

Biotechnological Potential of Lactic Acid Bacterial Strains Isolated from Fermented Milk: Antioxidant, Antimicrobial, Bile Salt Hydrogenase Activity, and Probiotic Potential

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To cite this article:

Yomna Ali Moustafa Marzok Elkhateeb. Biotechnological Potential of Lactic Acid Bacterial Strains Isolated from Fermented Milk: Antioxidant, Antimicrobial, Bile Salt Hydrogenase Activity, and Probiotic Potential. *International Journal of Biomedical Science and Engineering*. Vol. 10, No. 3, 2022, pp. 78-85. doi: 10.11648/j.ijbse.20221003.13

Received: July 16, 2022; **Accepted:** July 29, 2022; **Published:** October 11, 2022

Abstract: Friendly intestinal bacteria exert an important role in improving resistance to disease; but increased stress or consumption of antibiotic medications can lead to disturbance in balance of microflora. Probiotics used to return the balance between friendly bacteria and pathogenic bacteria in the gastrointestinal tract. Yogurt as a source of Lactic acid bacteria (LAB) or LAB mainly lactobacilli as probiotics have several therapeutic functions when administered as live microorganisms in adequate amounts. LAB strains have good potential for application in functional foods due to their Antioxidant activity. Antioxidants are necessary for preventing diseases induced by free radicals and called effective free radical scavengers. Aim of this study was to isolate strains from yoghurt and rayeb to evaluate their biotechnological potential including antimicrobial activity, antioxidant activity, bile salt hydrolase activity and probiotic potential. Statistical analyses were performed using SPSS at $P < 0.05$. A total of 50 bacterial strains were isolated from yoghurt and rayeb of 5 different companies (Almaraei, Nada, Nadeo, Alsafi and Activia) and used to examine their probiotic properties, antioxidant activity, bile salt dehydrogenase activity and antimicrobial activity. Based on morphological characters, 20 isolates were designated as being catalase negative, and rod-shaped while only nine isolates showed positive reaction with both gram stain and lactic acid production. Accordingly, representative isolates (nine isolates) were considered as presumptive *Lactobacillus* species. Assessment of Survival in the Gastrointestinal Tract (probiotic potential) revealed that 4 bacterial strains were acid tolerance, Strain 2 was the most acid resistance of all isolates, survived over a period of 4 h at pH 3 (Survival rate was 68.22%), 3 strains were bile salt resistance after 4 hours that the Survival rate was more than 50%, only 2 strains from the previous strains had BSH activity and antioxidant activity. Therefore, these bacterial strains had the ability to survive conditions mimic those present in gastrointestinal tract and had probiotic potential. These strains isolated from yoghurt and Rayeb Almaraei. The result of comparison between companies indicates that the best company which use starter cultures with probiotic potential was Almaraei.

Keywords: Yoghurt, Antimicrobial Activity, Antioxidant Activity, Bile Salt Hydrolase Activity, Probiotic Potential

1. Introduction

Food and lifestyle are important for human health. Several studies show health benefits of yogurt consumption or other fermented milk products due to Lactic acid bacteria (LAB) species which exert symbiotic relations during their growth and fermentation in milk. Thus, LAB species selected carefully to complement each other and to achieve efficiency in acid production. Some LAB species (which isolated from

human intestine) used in yogurt production to increase the number of LAB that resist gastrointestinal acidity. Friendly intestinal bacteria exert an important role to improve human resistance to disease; however, factors such as changes in lifestyle, diet, increased stress and consumption of antibiotic medications can all lead to changes in the balance of gut bacteria (microflora) [16, 19, 21].

Therefore, probiotics used to return the balance between friendly bacteria and pathogenic bacteria in the

gastrointestinal tract [39-41]. LAB have a long history of safe consumption, thus are Generally Recognized as Safe (GRAS) and used as probiotics. Yogurt as a source of LAB or LAB mainly lactobacilli as probiotics have several therapeutic functions when administered as live microorganisms in adequate amounts. Lactic acid bacteria (LAB) are gram-positive cocci or rods, catalase-negative, non-spore forming bacteria. LAB are strictly fermentative and produce mainly lactic acid and other metabolites [29, 30, 31, 34, 36, 44]. *Lactococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus* and *Bifidobacterium* are the important LAB genera. They are present in raw milk and the gastrointestinal tract. The relationship between intestinal microbiota and good health has only recently been investigated. [42, 43]. probiotics modulate the immune system, reduce serum cholesterol levels, enhance digestive and overall health by improving gut microbiota and promoting immunity. In general, the probiotic strains should also have desirable antibiotic resistance and sensitivity patterns, be antagonist toward potentially pathogenic microorganisms and have metabolic activities beneficial to the well-being of the host. Recently, research on antioxidants increased in various fields and many studies have been conducted to identify effective natural antioxidants. Lactic acid bacteria (LAB) have attracted much attention for decades that some lactobacilli possess antioxidant activity. Antioxidants inhibit the oxidative damage of free radicals to biological molecules. Therefore, antioxidants are necessary for preventing diseases induced by free radicals and called effective free radical scavengers [7-15]. Therefore, the objective of this study is to prove the health effects of yogurt, fermented milk and the bacterial cultures used in their production, by isolation of lactic acid bacteria (LAB) mainly from different types of traditionally dairy products of different companies, Comparing among all of isolated strains through screening for antioxidant activity, bile salt hydrogenase activity, antimicrobial activity, and probiotic properties.

2. Materials and Methods

2.1. Isolation of Lactic Acid Bacteria (LAB) and Bacterial Characterization

LAB were collected and isolated from yoghurt and rayeb (fermented milk) from five different companies (Almarai, Nada, Nadec, Alsafi and Activia). Samples were serially diluted and plated on to sterile de-Mann, Rogosa and Sharpe (MRS) agar. The MRS plates were incubated at 37°C for 48h. Bacterial plates were further purified by streaking continually on MRS agar media, single colonies were initially identified based on colony morphologies, microscopic examination, gram staining, milk coagulation test, and catalase production. For performing sugar fermentation test, overnight culture of each isolate centrifuged for 10 min at 10000 rpm, cells were resuspended in 5 mL MRS without glucose, containing bromocresol purple. One mL different sterilized sugar solutions were inoculated into different tubes. Incubated at 37°C for 24 h [3].

2.2. Assessment of Characteristics for Survival in the Gastrointestinal Tract (Probiotic Potential)

2.2.1. Screening of Isolated Strains for Acid Tolerance

Nine isolates from all isolated strains were grown in MRS broth at 37°C for 24 h. After incubation, the acid resistance was examined in MRS broth adjusted to pH 3. 0.1 ml of cultures were inoculated into MRS broth previously adjusted to pH 3 with HCl 1N and incubated at 37°C. Samples were taken after (0 h and 4 h) at pH 3 and plated on MRS agar. The plates were incubated at 37°C for 48 h. After the incubation period, viable bacterial colonies were counted. The survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial concentration after 0h. [38].

2.2.2. Screening of Isolated Strains for Bile-Salt Resistance

Bile tolerance was examined in MRS broth containing 0.3% (w/v) bile salt. A volume of 0.1ml of overnight cultures of isolated strains were inoculated into MRS broth without bile and into MRS broth containing 0.3% (w/v) bile salt then incubated at 37°C. Samples were taken after 4 h and plated on MRS agar. The plates were incubated at 37°C for 48 h. After the incubation period, viable bacterial colonies were counted, and the survival rate was calculated as the percentage of colonies grown on MRS agar compared to the initial bacterial concentration on MRS without bile salt which used as control. [38].

2.3. Screening of Isolated Strains for Bile-Salt Hydrolase (BSH) Enzyme

BSH activity was assayed qualitatively by determining bile salt deconjugation ability. Bile salt plates were prepared by adding 0.3% (w/v) bile salt to MRS agar. Ten microliters of overnight liquid cultures of isolates which showed bile resistance were spotted on agar plates and incubated for 72 h. The hydrolysis zone surrounding colonies was considered a positive result and indicated the BSH activity of isolates [6].

2.4. Screening of Isolated Strains for Antibacterial Activity

The antibacterial activities of the nine isolated *Lactobacillus* species were determined by disc diffusion method against two pathogenic indicator strains. One was Gram-positive bacteria, *Staphylococcus aureus* NRRLB767 and the other was Gram-negative bacteria, *Escherichia coli* ATCC 25955. The isolated strains were grown in MRS broth at 37°C for 48h. Cell suspensions were centrifuged at 5000 rpm for 15 min. The pH of the cell free supernatant was adjusted to pH 6.5-7.0 with 1N NaOH. This supernatant placed on wells which punched with a sterile cork drill over solidified Nutrient agar seeded with old culture of the two pathogenic indicator strains. Then, these plates were held for 2 hours at 4°C to allow diffusion of the supernatant and then incubated for 24 hours at 37°C. Zones of growth inhibition measured in cm. [5, 23].

2.5. Screening of Isolated Strains for Antioxidant Production

(0.2 ml) of each isolate were spread on MRS-agar plates

and incubated at 37°C for 48 hours. A sterilized filter paper was placed on the agar plate so that colonies and their metabolites were replicated on the paper. Incubation was further continued at 37°C for 24 hours. Then the filter paper was taken out and sprayed with a DPPH solution (80 µg/ml in ethanol) after drying. Strains showing a white-on-purple spot were regarded as antioxidant-producing strains [37].

2.6. Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software and Mstat-c Program. Descriptive statistics and ANOVA (One Way analysis) for the parametric variables were tested followed by LSD. All measurements are repeated three times [45, 46].

3. Results and Discussion

3.1. Isolation of Lactic Acid Bacteria (LAB) and Bacterial Characterization

A total of 50 bacterial strains were isolated from yoghurt and rayeb of 5 different companies (Almarai, Nada, Nadec, Alsafi and Activia) Figure 1. showed that 15 strains isolated from Almarai (10 from yoghurt and 5 from rayeb); 14 strains isolated from Nada company (9 from yoghurt and 5 from rayeb); 9 strains isolated from Nadec company (5 from yoghurt and 4 from rayeb); 7 strains isolated from Alsafi company (4 from yoghurt and 3 from rayeb); 5 strains isolated from Activia company (3 from yoghurt and 2 from rayeb).

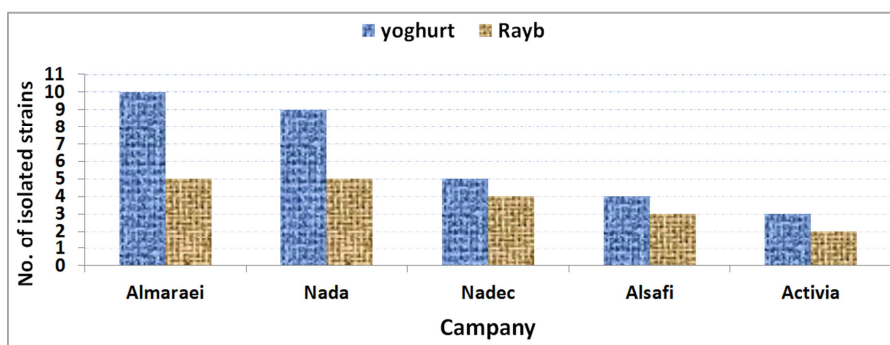


Figure 1. Number of isolates from different companies.

Figure 2 showed that 20 isolates were designated as being catalase negative due to no bubble production after addition of hydrogen peroxide.

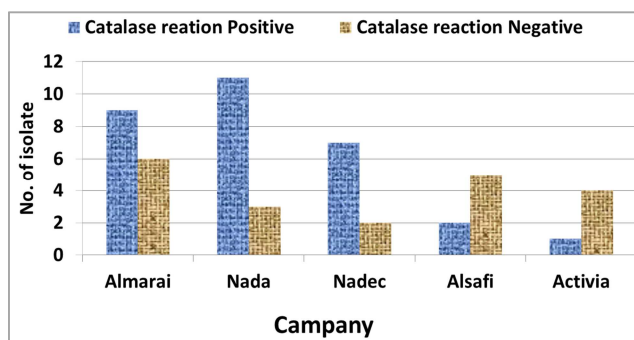


Figure 2. Catalase reaction for isolates.

Figure 3 showed that only nine isolates from the previous 20 isolates showed positive reaction with both gram stain (violet blue color) and milk coagulation test (isolated strains can coagulate skim milk as a result of lactic acid production).

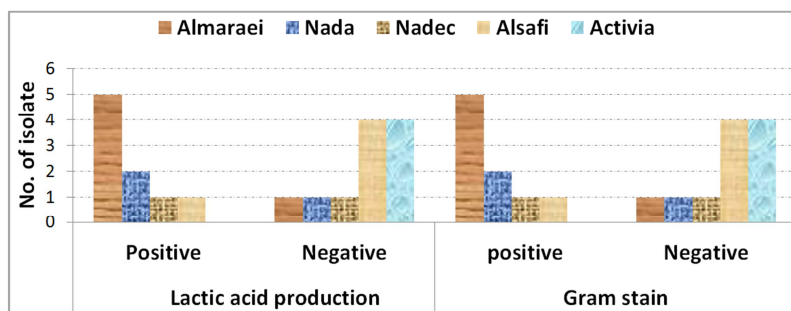


Figure 3. Lactic acid production and gram stain test for isolates.

Table 1 represent results of Sugar fermentation test

Isolated strains can ferment sugar, produce acid and gas, and change color of the broth medium to yellow color. Accordingly, representative isolates were considered as presumptive LAB [2]. Based on morphological characters,

nine isolates were rod-shaped and identified as Lactobacillus species and used to study their probiotic properties (acid tolerance and bile-salt resistance), bile salt hydrogenase activity, antimicrobial activity, and antioxidant activity.

Table 1. Sugar fermentation patterns of isolated strains.

No. of strain	1	2	3	4	5	6	7	8	9
Type of sugar									
Glucose	+ve								
Fructose	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
Mannose	+ve								
Galactose	+ve								
Lactose	+ve								
Maltose	+ve								
Sucrose	+ve								
Mannitol	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
Cellubiose	+ve								
Sorbitol	-ve								
Rhamnose	-ve								
Arabinose	+ve								
Xylose	+ve								
Rafinose	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve

3.2. Evaluation of Probiotic Potential

3.2.1. Screening of Isolated Strains for Acid Tolerance

Tolerance of isolated strains to low pH is an important factor to consider them probiotic bacteria. Resistance of strains to low pH means their survival in acidity of stomach and intestinal juice that pH of gastric acid in human stomach is 1.5 to 3.5 and this level is maintained by the proton pump H^+/K^+ ATPase. probiotics potential of isolated strains means the ability to survive under low pH (gastric conditions) and for bile salts and successfully provide the host their health benefits. The effects of low pH (3) on Viable count of isolated strains are presented in Table 2. There was a significant difference among strains and strain no. 1 which isolated from yoghurt of Almarai company was the most acid

resistance of all isolates, survived over a period of 4 h at pH 3 (Survival rate was 68.22%). The most acid tolerance isolates after that were strain 2 which isolated from Rayeb of Almarai company (Survival rate was 64.71%). Then, strain 3 (Survival rate was 60.94%) and strain 4 (Survival rate was 54.56%). The results showed a decrease in cell viability less than 50% within 4 h which means that the rest of isolates are sensitive to low pH value. The result revealed that there were only 4 strains were acid tolerance, 3 strains (no. 1, 3 and no. 4) isolated from yoghurt of Almarai company, and one strain (strain no. 2) isolated from Rayeb of the same company. This was consistent with the results by authors who found that Lactobacillus strains isolated from the feces of breast-fed infants and children had high survivability under acidic conditions. [22-25].

Table 2. Acid tolerance of isolated strains from yoghurt and Rayeb.

No. of strain	Source of strain	pH 3				Viable count after 4hr				Survival rate%at 4hr (SR%)			
		Control											
1	yoghurt Almarai	6.01	h	±	0.01	4.10	b	±	0.10	68.22	a	±	1.55
2	Rayeb Almarai	6.15	g	±	0.05	3.98	b	±	0.08	64.71	b	±	0.78
3	yoghurt Almarai	7.22	d	±	0.02	4.40	a	±	0.02	60.94	c	±	0.11
4	yoghurt Almarai	7.33	c	±	0.10	4.00	b	±	0.10	54.56	d	±	0.62
5	Rayeb Almarai	6.48	f	±	0.08	3.15	d	±	0.05	48.61	e	±	0.17
6	yoghurt Nada	7.90	b	±	0.05	3.53	c	±	0.06	44.72	f	±	0.50
7	Rayeb Alsafi	7.02	e	±	0.02	2.57	e	±	0.07	36.61	g	±	0.90
8	Rayeb Activia	8.02	a	±	0.02	2.60	e	±	0.10	32.42	h	±	1.17
9	yoghurt Nadec	6.20	g	±	0.10	1.50	f	±	0.10	24.18	i	±	1.23
LSD at $\alpha = 0.05$		0.109				0.133				1.550			

3.2.2. Screening of Isolated Strains for Bile-Salt Resistance

The effects of bile on Viable count of isolated strains are presented in Table 3. The results indicate that all isolates grew in the presence of 0.3% bile but there was a significant difference among strains. Only 3 isolates revealed bile

resistance after 4 hours that the Survival rate was more than 50%. While survival rate of the other 6 isolates was less than 50% which means sensitive or no resistant isolate. Strain 1 was the most bile resistance of all isolates, survived over a period of 4 h (Survival rate was 90.19%). The most bile resistance isolates after that were strain 2 (Survival rate was

67.27%) then strain 3 (Survival rate was 67.26%). The result revealed that there were only 3 strains were bile-salt resistance, 2 strains (no. 1 and no. 3) isolated from yoghurt of Almarai company, and one strain (strain no. 2) isolated from Rayeb of the same company. This was consistent with the previous results which proved that the average of bile salts

concentration was 0.3%, and that food intestinal retention about 4–6 h. Previous studies observed the reduction in log CFU/mL from an initial count log CFU/mL 8.54–6.09. Other studies approved that acid and bile tolerance was strain-specific and there was extreme variability in resistance within species [25–28].

Table 3. Bile-salt resistance of isolated strains from yoghurt and Rayeb.

No. of strain	Source of strain	Bile salt 0.3%											
		Control (without bile salt)				Viable count after 4hr				Survival rate at 4hr% (SR%)			
1	yoghurt Almarai	7.54	c	±	0.04	6.80	a	±	0.03	90.19	a	±	0.07
2	Rayeb Almarai	7.79	b	±	0.09	5.24	c	±	0.04	67.27	b	±	0.26
3	yoghurt Almarai	7.88	a	±	0.04	5.30	b	±	0.03	67.26	b	±	0.03
4	yoghurt Almarai	7.01	f	±	0.04	3.45	d	±	0.05	49.22	c	±	0.44
5	Rayeb Almarai	7.14	e	±	0.04	3.31	e	±	0.04	46.36	d	±	0.30
6	yoghurt Nada	7.16	e	±	0.02	3.27	e	±	0.02	45.67	e	±	0.15
7	Rayeb Alsafi	6.99	f	±	0.01	3.11	f	±	0.04	44.49	f	±	0.51
8	Rayeb Activia	7.33	d	±	0.03	2.44	g	±	0.04	33.29	g	±	0.41
9	yoghurt Nadec	7.03	f	±	0.03	1.89	h	±	0.03	26.88	h	±	0.32
LSD at $\alpha = 0.05$		0.077				0.054				0.545			

3.3. Screening of Isolated Strains for Bile-Salt Hydrolase (BSH) Enzyme

Figure 4 showed a positive result and indicated the BSH activity of isolated strain no. 1 and no. 2. BSH activity was assayed qualitatively by determining bile salt deconjugation ability. The three isolates which showed bile resistance were spotted on agar plates and incubated for 72 h. The hydrolysis zone surrounding colonies was considered a positive result and indicated the BSH activity of isolates [1, 26]. This was consistent with the results of previous studies which found that resistance of Lactic Acid Bacterial strains to bile salt can be related to the activity of enzyme BSH, which is responsible for the degradation of bile salts lowering its toxic effect. Bile salts are formed in the liver and stored in gall bladder as conjugated bile salts. Deconjugation by BSH enzyme is a detoxification mechanism against bile salt toxicity for the cell that results in free bile acids which are excreted from the body. BSH enzyme activity is considered a protective mechanism. [6, 17, 18]

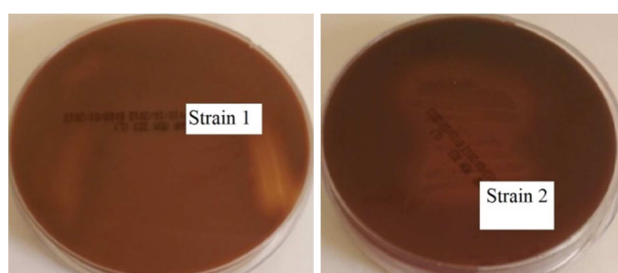


Figure 4. BSH activity of Lactic Acid Bacterial strains isolated from yoghurt and fermented milk.

Results of Characteristics Assessment for Survival in the Gastrointestinal Tract (probiotic potential) revealed that 4 bacterial strains were acid tolerance, 3 strains were bile salt resistance, only 2 strains from the previous strains had BSH activity. Therefore, these bacterial strains had the ability to

survive conditions mimic those present in gastrointestinal tract and had probiotic potential. These strains isolated from yoghurt and Rayb Almarai. The result of comparison between companies indicates that the best company which use starter cultures with probiotic potential was Almarai.

3.4. Screening of Isolated Strains for Antibacterial Activity

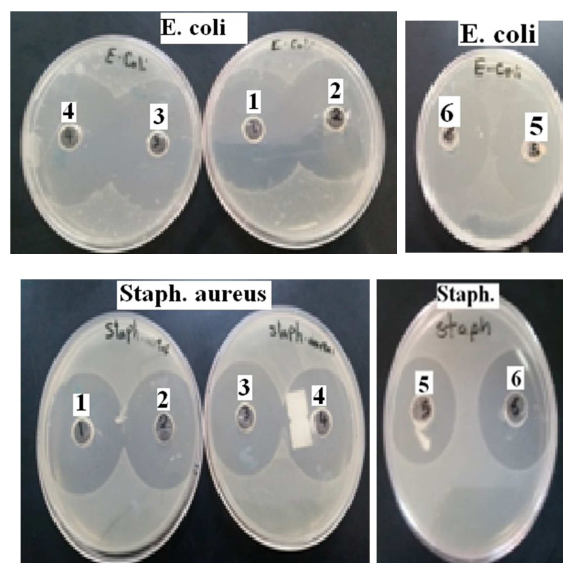


Figure 5. Antimicrobial activity of isolated strains against *Escherichia coli* and *Staphylococcus aureus*.

All nine isolated strains were tested to antibiotic activity by the well diffusion method and the results based on the monitoring of inhibition zone against two pathogenic indicator strains. One was Gram-positive bacteria, *Staphylococcus aureus* NRRLB767 and the other was Gram-negative bacteria, *Escherichia coli* ATCC 25955. Figure 5 showed that isolated strains have good antibacterial effect against two pathogenic indicator strains. Similarly, other studies have demonstrated the antibacterial activity of LAB strains against food borne pathogens, both gram-positive and

gram-negative bacteria [5, 20, 33, 35]. The biological properties could be attributed to other bioactive secondary metabolites. According to this result, applications of isolated strains in food processing would be valuable, used to extend food shelf life and other preservation strategies. These results prove that isolated bacteria had the ability to inhibit pathogenic bacteria due to its production of antimicrobial substance. Results from this study indicated that most cells of these yoghurt bacteria would release their intracellular constituents in the GI tract and had probiotic property [4].

Based on the diameter of inhibition zones, Table 3 showed that only 6 isolates had antimicrobial activity, there were significant differences among strains and strain no. (1) had the highest activity against pathogenic bacteria (*E. coli*) and (*Staph.aureus*), the inhibition zones for the indicator strains were (4.2 cm) and (3.30 cm) respectively.

Table 4. Antimicrobial activity of isolated strains against *Escherichia coli* and *Staphylococcus aureus*.

No. of strains	<i>Escherichia coli</i>				<i>Staphylococcus aureus</i>			
1	4.2	a	±	0.1	3.30	a	±	0.20
2	3.9	bc	±	0.1	3.10	ab	±	0.10
3	4.0	ab	±	0.1	3.03	b	±	0.03
4	4.0	ab	±	0.2	2.70	c	±	0.20
5	3.6	c	±	0.3	2.37	d	±	0.02
6	3.0	d	±	0.2	2.77	c	±	0.02
7	-ve				-ve			
8	-ve				-ve			
9	-ve				-ve			
LSD at $\alpha = 0.05$	0.3182				0.2179			

3.5. Screening of Isolated Strains for Antioxidant Production

Guided by the screening method with DPPH, Table 5. revealed that only 2 strains which isolated from yoghurt and Rayeb Almarai showed a positive antioxidant production and regarded as antioxidant-producing strains. This was consistent with the results of previous studies which found that the DPPH free radical is a radical with a deep purple color when mixed with an antioxidant, its color turns and reduced to color of the corresponding hydrazine. Strains showing a white-on-purple spot were regarded as antioxidant-producing strains. Other study searched for the development of natural antioxidant agents to prevent the illness effect of free radicals and suggests that LAB strains have good potential for application in functional foods due to their antioxidant activity. [13-15, 32, 37].

Table 5. Detection of antioxidant-producing strains.

No. of strain	Source of strain	antioxidant production
1	yoghurt Almarai	+ve
2	Rayeb Almarai	+ve
3	yoghurt Almarai	-ve
4	yoghurt Almarai	-ve
5	Rayeb Almarai	-ve
6	yoghurt Nada	-ve
7	Rayeb Alsafi	-ve
8	Rayeb Activia	-ve
9	yoghurt Nadec	-ve

4. Conclusion and Recommendation

The composition of gut microbiota is linked to dietary patterns. Ingested bacteria can participate in the prevention or treatment of emerging diseases. Evidence suggests that probiotic milk products enhance digestive and overall health by improving gut microbiota. The current study showed that some traditional dairy products may be the natural sources for bacteriocin-producing LAB which can be used as probiotics. Probiotics, such as lactic acid bacteria, are active bacterial cultures with unique characteristics that allow them to survive in the gastrointestinal tract and compete with other enteric microorganisms. Thus, they help to maintain the natural balance of the microbiota and overall health. The beneficial properties of probiotic microorganisms are highly dependent on strains that there is a difference in the potent of probiotic property among strains which isolated from different sources and different companies. These findings should encourage consumption of fermented dairy products especially yogurt and rayeb as part of the total daily food consumption due to their various health benefits that they contain LAB which had probiotic potential. Present study approved that acid and bile tolerance of lactic acid bacterial strains was strain-specific and there was extreme variability in resistance within species. Therefore, to ensure that the intestines always contain probiotics in sufficient numbers to display their health effect, consumers should use fermented dairy products as part of their total daily diet from different sources and different companies. Therefore, eating yogurt or rayeb may help with digestion, but for reaching this positive effect, consumer not only have to eat it with live bacteria, but should also eat it daily that only small amount of the bacteria they contain can withstand the acidity of stomach and reach the intestine. As well, the bacteria will pass through digestive system within a day or two, so to always ensure their presence in intestine, consumer should eat them every day. Finally, on using fermented dairy products, try to use the products of different companies because each company use different strain as starter culture and the results proved that probiotic potential was strain-specific and variable within species.

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