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Comparative studies of the antimicrobial, antioxidant activities, vitamins, organic acid and short-chain fatty acids of postbiotics derived from five probiotic strains

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Abstract

The current study was conducted to determine and investigate the properties of postbiotics like antimicrobial activity, antioxidants, vitamins, organic acids, and short-chain fatty acids produced by some probiotics. The antimicrobial activity of all postbiotics from tested probiotic strains showed antimicrobial effects against most indicator strains. All tested strains showed a high inhibition zone against *C. albicans* and ranged from 13.5 to 27 mm. The results show that *L. acidophilus* and *L. helveticus* had the largest inhibitory effects on tested pathogenic bacteria and fungi. Furthermore, the results show that the postbiotics of all probiotics have antioxidant activity, and the *L. plantarum* strain had the highest antioxidant activity 234.38µg TE/ml, followed by *L. rhamnosus* GG 229.52µg TE/ml and *B. bifidum* 227.58µg TE/ml. On the other hand, the results indicated that postbiotics produced by various strains contain many organic acids in different concentrations, and butyric acid was not detected in all postbiotics of the tested strains. In addition, different amounts of vitamin B group (µg/ml) were detected in all tested postbiotics, and vitamin B6 was the highest vitamin produced by all probiotic strains in the study. Therefore, our findings suggest that the novel postbiotic compounds detected in this study are in preparation for the next research on their nutritional evaluation to identify the best strains used as potential functional dairy products.

*Keywords***:** Postbiotic, probiotic, antioxidant, antimicrobial, vitamins, short-chain fatty acids.

1. Introduction

Probiotics and postbiotics have gradually become the focus of the scientific and nutrition communities [1]. Postbiotics were defined as a preparation of inanimate microorganisms and/or their components that confer health benefits on the host directly or indirectly [2, 3]. Therefore, the term postbiotic refers to substances such as proteins, vitamins (B-group)**,** peptides, carbohydrates**,** organic acids, polysaccharides, enzymes, or any other soluble factor (products or metabolic by-products) of microbial metabolisms derived after the microorganisms are dead or inanimate [4, 5]. In addition, the postbiotic must be derived from well-defined food-grade microorganisms released after cell lysis, during the growth and fermentation of complex microbiological cultures, food, or gut [6, 7]. The addition of postbiotics to dairy products may have significant negative effects on their sensory characteristics and is a novel method to enhance the safety of dairy products [8, 9]. Probiotic bacteria produce a large number of antioxidant enzymes [10]. Typical nutrients like vitamin B12, vitamin K, and folate, as well as several amino acids that can be produced by gut bacteria, are examples of postbiotics [11]. Other types include lipopolysaccharides, enzymes, short-chain fatty acids, bacterial lysates, and cell-free supernatants. Compared to probiotics, postbiotics have several advantages: they are more stable, have a longer shelf life, safer and better environmental tolerance.

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Moradi et al. [12] mentioned that postbiotics are more resistant to food processing and they can remain active for longer periods. Noori et al [13] found that massive amounts of postbiotics were applied in the food industry to produce functional foods. In general, postbiotics have shown all the beneficial effects of probiotics [14, 15]. In addition, research focused on the biological activities of postbiotics for preventing pediatric acute gastroenteritis by using fermented cow's milk with *Lacticaseibacillus paracasei* [16]. This study aimed to compare the organic acids, short-chain fatty acids, vitamin content in postbiotics produced by *L. helveticus, L. acidophilus, L. rhamnosus* GG and *B. bifidum*, prelude to select the best for nutritional evaluation, followed by the dairy industrial application.

2. Materials and Methods

2.1. Sources of all microbial strains

The sources of all microbial strains, including probiotics, pathogenic and food spoilage microorganisms are illustrated in Table 1.

Table 1. The sources of microbial strains used in the study.

Strains	Sources
Probiotic strains	
- *Bifidobacterium bifidum* - *Lactobacillus plantarum* DSA 20174	Provided by Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Egypt
Lactobacillus acidophilus	obtained from Chr. Hansens's Lab., Denmark
Lactobacillus helveticus CNRZ 32	Provided by the Centre National de Recherché Zoo Technique, Jouy-en-Josas, France
Lactobacillus rhamnosus GG	Provided by Afify et al., [17] Food Sciences and Nutrition dept., NRC.
Pathogenic and food spoilage microorganisms	
- *Escherichia coli* strain E11 (accession number KY780346.1) - *Salmonella enterica* strain SA19992307 (accession number CP030207.1) - *Bacillus cereus* strain 151,007-R3-K09-40-27 F (accession number KY820914.1)	Were isolated and identified by Al-Gamal et al., [18]
Listeria monocytogenes	Supplemented from the collection of Dairy Microbiological Lab., NRC, Egypt
Staphylococcus aureus	Is a clinical isolate
- *Aspergillus flavus* 3357 - *Saccharomyces cerevisiae* Y-2223	Provided by the Northern Regional Research Laboratory, Illinois, USA (NRRL).

2.2. Preparation of postbiotic by probiotic strains

All of the probiotic strains were grown in MRS broth for 24 h at 37ºC. The cells were separated by centrifugation at 10.000×g for 10 min. The supernatants were heated for 10 minutes at 100°C. The harvested cell-free supernatant (postbiotic) was kept at 4°C until used for chemical analysis.

2.3. The antimicrobial activity by agar well diffusion assay

The antimicrobial activity of the crude extract was evaluated through disc diffusion assays as recommended in the British Society for Antimicrobial Chemotherapy guidelines [19]. Briefly, a typical colony was picked and introduced in 5 ml of tryptone soy broth from the overnight incubated culture. The broth culture was incubated at 35°C until visible turbidity reached 0.5 "McFarland" standard solution. Then, nutrient agar plates were inoculated with sterile cotton swabs in three directions to give a semi-confluent growth after overnight incubation. Within 15 minutes, discs with tested substances were

applied to the dried surface of the inoculated agar plates. After incubation at 35°C for 20 h, inhibition zone diameters (mm) were recorded.

2.4. Determination of antioxidant activity of the postbiotics

Free radical scavenging capacity was determined using the stable 1,1-diphenyl-2-picryl-hydrazyl (DPPH•). The final concentration was 50 μM for DPPH•, and the final reaction volume was 3.0 mL. The absorbance at 517 nm (A) was measured against a blank of pure methanol at 60 min. The percent inhibition of the DPPH free radical was calculated by the following equation:

$$\textbf{Inhibition (\%)} = 100 \times (A_{control} - A_{sample})/A_{control}$$

In addition, the antioxidant activity was determined using a calibration curve prepared with Trolox acid and expressed as mg of Trolox equivalent (TE) per gram of sample [20].

2.5. Detection of organic acids

HPLC analysis was carried out using an Inert Sustain. The separation was carried out using an Eclipse AQ-C18 HP column (4.6 mm x 150 mm i.d., 3 μm). The mobile phase consisted of 0.005N sulfuric acid. The mobile phase was programmed consecutively in a linear gradient for flow rate as follows: 0-4.5 min (0.8 ml/min); 4.5-4.7 min (1 ml/min); 4.7-4.71 min (1 ml/min); 4.71-8.8 (1.2 ml/min); 8.8-9 (1.3 ml/min); 9-23 (1.3 ml/min); 23-25 (0.8 ml/min). The diode array detector (DAD) was monitored at 210 nm. The injection volume was 5 μl for each of the sample solutions. The column temperature was maintained at 55 °C.

2.6. Detection of vitamins

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using ZORBAX SB-C8 (4.6 mm x 150 mm i.d., 5 μm). The mobile phase consisted of 50 mM sodium phosphate (pH 2.5)/MeOH (90:10) (A) and sodium phosphate (pH 2.5)/MeOH (10:90) (B) at a flow rate of 1 ml/min. The mobile phase was programmed in the case of water-soluble vitamins consecutively in a linear gradient and the injection volume was 5 uL. The multi-wavelength detector was monitored at 270 nm.

3. Results and Discussion

3.1. Antimicrobial activity

The antagonistic activity of five postbiotic of probiotic strains on pathogenic bacteria and fungi was determined using a well diffusion assay. Generally, as presented in Table 2, five postbiotics obtained from tested probiotic strains showed zones of inhibition against most indicator strains, but with different diameters. The diameter of inhibition zones in the case of *B. cereus* were 14, 13.5, 6, and 6 mm for *L. acidophilu*s, *L. helveticus*, *L. plantarum*, and *rhamnosus* GG postbiotics, respectively. Retarding of *Staphylococcus aureus* growth was obtained at 7, 8.5, 6.5, and 6.5 due to *L. acidophilu*s, *L. helveticus*, *L. plantarum*, and *L. rhamnosus* GG, respectively. Just *L. acidophilus and L. helveticus hindered listeria monocytogenes*, while *L. plantarum*, *L. rhamnosus* GG, and *B. bifidum* did not show any zones of inhibition. Gram-negative bacteria were affected by all five LAB strains, but with smaller zones of inhibition when compared with Gram-positive strains. The greatest zones of inhibition were obtained for *Salmonella enterica* (8.5 mm) and *Pseudomonas aeruginosa* (9 mm) because of *L. acidophilu*s. There was no significance among all lactic acid bacteria strains in the case of gram-negative bacteria. The obtained antifungal activity of the tested Lactic acid bacterial strains showed stronger potential than bacteria. However, just *L. acidophilus* (7mm), *L. helveticus* (6 mm) and *L. plantarum* (9 mm) prevented *Aspergillus flavus* from proper radial growth. *Candida albicans* was greatly inhibited at 27, 25, 24.5, 13.5, and 15.5 mm, respectively, due to the treatment with *L. acidophilus* and *B. bifidum*. Probiotic bacteria produce antimicrobial compounds such as organic acids and bacteriocins [21]. Contrary to our results, Noori et al., [13] found that *L. acidophilus* and *L. plantarum* were unable to eliminate the pathogenic bacteria; however, *L. casei* and *L. rhamnosus* postbiotics exhibited excellent antimicrobial activity**.** Magnusson et al., [22] reported that the antimicrobial activity *L. plantarum* depends on bacteriocins , organic acids and other inhibitory substances. Russo et al., [23] and Dinev et al., [24] confirmed the previous research and added that *L. plantarum*

has inhibitory activity against Gram-negative pathogens and many moulds and yeasts, including pathogenic and mycotoxigenic strains. Al-Gamal et al., [18] found that both *L. helveticus* and *L. plantarum* have the strongest antagonism against some pathogens.

Table 2: The antimicrobial activity of postbiotic extracts by disc diffusion assay

Pathogenic and spoilage strains	Inhibition zone (mm)						P-value
	LAB 1	LAB 2	LAB3	LAB4	LAB5	C+	
B. cereus	14.0 ± 0.00 ^{B}	13.5 ± 0.00^{C}	6.0 ± 0.00^{D}	6.0 ± 0.00^{D}	ND	25.0 ± 0.00^{A}	-
S. aureus	7.0 ± 0.00^{C}	8.5 ± 0.51 ^{B}	6.33 ± 0.34^{C}	6.50 ± 0.00^{C}	ND	22.0 ± 0.00^{A}	0.000
L. monocytogenes	11.33 ± 0.34^{B}	9.33 ± 0.34^{C}	ND	ND	ND	35.0 ± 0.00^{A}	0.000
E. coli	7.0± 0.00^{B}	7.0 ± 0.00^{B}	6.67 ± 0.34 ^{B}	6.0 ± 0.00^{B}	7.0 ± ^{B}	36.67 ± 1.36^{A}	0.000
Sal. enterica	8.33 ± 0.34^{B}	8.17 ± 0.17^{B}	6.0 ± 0.00^{C}	7.5 ± 0.29^{BC}	6.0 ± ^{C}	36.33 ± 1.22^{A}	0.000
Ps. aeruginosa	9.0 ± 0.00^{B}	8.17 ± 0.17^{B}	6.0 ± 0.00^{C}	6.33 ± 0.34^{C}	6.67 ± 0.34^{C}	32.0 ± 0.59^{A}	0.000
Asp. flavus	7.0 ± 0.00^{B}	6.0 ± 0.00^{C}	ND	ND	ND	26.0 ± 0.00^{A}	-
C. albicans	27.0 ± 0.00^{A}	25.0 ± 0.00^{B}	14.67 ± 0.34^{C}	13.33 ± 0.34^{D}	15.33 ± 0.34^{C}	10.33 ± 0.68^{E}	0.000

Data expressed as Mean ± SE; Values that share a letter within the same row are not significantly different; **ND**: Not detected

LAB 1= *L. acidophilus* **LAB 2=** *L. helveticus* **LAB 3=** *L. plantarum* **LAB 4=** *L. rhamnosus GG* **LAB 5=** *B. bifidum* **C+=** Positive control

3.2. Antioxidant activity of the postbiotics extracted from tested probiotic strains

The data that are presented in Table 3 and Figure 1 show the DPPH scavenging activity of different postbiotics. For instance, the antioxidant activity of *L. plantarum* showed 234.38 µg TE/ml media, followed by *L. rhamnosus* GG (229.52 µg TE/ml), *B. bifidum* (227.58 µg TE/ml media), *L. helveticus* (225.44 µg TE/ml media) and *L. acidophilus* (220.97 µg TE/ml media). Our results are similar to those of [25, 26, 27] who reported that the antioxidant activity increased in juices inoculated by *L. acidophilus* and *L. plantarum*. Dilna et al., [28] confirmed the important of antioxidant activity, helpful in the food and feed industry and positively affect human health. Furthermore, Izuddin et al., [29] assessed the antioxidant features of *L. plantarum*-derived postbiotics.

Table 3. Antioxidant activity of the postbiotics extracted from tested probiotic strains

Tested probiotic strains	Antioxidant activity (µg TE/ml media)	S.d
L. acidophilus	220.97^{d}	2.11
L. helveticus CNRZ 32	225.44^{c}	1.40
L. plantarum DSA 20174	234.38^{a}	1.57
L. rhamnosus GG	229.52^{b}	0.53
B. bifidum	227.58^{bc}	1.16

The averages followed by the different letter indicate statistical significant at level 5% of probability

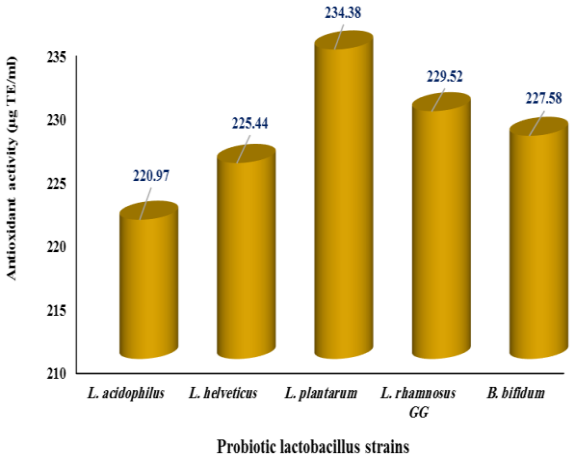


Figure 1. Antioxidant activity of different postbiotics extracted from probiotics against DPPH (µg TE/ml media).

3.3. The amount of organic acid and S short chain fatty acids in postbiotics

Organic acids, including SCFA (acetic acid, propionic acid and butyric acid), produced in postbiotics by the probiotic strains is presented in Table 4 and Fig. 2. Lactic acid was produced by *L. acidophilus* (13927.58 µg/ml), followed by *L. helveticus* (10901.39 µg/ml). The highest levels of formic acid, citric acid and acetic acid were recorded by *L. helveticus* (213.04 µg/ml), *L. rhamnosus* (745.13 µg/ml), and *L. rhamnosus* (11355.13 µg/ml). The highest concentrations of propionic acid and succinic acid were produced by *L. rhamnosus* (1520.23 µg/ml) and *L. acidophilus* (3016.19 µg/ml), respectively. It was observed that all the strains used did not produce butyric acid. Jankowsket et al., [30] found that the production of acetic acid was lower than that of lactic acid in the postbiotic of different strains of *L. plantarum*. On the other hand, Zalan et al., [31] mentioned that lactic acid is well known for its antimicrobial activity against pathogenic bacteria. Besides, Van Thu et al., [32] reported the production of acetic acid and lactic acid by the combination of postbiotics produced by various *L. plantarum* strains.

Table 4. Organic acids content (µg/ml) of postbiotics.

Organic acids	*L. acidophilus*	*L. helveticus*	*L. plantarum*	*L. rhamnosus*	*B. bifidum*
Formic acid	2. 39	213.04	ND	12.15	83.20
Lactic acid	13927.58	10901.39	3711.84	1022.16	2901.72
Citric acid	134.57	204.33	390.09	745.13	481.61
Acetic acid	5898.18	5663.19	8111.85	11355.13	9239.13
Succinic acid	3016.19	2920.55	185.55	525.85	195.46
Propionic acid	105.89	740.61	706.66	1520.23	554.09
Butyric Acid	ND	ND	ND	ND	ND

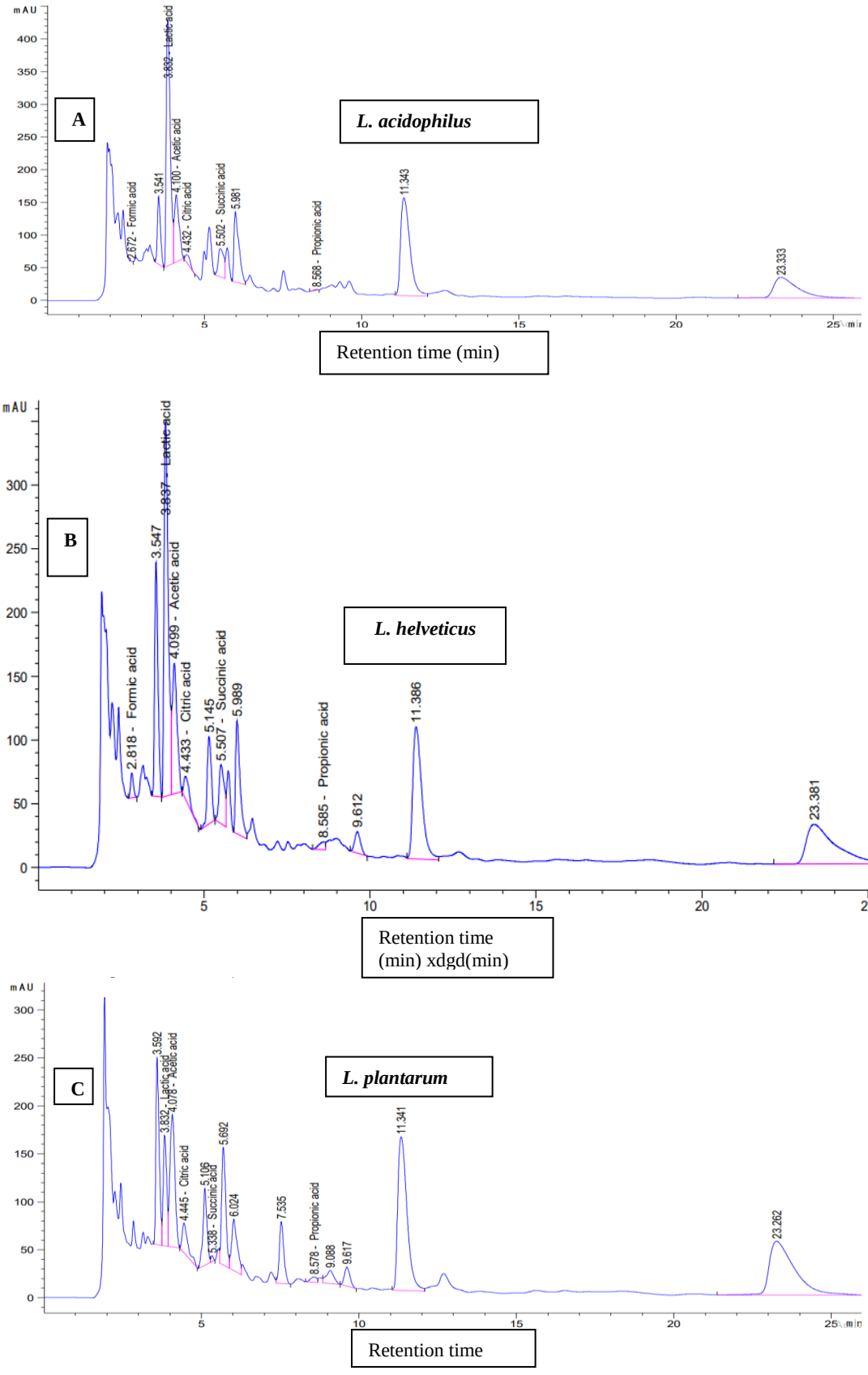

A
L. acidophilus
mAU
2.672 - Formic acid
3.541
3.832 - Lactic acid
4.100 - Acetic acid
4.432 - Citric acid
5.502 - Succinic acid
5.981
8.568 - Propionic acid
11.343
23.333
Retention time (min)
B
L. helveticus
2.818 - Formic acid
3.547
3.837 - Lactic acid
4.099 - Acetic acid
4.433 - Citric acid
5.145
5.507 - Succinic acid
5.989
8.585 - Propionic acid
9.612
11.386
23.381
Retention time (min) xdgd(min)
C
L. plantarum
3.592
3.832 - Lactic acid
4.078 - Acetic acid
4.445 - Citric acid
5.106
5.338 - Succinic acid
5.692
6.024
7.535
8.578 - Propionic acid
9.088
9.617
11.341
23.262
Retention time

Fig. 2. Continue

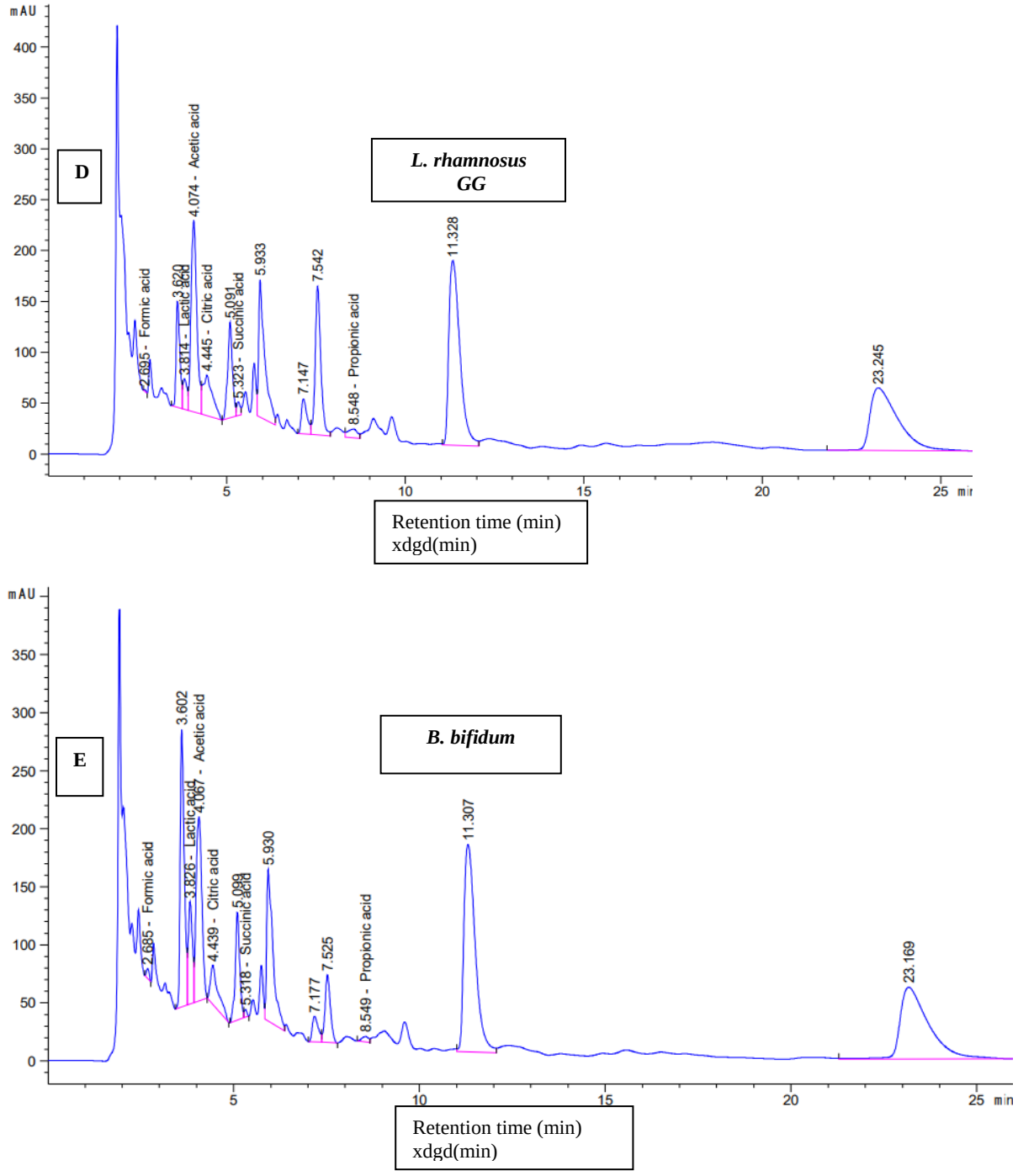


Figure 2. (A, B, C, D and E) Organic acids detected in postbiotics extracted from tested probiotic *Lactobacillus* strains
Figure 2 A. Organic acids detected in postbiotics of *Lactobacillus acidophilus*
Figure 2 B. Organic acids detected in postbiotics of *Lactobacillus helveticus*
Figure 2 C. Organic acids detected in postbiotics of *Lactobacillus plantarum*
Figure 2 D . Organic acids detected in postbiotics of *Lactobacillus rhamnosus*
Figure 2 E. Organic acids detected in postbiotics of *B. bifidum*

3.4. The amount of vitamin B in postbiotics

The vitamin content of the different postbiotic strains are shown in Table 5 and Fig. 3. All tested strains produced different levels of vitamin B1, B2, B6, B9 and B12. From five strains, *L. rhamnosus* GG showed the highest B1 level 27.61 µg/ml. The highest conc. of B2, B6, B9 were produced by *L. helveticus* (2.24 µg/ml), *L. helveticus* (266.09 µg/ml), and *L. acidophilus* (28.45 µg/ml), respectively. B-group vitamins have the ability to dissolve in water and play an important role in the metabolism of carbohydrates, proteins and lipids [33]. In this regard, it was previously described that LAB produce a variety of amounts of B-group vitamins Del Valle et al., [34] and LeBlanc et al., [35] found that *L. rhamnosus* GG was a good folate and riboflavin producer and *B. longum* and *B. bifidum* were low but significant producers of intracellular thiamine. Hill et al., [36] reported that vitamin B12 (Cobalamin) produced by a few bacteria, such as some kind of *Lactobacillus* and *Propionibacterium*. Probiotic bacteria, mostly belonging to the genera *Lactobacillus* and *Bifidobacterium*, confer a number of health benefits, including vitamin production [37, 38, 39]. Some LABs are able to synthesize B vitamins such as riboflavin [40]. Currently, the strains of *Lactobacillus* with the greatest relevance for the manufacturing of functional foods [41]. *Lactobacillus plantarum* CRL 725 was able to significantly increase the initial concentration of riboflavin in soymilk [34]. Strains of Bifidobacteria were able to produce elevated concentrations of thiamin in soymilk and fermented milks [42, 43].

Table 5. Vitamins B content (µg/ml) of postbiotics extracted from tested probiotic strains.

Postbiotics	Vitamins B content (µg/ml)				
	B1	B2	B6	B9	B12
L. acidophilus	20.49	1.90	191.13	28.45	ND
L. rhamnosus GG	27.61	1.99	154.60	16.32	ND
L. helveticus	22.07	2.24	266.09	25.11	ND
L. plantarum	17.61	1.97	237.22	22.5	ND
B. bifidum	23.91	1.29	259.13	13.69	ND

ND= not detectable

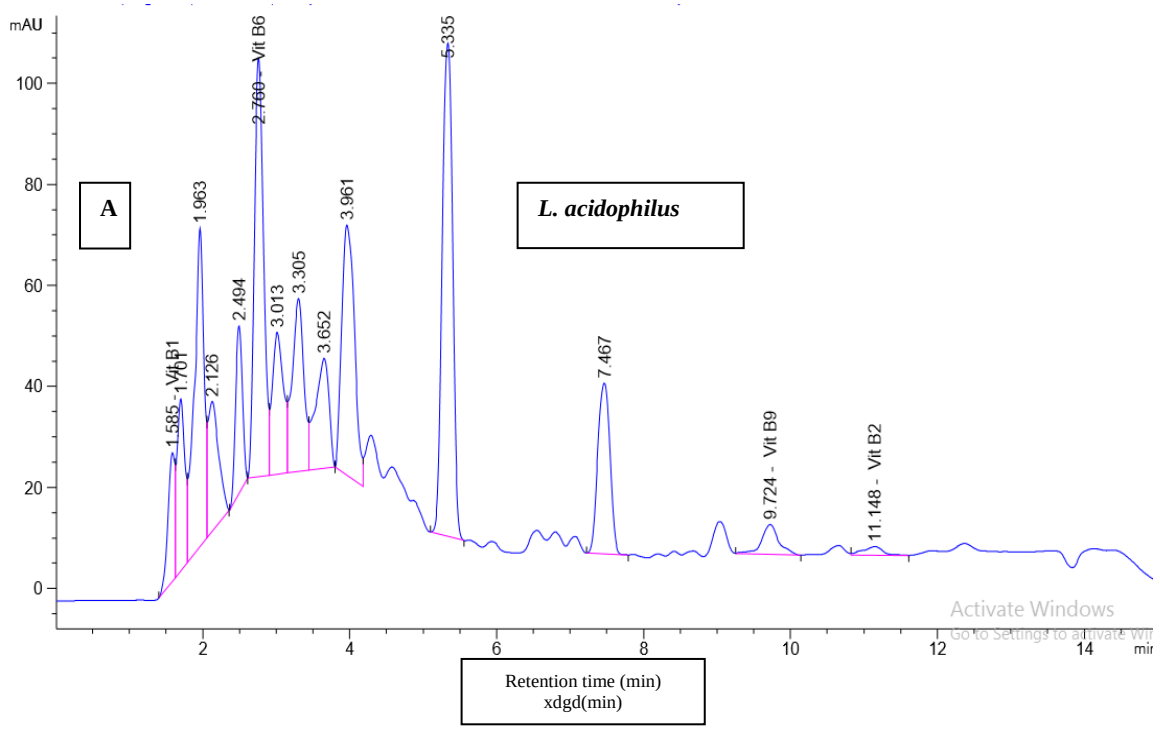
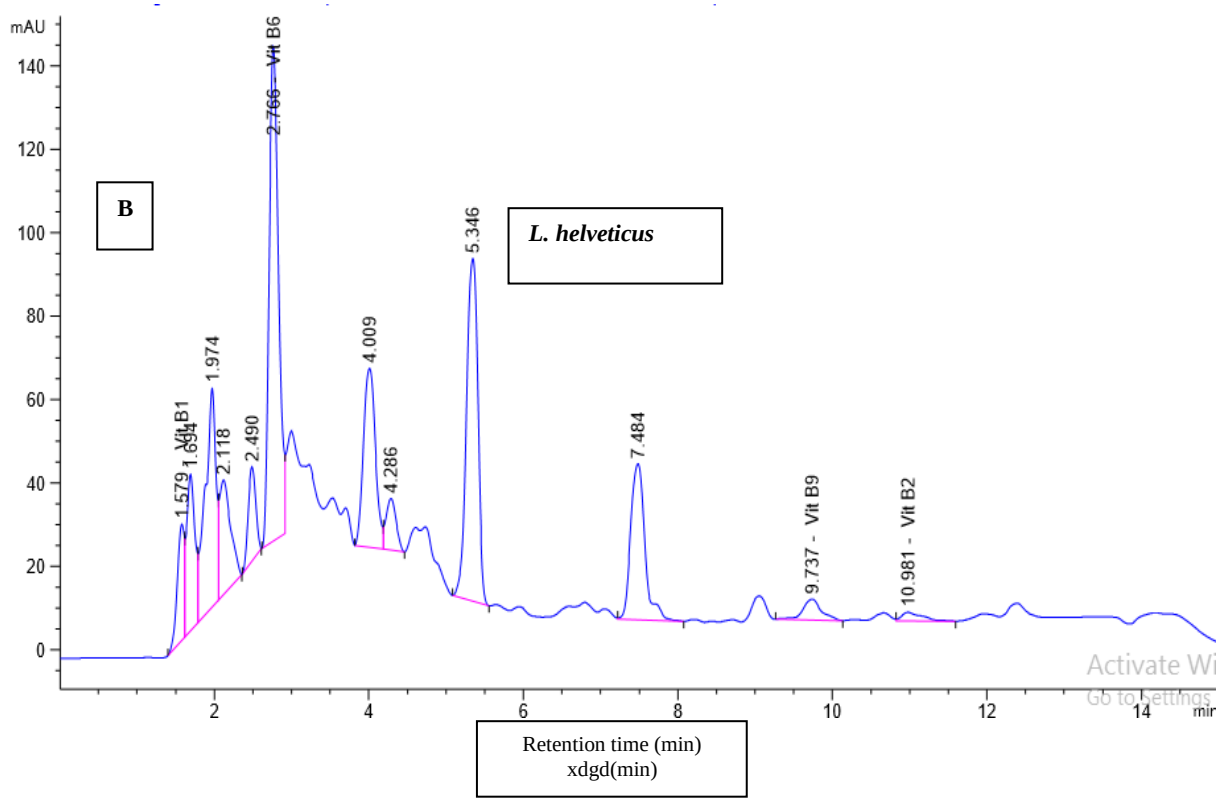
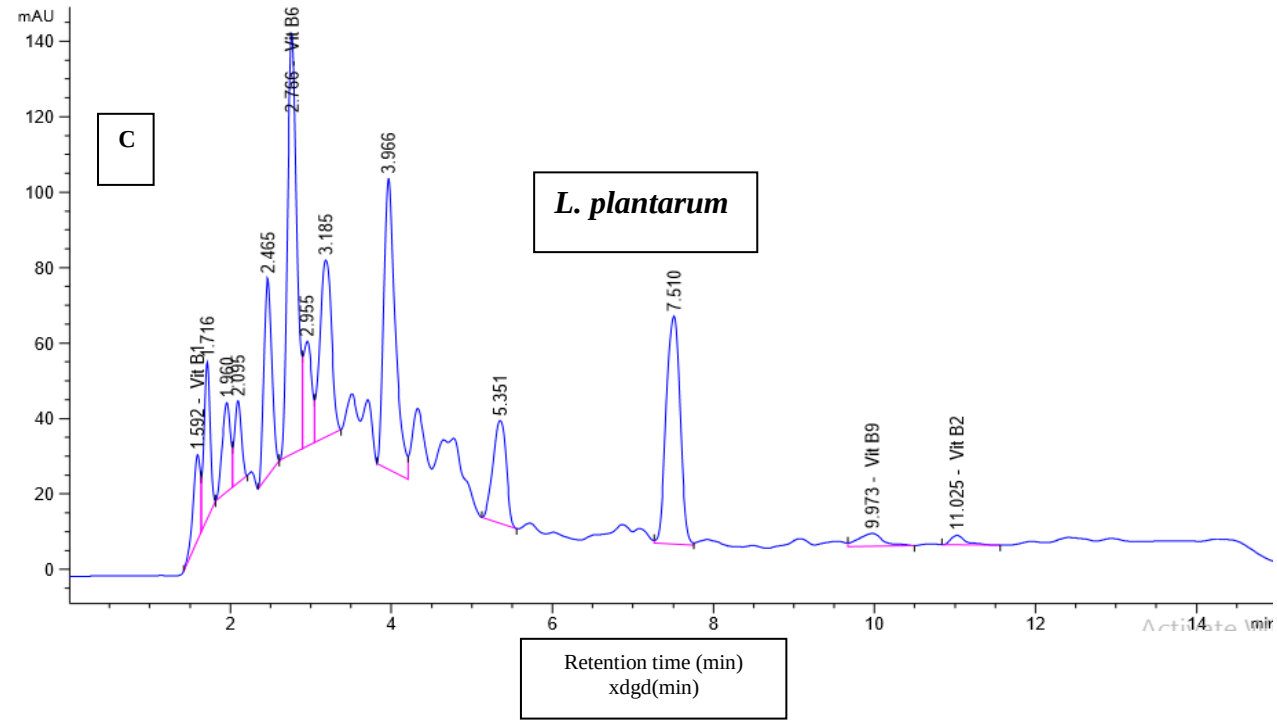


Fig. 3. Continue





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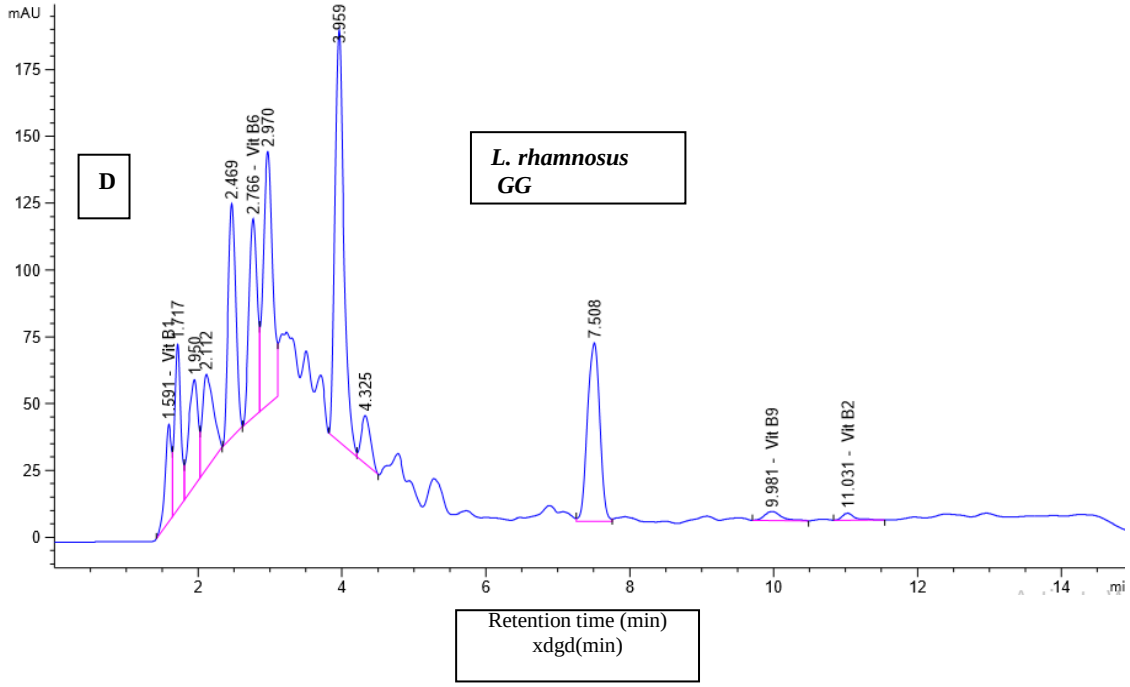

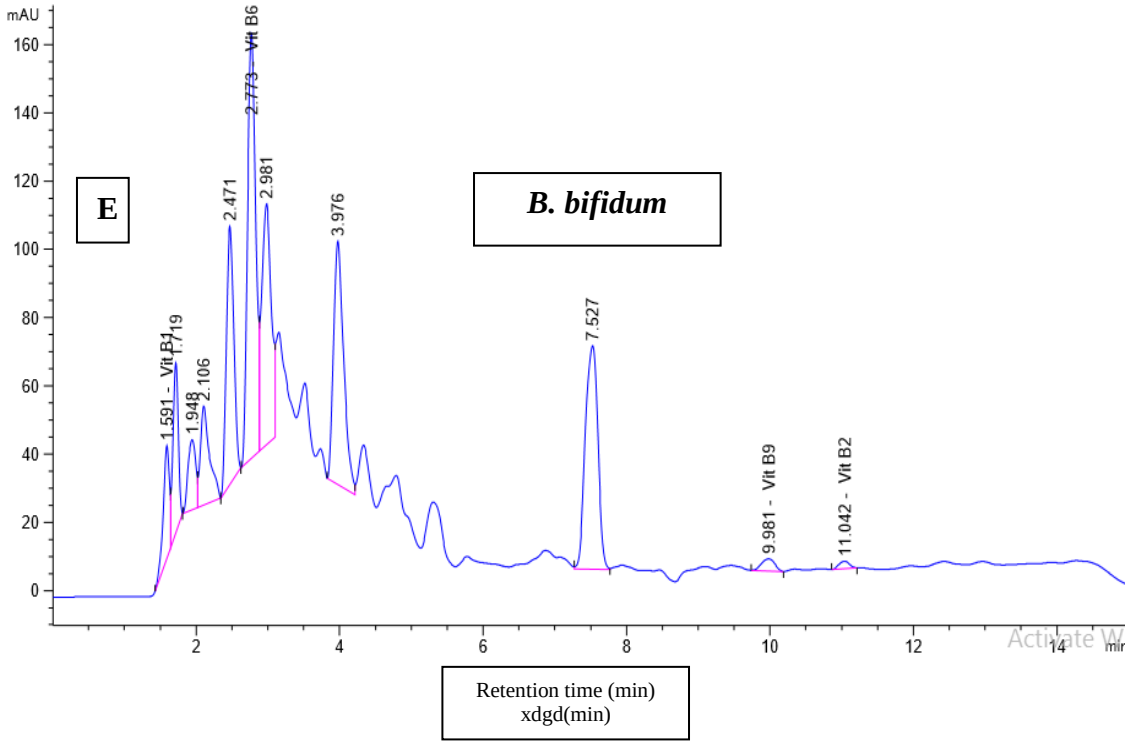

Figure 3. (A, B, C, D and E) Vitamins detected in postbiotics of probiotic *Lactobacillus* strains
Figure 3 A. Vitamins detected in postbiotics of *Lactobacillus acidophilus*
Figure 3 B. Vitamins detected in postbiotics of *Lactobacillus helveticus*
Figure 3 C. Vitamins detected in postbiotics of *Lactobacillus plantarum*
Figure 3 D, Vitamins detected in postbiotics of *Lactobacillus rhamnosus*

4. Conclusions

In this research, we selected different postbiotics from five probiotic bacteria and evaluated their antimicrobial, antioxidant activities, and organic acid contents. We found that several beneficial organic compounds were present in the postbiotics resulting from five probiotic strains used. Our results confirm that these postbiotics contain all the important and beneficial components for human in varying amounts. Therefore, in our next study, we plan to evaluate the nutritional value of these postbiotics to determine their potential for use in dairy products.

5. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

6. Acknowledgments

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