, inulin (Johnson Matthey Co., Alfa Aesar, MA, USA) or raffinose (Sigma–Aldrich, St. Louis, MO, USA), then inoculated with the probiotic bacteria. To simulate the high-temperature treatment during food processing, PEH was treated at 121°C for 15 minutes [heated pectin enzyme of 24-hour hydrolysate (HPEH-24)] and added into the glucose-free MRS broth at 2% (w/v). The MRS broths were inoculated with *L. acidophilus* or *B. bifidum* at the level of the 10^4 CFU/mL. After incubation at 37°C for 24 hours or 48 hours in anaerobic jars with Anaero Pack (MCG), the probiotic bacterial populations were enumerated on commercial MRS agar after incubation at 37°C for 48 hours anaerobically. MRS broths without glucose or with 2% glucose were used as controls. The pH values of the broths were also measured as an indicator of the fermentation of the inoculated probiotic bacteria.

2.10. Statistical analysis

All experiments were conducted at least twice and triplicate samples were used for each test. Data were collected and analyzed by using one-way analysis of variance and Duncan's test. The significant differences between tests were set at $p < 0.05$. All statistical analyses were performed using SPSS (version 12.0; SPSS Inc., St. Armonk, NY, USA).

3. Results and discussion

3.1. Sugar composition and molecular weight distribution

Monosaccharide contents of PEH-6, PEH-12, PEH-24, and PEH-48 determined by gas chromatography are shown in Table 1. Among them, glucose was the most abundant monosaccharide in all PEHs, followed by mannose, galactose, and arabinose. The results of different hydrolysis time intervals revealed monosaccharide concentrations increased through the hydrolysis time. The concentrations of monosaccharide in PEHs were ranked as PEH-48 > PEH-24 > PEH-12 > PEH-6, which ranged from 29.87% at 6 hours to 71.81% at 48 hours. In

Table 1 – Molecular weight (kDa) and sugar content (%) of pectin enzyme hydrolysate.

	PEH-6	PEH-12	PEH-24	PEH-48
Molecular weight	1.80	1.71	1.00	< 1.00
Sugar content [a]				
Rhamnose	Tr	Tr	Tr	Tr
Fucose	Tr	Tr	Tr	Tr
Arabinose	0.93	1.92	2.39	2.93
Xylose	Tr	Tr	Tr	Tr
Mannose	4.92	7.51	9.12	12.64
Galactose	3.77	4.11	4.27	5.15
Glucose	20.25	23.83	35.21	51.09

PEH = pectin enzyme hydrolysate; Tr = trace amount (< 0.01).
[a] Expressed as g/100 g dry weight.

addition, longer hydrolysis time lowered the average molecular weights of PEHs. The molecular weight of unhydrolyzed pectin was 353 kDa [17,18] and the molecular weights of PEH-6 and PEH-48 were 1.80 kDa and < 1 kDa, respectively (Table 1). This reduction of molecular weight could be caused by the higher proportion of monosaccharides and smaller oligosaccharides generated during hydrolysis. Since the molecular weight of a glucose unit is 180, PEH-24 with molecular weight at 1 kDa was equal to 5–6 glucose moieties. Considering the proportion of monosaccharide (50.99%), PEH-24 should be a mixture of mono- and oligosaccharides containing 5–10 sugar moieties.

3.2. *The growth effects of PEH on* L. acidophilus *and* B. bifidum *in vitro*

Growth of *L. acidophilus* and *B. bifidum* in the glucose-free MRS media containing PEHs of different hydrolysis time intervals (PEH-6, PEH-12, PEH-24, or PEH-48) at different concentrations [1%, 2%, or 4% (w/v)] are shown in Table 2. There was no significant difference ($p > 0.05$) between the media containing PEHs of different hydrolysis time intervals but significant difference ($p < 0.05$) was found among the media containing different concentrations of PEHs. Both *L. acidophilus* and *B. bifidum* were found to have highest growth in the media containing 4% PEH, followed by 2% and 1% (w/v). Compared with the control glucose-free MRS, even the media containing only 1% PEH showed significantly greater growth ($p < 0.05$) of the two probiotic bacteria than the control. Both incubation times (24 hours and 48 hours) showed the same growth trend for both probiotic bacteria. However, probiotic counts were lower after 48 hours than 24 hours, particularly for *B. bifidum*. The reductions of probiotic counts from 24 hours to 48 hours were lower in the media containing PEH than controls, particularly in the media containing higher concentrations of PEH. These results indicate that the probiotics might exhaust nutrients from the media after 24 hours incubation and cell death becomes greater than multiplication. In addition, higher PEH concentrations offered some protection to retard the cell death.

Most strains of *L. acidophilus* could ferment mono- and disaccharides such as amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, and trehalose [23]. Olano-Martin et al [24] reported that the oligosaccharide from low methylated apple pectin also promoted the growth of lactobacilli. In that study, lactobacilli showed higher growth rates in the media using low methylated apple pectin as a carbon source. Species of *Bifidobacterium*, with the assistance of intracellular enzymes, could degrade polysaccharides into monosaccharides such as glucose and fructose phosphates and used them as a nutrition source [23]. In this study, both *L. acidophilus* and *B. bifidum* could use PEH as a carbon source. However, the growth stimulation of PEH on these two probiotics was not exactly the same. The growth rate of *L. acidophilus* was slower than that of *B. bifidum* at 24 hours incubation but populations were higher at 48 hours. The faster growth of *B. bifidum* at 24 hours incubation than *L. acidophilus* could be a result of the activities of the intracellular enzymes described above. However, this fast growth could cause *B. bifidum* to exhaust the nutrients in the first 24 hours

Table 2 – The populations (log CFU/mL) of *Lactobacillus acidophilus* or *Bifidobacterium bifidum* inoculated at 4 log CFU/mL in the glucose-free medium with different hydrolysis times or concentrations of PEH after 24 h or 48 h incubation time.

Incubation time	Supplements	*L. acidophilus**				*B. bifidum**			
		PEH-6	PEH-12	PEH-24	PEH-48	PEH-6	PEH-12	PEH-24	PEH-48
24 h	1% PEH	7.67 ± 0.15^{aC}	7.48 ± 0.19^{aC}	7.69 ± 0.19^{aC}	7.43 ± 0.23^{aC}	8.72 ± 0.19^{aB}	8.54 ± 0.18^{aC}	8.68 ± 0.16^{aC}	8.51 ± 0.08^{aC}
	2% PEH	7.83 ± 0.14^{aB}	7.71 ± 0.13^{aB}	7.79 ± 0.15^{aB}	7.83 ± 0.05^{aB}	8.83 ± 0.12^{aB}	8.79 ± 0.15^{aB}	9.01 ± 0.13^{aA}	8.99 ± 0.12^{aA}
	4% PEH	8.23 ± 0.24^{aB}	8.19 ± 0.17^{aB}	8.27 ± 0.21^{aA}	8.33 ± 0.12^{aA}	9.02 ± 0.28^{aA}	8.95 ± 0.14^{aA}	8.88 ± 0.15^{aA}	9.0 ± 0.22^{aA}
	Control[a]	6.64 ± 0.27^{D}				6.55 ± 0.28^{D}			
	2% glucose	8.49 ± 0.23^{A}				8.93 ± 0.23^{A}			
48 h	1% PEH	4.41 ± 0.25^{aC}	4.56 ± 0.16^{aC}	4.54 ± 0.14^{aC}	4.50 ± 0.27^{aC}	5.15 ± 0.08^{aC}	4.90 ± 0.24^{bD}	4.75 ± 0.14^{bD}	4.77 ± 0.15^{bD}
	2% PEH	5.57 ± 0.18^{aB}	5.39 ± 0.25^{aB}	5.41 ± 0.13^{aB}	5.38 ± 0.19^{aB}	5.42 ± 0.24^{aC}	5.23 ± 0.17^{cC}	5.39 ± 0.21^{abC}	5.16 ± 0.14^{cC}
	4% PEH	7.56 ± 0.18^{aA}	7.35 ± 0.21^{bA}	7.53 ± 0.16^{aA}	7.34 ± 0.24^{aA}	6.69 ± 0.17^{abB}	6.81 ± 0.20^{aB}	6.85 ± 0.16^{aB}	6.35 ± 0.11^{bB}
	Control[a]	4.05 ± 0.22^{D}				4.16 ± 0.22^{E}			
	2% glucose	7.38 ± 0.21^{A}				7.05 ± 0.18^{A}			

[a]Control was a glucose-free medium.
[b]Average ± standard deviation with different small case letters in the same row are significantly different ($p < 0.05$*) and average ± standard deviation with different capital letters are significantly different ($p < 0.05$*) between bacterial populations within the same incubation time.

and substantially lower its population after 48 hours incubation.

Since the growth-enhancing effect of PEHs was not significantly different from different hydrolysis time intervals and PEH-24 was the most convenient to collect, PEH-24 was used for the following tests, including comparing with commercial prebiotics, acid tolerance, and survival in a food system.

3.3. *Effects of PEH on TTA and medium pH during incubation of* L. acidophilus *and* B. bifidum

A greater decrease in pH and increase in TTA were obtained when higher concentrations or longer hydrolysis intervals of PEH were added (Table 3). The lowest pH and the highest TTA were found in the media containing 4% PEH-48, while the negative control media showed a neutral pH and almost undetectable TTA. The higher PEH concentrations caused significantly lower pH values and higher TTA ($p < 0.05$). Lower pH values and higher TTA were observed at 48 hours incubation time for both probiotic bacteria. This indicates that greater fermentation of *L. acidophilus* and *B. bifidum* occurred in the media containing higher PEH concentrations and longer hydrolysis intervals, particularly for the hydrolysis intervals longer than 12 hours.

Although the glucose concentration was higher in the PEHs obtained from the longer hydrolysis time intervals, both pH values and TTA were not significantly different within the group of PEH-6 and PEH-12, or within the group of PEH-12 and PEH-24, but a significant difference was revealed between these two groups. In addition, PEHs obtained from different hydrolysis time intervals at the same concentration showed no significant difference ($p > 0.05$) for the populations of *L. acidophilus* and *B. bifidum*. However, significantly lower pH values and higher TTA were obtained from the longer hydrolysis intervals. These results indicated that the smaller molecular weight of the PEHs obtained from the longer hydrolysis time may not be sufficient to generate higher growth but enough for more active fermentation.

Lower pH values and higher TTA were found in the media inoculated with *L. acidophilus* than the media inoculated with *B. bifidum* in our study. These results were similar to a previous study [22], which also explained that *L. acidophilus* produced significantly higher amounts of lactic acid than *B. bifidum*. These results indicated *L. acidophilus* performed greater PEH fermentation than *B. bifidum* and could contribute to the higher populations of *L. acidophilus* than *B. bifidum* at 48 hours incubation. Therefore, the higher populations of *B. bifidum* at 24 hours incubation could result from the higher enzymatic ability of *B. bifidum* to hydrolyze oligosaccharides into monosaccharides and the greater ability to utilize monosaccharides [22].

Previous studies reported that pectin-derived oligosaccharides had prebiotic activities and stimulated the growth of *Bifidobacterium* [24,25]. Another study [26] reported that extracted pectin oligosaccharide of apple pomace enhanced the growth of *Bifidobacterium* and increased the concentration of short-chain fatty acids in human feces. Our study showed similar results, that PEH enhanced the growths and fermentation of *L. acidophilus* and *B. bifidum*.

In the comparison test with the commercial prebiotics, the lowest pH values were found in the media containing 2% glucose or 2% PEH-24 (Table 4). In addition, media containing PEH or HPEH had a lower pH than those containing inulin and raffinose. These results suggest stronger fermentation occurred in the media containing PEH or HEPH than inulin or raffinose.

3.4. *Effects of PEH on acidity tolerance and survival ability of the tested probiotics*

When pH was reduced to 3.2, populations of the tested probiotics did not decrease significantly ($p > 0.05$) for all treatments (Table 5). However, when pH was reduced to 2.5, the probiotic in the negative control did not survive and the probiotic populations in the media supplemented with PEH were significantly higher ($p < 0.05$) than those supplemented with glucose. When pH was reduced to 2.0, only the probiotics in the medium supplemented with PEH survived.

In nonfat milk, there was no obvious decrease in probiotic populations in the first 6 days of storage (Table 6). Probiotic populations began to decrease on Day 10 and Day 15 for the negative control and the nonfat milk added with glucose, respectively. In addition, probiotic populations continued decreasing in the negative control and glucose groups but maintained at the same level throughout storage for up to 20 days in the media containing PEH. These results clearly show PEH possesses an ability to assist the tested probiotics in acid tolerance and survival in a food system, both of which are important characteristics for an effective prebiotic [23,27,28]. In addition, the tested probiotics showed significantly higher acid tolerance and survival populations in the media supplemented with PEH than glucose. This indicated that PEH should contain some oligosaccharides which assisted the probiotics in acid tolerance and survival ability, while glucose did not.

3.5. *Effects of different prebiotics on the growth of* L. acidophilus *and* B. bifidum

Bacterial populations were significantly higher ($p < 0.05$) in the media containing PEH, HPEH, or commercial prebiotics than the negative control glucose-free MRS (Figure 1). Comparing two commercial prebiotics with PEH, the media containing 2% PEH-24 showed significantly higher counts ($p < 0.05$) of *L. acidophilus* than the ones containing inulin [degree of polymerization (DP) 12] or raffinose (DP 3) at 24 hours incubation (Figure 1A). However, the media containing 2% inulin or raffinose showed higher counts of *L. acidophilus* than the ones containing PEH-24 at 48 hours incubation. For the media containing HPEH-24, *L. acidophilus* population was significantly lower than PEH-24 at 24 hours but not at 48 hours.

Similar results were obtained for the growth of *B. bifidum* (Figure 1B). At 24 hours incubation, the *B. bifidum* counts were significantly higher ($p < 0.05$) in the media containing the commercial prebiotics or PEH than the negative control. However, the highest count was obtained in the medium containing HPEH-24 (9.09 log CFU/mL) which was significantly higher ($p < 0.05$) than the media containing raffinose (7.99 log CFU/mL) or inulin (7.79 log CFU/mL). At 48 hours incubation, the media containing PEH-24 had a significantly higher count

Table 3 – The pH values and titratable acidity of the glucose-free medium with different hydrolysis times or concentrations of PEH and incubation of *Lactobacillus acidophilus* or *Bifidobacterium bifidum* inoculated at 4 log CFU/ml for 24 hours or 48 hours.

Incubation time	*L. acidophilus**							
	pH values				Titratable acidity			
	Supplements	PEH-6	PEH-12	PEH-24	PEH-48	PEH-6	PEH-12	PEH-24	PEH-48
24 h	1% PEH	5.03 ± 0.05^{cC}	4.90 ± 0.05^{bC}	4.74 ± 0.04^{aB}	4.78 ± 0.03^{aB}	0.72 ± 0.07^{bD}	$0.76 \pm 0.0.7^{bD}$	1.09 ± 0.09^{aBC}	0.99 ± 0.04^{aC}
	2% PEH	4.81 ± 0.01^{cB}	4.78 ± 0.14^{cB}	4.65 ± 0.06^{bA}	4.61 ± 0.01^{aA}	0.92 ± 0.04^{bC}	1.04 ± 0.14^{bBC}	1.26 ± 0.05^{aB}	1.35 ± 0.04^{aB}
	4% PEH	4.63 ± 0.02^{bA}	4.67 ± 0.05^{bA}	4.49 ± 0.20^{aA}	4.51 ± 0.02^{aA}	2.06 ± 0.06^{bA}	2.03 ± 0.04^{bA}	2.13 ± 0.05^{aA}	2.12 ± 0.04^{aA}
	Control	6.30 ± 0.03^{D}				0.06 ± 0.02^{E}			
	2% glucose	4.53 ± 0.11^{A}				2.08 ± 0.09^{A}			
48 h	1% PEH	4.81 ± 0.01^{cC}	4.84 ± 0.04^{cC}	4.65 ± 0.01^{aC}	4.77 ± 0.02^{bC}	1.04 ± 0.04^{cC}	1.00 ± 0.04^{cC}	1.24 ± 0.02^{aC}	1.10 ± 0.01^{bC}
	2% PEH	4.39 ± 0.04^{bB}	4.33 ± 0.02^{aA}	4.33 ± 0.02^{aA}	4.32 ± 0.01^{aA}	1.40 ± 0.15^{bBC}	1.97 ± 0.02^{aB}	1.95 ± 0.06^{aB}	1.99 ± 0.04^{aB}
	4% PEH	4.30 ± 0.02^{aA}	4.31 ± 0.03^{bA}	4.31 ± 0.03^{bA}	4.28 ± 0.02^{aA}	2.16 ± 0.15^{aA}	2.10 ± 0.12^{aA}	2.14 ± 0.02^{aA}	2.23 ± 0.17^{aA}
	Control	6.28 ± 0.04^{D}				—			
	2% glucose	4.29 ± 0.04^{A}				2.20 ± 0.07^{A}			
Incubation time	*B. bifidum**							
	pH values				Titratable acidity			
	Supplements	PEH-6	PEH-12	PEH-24	PEH-48	PEH-6	PEH-12	PEH-24	PEH-48
24 h	1% PEH	5.53 ± 0.13^{bD}	5.34 ± 0.05^{bD}	5.04 ± 0.04^{aC}	5.04 ± 0.03^{aC}	0.53 ± 0.09^{cE}	$0.68 \pm 0.0.2^{bE}$	0.95 ± 0.01^{aD}	0.93 ± 0.01^{aD}
	2% PEH	5.05 ± 0.01^{cC}	4.89 ± 0.07^{bB}	4.68 ± 0.02^{aA}	4.68 ± 0.05^{aA}	0.92 ± 0.04^{bD}	1.14 ± 0.02^{aC}	1.62 ± 0.05^{aB}	1.60 ± 0.06^{aB}
	4% PEH	4.79 ± 0.06^{cB}	4.75 ± 0.01^{cB}	4.63 ± 0.08^{bA}	4.55 ± 0.03^{aA}	1.63 ± 0.06^{cB}	1.70 ± 0.07^{cB}	1.85 ± 0.05^{bA}	2.15 ± 0.04^{aA}
	Control	6.34 ± 0.03^{E}				0.06 ± 0.02^{F}			
	2% glucose	4.59 ± 0.04^{A}				2.09 ± 0.09^{A}			
48 h	1% PEH	5.45 ± 0.05^{bD}	5.29 ± 0.04^{bD}	5.00 ± 0.01^{aC}	5.00 ± 0.02^{aC}	0.54 ± 0.06^{cD}	0.69 ± 0.04^{bD}	1.09 ± 0.06^{aC}	1.07 ± 0.02^{aC}
	2% PEH	5.05 ± 0.01^{cC}	4.85 ± 0.02^{bC}	4.65 ± 0.02^{aB}	4.68 ± 0.01^{aB}	0.96 ± 0.06^{cC}	1.17 ± 0.06^{bC}	1.66 ± 0.09^{aB}	1.62 ± 0.03^{aB}
	4% PEH	4.72 ± 0.02^{bB}	4.70 ± 0.02^{bB}	4.50 ± 0.03^{aA}	4.45 ± 0.02^{aA}	1.71 ± 0.15^{bB}	1.75 ± 0.02^{bB}	2.14 ± 0.02^{aA}	2.25 ± 0.05^{aA}
	Control	6.30 ± 0.04^{E}				0.08 ± 0.04^{E}			
	2% glucose	4.39 ± 0.07^{A}				2.35 ± 0.10^{A}			

Average ± standard deviation with different small case letters in the same row are significantly different ($p < 0.05$) and average ± standard deviation with different capital letters are significantly different ($p < 0.05$*) between bacterial populations within the same incubation time.

Table 4 – The pH values in the glucose-free media with 2% different supplements incubated with *Lactobacillus acidophilus* or *Bifidobacterium bifidum* inoculated at 4 log CFU/mL for 24 hours or 48 hours.

Supplements	*L. acidophilus*	*B. bifidum*
24 h*		
Control	6.35 ± 0.01^{C}	6.49 ± 0.01^{C}
Glucose	4.79 ± 0.02^{B}	4.38 ± 0.03^{A}
Inulin	6.31 ± 0.02^{C}	6.41 ± 0.01^{C}
Raffinose	6.15 ± 0.01^{C}	6.43 ± 0.03^{C}
PEH-24	4.73 ± 0.02^{B}	4.86 ± 0.04^{B}
HPEH-24	4.71 ± 0.01^{B}	4.88 ± 0.02^{B}
48 h		
Control	6.28 ± 0.01^{D}	6.43 ± 0.03^{D}
Glucose	4.26 ± 0.03^{A}	4.30 ± 0.01^{A}
Inulin	5.96 ± 0.02^{C}	6.24 ± 0.02^{D}
Raffinose	6.12 ± 0.01^{CD}	6.40 ± 0.02^{D}
PEH-24	4.65 ± 0.02^{B}	4.75 ± 0.04^{B}
HPEH-24	4.66 ± 0.01^{B}	4.77 ± 0.03^{B}

HPEH-24 = heated pectin enzyme of 24-h hydrolysate; PEH = pectin enzyme hydrolysate.
Average ± standard deviation with the different letters in the same incubation time are significantly different ($p < 0.05$).

of *B. bifidum* than inulin or raffinose ($p < 0.05$), but no significant difference was found between the HPEH medium and raffinose.

Goderska et al [22] reported that inulin could support the growth of *B. bifidum* DSM 20082 but the *B. bifidum* population was about 1 log CFU/mL lower than the media containing 2% glucose (3.41×10^8 CFU/mL vs. 2.16×10^9 CFU/mL). The growth trend of *B. bifidum* and *L. acidophilus* in our study was similar with these results. In addition, the *L. acidophilus* populations and the pH values in our study were similar to those of Goderska et al [22], in which *L. acidophilus* populations and the pH values were 6.28×10^6 CFU/mL and 5.9 at 48 hours incubation, respectively. However, the *B. bifidum* populations were lower in our study, particularly in the media using inulin or raffinose. This could be due to the different media used in the experiments. MRS medium with 0.05% L-cysteine hydrochloride added was used in our study, but DSMZ 58 medium with 4% NaCl was used in the previous study [22]. Another possibility is that the saccharide utilization of *Bifidobacterium* was dependent on the species or even strains [29] and the strains of *B. bifidum* used in our study and the study of Goderska et al [22] could be different.

A previous study [30] reported the majority of *Bifidobacterium* strains fermented fructo-oligosaccharides, as well as

Table 5 – Populations (log CFU/mL) of *Lactobacillus acidophilus* or *Bifidobacterium bifidum* in media supplemented with PEH or glucose at various pH.

Supplements/pH	Original population	pH 7	pH 3.2	pH 2.5	pH 2
*L. acidophilus**					
2% PEH	6.80 ± 0.86^{A}	6.61 ± 0.28^{aA}	6.04 ± 1.11^{aA}	5.12 ± 0.41^{aB}	2.03 ± 0.31^{C}
Control[a]		6.66 ± 0.36^{aA}	6.12 ± 0.41^{aA}	n.d.	n.d.
2% glucose		6.60 ± 0.86^{aA}	6.32 ± 1.11^{aA}	2.31 ± 0.72^{bB}	n.d.
B. bifidum					
2% PEH	6.67 ± 1.16^{A}	6.98 ± 0.20^{aA}	6.64 ± 0.67^{aA}	4.42 ± 0.22^{aB}	1.72 ± 0.13^{C}
Control[a]		6.93 ± 0.70^{aA}	5.85 ± 0.67^{bB}	n.d.	n.d.
2% glucose		6.31 ± 0.47^{bA}	6.53 ± 0.74^{aA}	2.23 ± 0.52^{bB}	n.d.

*Average ± standard deviation with the different small case letters in the same pH within the same bacterial species are significantly different ($p < 0.05$) and average ± standard deviation with the different capital case letters in the same media within the same bacterial species are significantly different ($p < 0.05$).
n.d. = not detected.
[a] Control: glucose-free MRS broth.

Table 6 – Populations (log CFU/mL) of *Lactobacillus acidophilus* or *Bifidobacterium bifidum* in nonfat milk supplemented with PEH or glucose during storage.

	Days							
Supplements	0	2	4	6	8	10	15	20
*L. acidophilus**								
2% PEH	6.89 ± 0.02^{A}	6.77 ± 0.02^{aA}	6.84 ± 0.04^{aA}	6.84 ± 0.03^{aA}	6.84 ± 0.03^{aA}	6.83 ± 0.03^{aA}	6.85 ± 0.02^{aA}	6.25 ± 0.02^{aA}
Control[a]		6.59 ± 0.03^{aA}	6.86 ± 0.03^{aA}	6.23 ± 0.02^{aA}	6.23 ± 0.02^{aA}	5.59 ± 0.01^{bB}	4.67 ± 0.04^{bB}	3.47 ± 0.03^{cD}
2% glucose		6.71 ± 0.03^{bA}	6.88 ± 0.03^{aA}	6.30 ± 0.03^{aA}	6.30 ± 0.03^{aA}	6.79 ± 0.01^{aA}	5.76 ± 0.01^{bB}	4.64 ± 0.02^{bC}
B. bifidum								
2% PEH	6.87 ± 0.03^{A}	6.70 ± 0.01^{aA}	6.35 ± 0.05^{aA}	6.16 ± 0.04^{aA}	6.16 ± 0.04^{aA}	6.12 ± 0.02^{aA}	6.08 ± 0.01^{aA}	6.03 ± 0.01^{aA}
Control[a]		6.52 ± 0.02^{cA}	6.35 ± 0.04^{aA}	6.32 ± 0.02^{aA}	6.32 ± 0.02^{aA}	5.06 ± 0.06^{bB}	4.17 ± 0.02^{cC}	3.92 ± 0.01^{cD}
2% glucose		6.62 ± 0.01^{bA}	6.36 ± 0.01^{aA}	6.23 ± 0.01^{aA}	6.23 ± 0.01^{aA}	6.14 ± 0.06^{aA}	5.12 ± 0.05^{bB}	4.27 ± 0.01^{bC}

Average ± standard deviation with the different small case letters in the same day within the same bacterial species are significantly different ($p < 0.05$) and average ± standard deviation with the different capital case letters in the same treatment within the same bacterial species are significantly different ($p < 0.05$*).
[a] Control was the nonfat milk only.

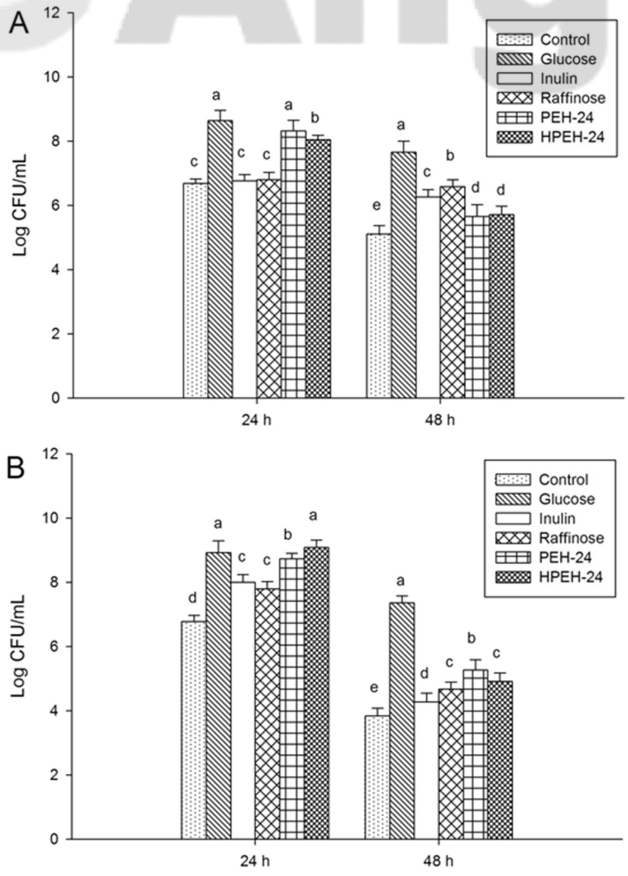

Figure 1 – The populations of (A) *Lactobacillus acidophilus* and (B) *Bifidobacterium bifidum* in the glucose-free MRS media supplemented with 2% of different prebiotics. Different letters on the bars in the same incubation time indicate significantly different ($p < 0.05$).

low-polymerized inulin, but did not ferment highly polymerized inulin. The same report also showed a better growth of probiotics in media supplemented with inulin with a DP > 10. However, Bruno et al [27] reported that galacto-oligosaccharides and fructo-oligosaccharides with lower DP showed a better ability to support the growth of bifidobacteria than the carbohydrates with higher DP. In our study, the PEHs obtained from longer hydrolysis time intervals had a higher percentage of monosaccharide and lower molecular weights, and thus, had a lower DP. However, the amounts of PEHs added to the media were more critical to the probiotic growth than the molecular weight or DP of PEHs. Thus, using more enzyme or longer hydrolysis time to obtain low DPs of PEH is not necessary. Also, an abundant source which can consistently offer nonexpensive pectin should be the key factor and orange peel is an excellent candidate based on our study. As well as citrus pectin, oligosaccharides obtained from other fruits were shown to possess prebiotic functions, such as the oligogalacturonide obtained from apple pectin which has also demonstrated the ability to stimulate the growth of *Bifidobacterium*, although pH reduction was not high (from 6.9 to 6.2 after 48 h) [22].

This study found that PEH-24 promoted the growth of *L. acidophilus* slowly but the bacterial population remained high at 48 hours. Conversely, PEH-24 increased the growth of *B. bifidum* at a faster rate, but bacteria population was lower at 48 hours incubation. The reason behind this may be related to the higher growth of *B. bifidum*, which in turn reached the decline phase faster and showed greater cell death than multiplication. Even though the media containing glucose showed the best growth of the tested probiotics, glucose can be absorbed by the body and offer calories. However, pectin oligosaccharides cannot be absorbed by the body and thus do not offer calories. Also, as a nutrition source for probiotics, pectin could protect *L. acidophilus* from gastric acid and pancreatic juice, increasing the probability of *L. acidophilus* survival and colonization in the gastrointestinal tract [28]. Our results clearly showed PEH offers a protective ability to the tested probiotics in acidic environments and glucose did not possess this ability. Moreover, pectin oligogalacturonide possesses antioxidant and anticancer abilities and could be used as a food additive to improve health [17,18]. Hence, PEH is an excellent material as a food supplement which offers both antioxidant and prebiotic functions.

In conclusion, our study showed higher PEH concentrations resulted in significantly ($p < 0.05$) higher probiotic populations with reduced pH and higher TTA. PEH also offers acid tolerance and longer survival time for the tested probiotics. PEHs of longer hydrolysis time intervals did not show a significant difference in probiotic growth. In comparison with other prebiotics, the addition of 2% PEH-24 significantly increased the probiotic population ($p < 0.05$).

Conflicts of interest

The authors have no conflicts of interest to declare.

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