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Potential role of probiotic bacteria as antioxidants agent

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Abstract

Oxidants are free radicals that find in the environment, but they are also produced naturally in our body. Antioxidants have an important role in the body health and protected from many diseases caused by free radicals. The unique characters of probiotic bacteria make it used extensively in the field of medicine antioxidant, anticancer, anti-microbial, anti-diabetic, anti-inflammatory properties and hepatoprotective agent. This study was designed to explore the influence of lactic acid bacteria as antioxidative agents. Antioxidative properties for all tested stains were investigated through determination scavenging activity of α, α -Diphenyl- β -Picrylhydrazyl (DPPH), hydroxyl radicals (OH^\cdot) and inhibition of lipid peroxidation in rat liver homogenate in vitro. The present findings revealed that, *Lactobacillus plantarum* subsp. *plantarum* DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T possessed the highest antioxidant properties, tolerated acidity up to 4 and revealed viability under bile salt concentration up to 0.5%. The results of this study provide new insights into the lactic acid bacteria with antioxidative properties as they tolerated acidity up to and revealed viability under bile salt concentration.

Keywords: antioxidant activity, lactic acid bacteria, acid and bile tolerance.

1 Introduction

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) formation and scavenging by antioxidants. Excess generation of ROS can cause oxidative damage to biomolecules resulting in

lipid peroxidation, mutagenesis and carcinogenesis (Khan and Sultana, 2009).

Probiotics are live microorganisms thought to be beneficial to the host organism. According to the currently adopted definition by FAO/WHO, probiotics are: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host". Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microbes used as probiotics; but certain yeasts and bacilli may also be helpful. Lactic acid bacteria (LAB), which are widely used in the food industry, are generally recognized as safe (Liu and Pan 2010). LAB, particularly lactobacilli, has recently received increasing attention because of their specific role in maintaining human health and in decreasing the risk of ROS accumulation (Choi et al., 2006) and (Lee et al., 2005).

Lactic acid bacteria have various potential biological functions (De Vrese, and Schrezenmeir, 2008). Several studies have provided evidence that certain lactic acid bacteria possess antioxidative activity (Mikelsaar and Zilmer, 2009). Saide and Gilliland, (2005) suggested that some Lactobacilli strains are a source of dietary antioxidants. In our previous studies, we also demonstrated that lactic acid bacteria possess antioxidative ability through one or multiple mechanisms, including metal ion chelation, radical scavenging, inhibition of lipid peroxidation and activity reduction in vitro (Lin and Chang, 2000). The present study attempt to evaluate the antioxidant activity of different probiotic bacteria.

2 Materials and Methods

Bacterial strains and culture

Lactobacillus plantarum subsp. *plantarum* DSMZ 20174, *Bifidobacterium bifidum*, *Lactobacillus rhamnosus* ATCC 7469, *Bifidobacterium longum* subsp. *longum* DSMZ 200707, *Streptococcus lactis* subsp. *cremoris*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080. *Streptococcus thermophilus*, *Lactobacillus acidophilus* DSMZ 20079 T and, *Lactobacillus fermentum* DSMZ 20049, were kindly provided from microbiological resources center (Cairo MIRCEN), Faculty of Agriculture Ain Shams University, Faculty of Agriculture Kafr El-Sheikh University and Faculty of Science Tanta University. All strains were cultured in De Man-Rogosa- Sharpe (MRS) broth at 37°C for 24 h.

Effect of bile salts and pH on bacterial growth.

Bile tolerance

Bile tolerance of all the investigated strains were studied described by Gilliland et al., (1984). Briefly, MRS broth supplemented with different concentrations of bile salts (0.3 and 0.5% ox-bile salts, Oxoid) was inoculated with 1% inoculums (1×10^9 CFU) and incubated at 37°C for 1h, then samples were drawn, plated on MRS plates and incubated at 37°C for 24h. Growth on the plates indicated their tolerance to bile salts. The control comprised of MRS without bile salt.

Acid tolerance

MRS with different pH (2.0, 3.0, and 4.0 adjusted with HCl) was prepared according to Pereira and Gibson (2002). Then was inoculated with 1% inoculums (1×10^9 CFU) and incubated grown overnight. Samples were drawn, plated on MRS plates and incubated at 37°C for 24h.

Antioxidant activities of the probiotic bacterial strains

Hydroxyl radical scavenging capacity of the probiotic bacteria:

Hydroxyl radical (OH^\cdot) scavenging assay was performed according to Wang et al., (2008). OH^\cdot was generated by Fenton reaction in the system of FeSO_4 and H_2O_2 . The reaction mixture was consisted of 0.5 ml FeSO_4 (8 mM), 0.8 ml H_2O_2 (6 mM), and 0.5 ml distilled water. 1.0 ml of each of the bacterial treatments and 0.2 ml sodium salicylate (20 mM) were added and incubated at 37 °C for 1 h. The absorbance of the mixture was recorded at 562 nm. The scavenging activity was calculated using the following Equation:

$$\text{Scavenging or inhibition rate \%} = [1 - (A1 - A2)/A0] \times 100$$

Where: A0 is the absorbance of the control (without sample), A1 is the absorbance of the sample addition, A2 is the absorbance without sodium salicylate.

Determination of 2,2-diphenyl-picrylhydrazyl (DPPH) radical scavenging activity of the probiotic bacteria

The capacity of the probiotic bacterial treatments to reduce the 2, 2-diphenyl-picrylhydrazyl (DPPH) stable free radical was assessed using the method described by Blois (2002). DPPH (2, 2 diphenyl-1-picrylhydrazyl): (0.025 g/L) 0.025 g was dissolved in 1 liter methanol. Added 0.1 ml of sample extract (after 24, 48, 72 and 96 h incubation) to 3.9 ml of DPPH solution. Total mixture was incubated at room temperature for 30 min in the dark conditions. The absorbance was recorded at 515 nm Hsu et al., (2006). The percentage of DPPH scavenging activity was calculated according to absorbance difference as has been depicted in equation:

$$\text{DPPH scavenging \%} = [1 - (A1 - A2)/A0] \times 100$$

Where, A0 is the absorbance of DPPH alone. A1 is the absorbance of DPPH + sample. A2 is the absorbance of the sample only.

Inhibition of lipid peroxidation in rat liver homogenate by the probiotic bacterial strains

The inhibition effect on lipid peroxidation was determined according to the thiobarbituric acid method with some modifications Chu-Chyn et al., (2009); $\text{FeCl}_2 - \text{H}_2\text{O}_2$ was used to induce the liver homogenate peroxidation. In this method, One millilitre of liver homogenate (each 100 ml homogenate solution contains 1.0 g rat liver), 100 μL PBS buffer, 1mM FeCl_3 , 200 μL ascorbic acid and cell-free extracts of probiotic bacterial strains were mixed. The mixture was incubated at 37 °C for 60 min. The inhibition effect on lipid peroxidation was determined according to thiobarbituric acid assay described by Buege and Aust, (1978). The percentage of inhibition effect was calculated according to Eq. (3):

$$\text{The level of inhibition (\% on lipid peroxidation)} = [A1 - A2/A0] \times 100\%$$

Where: A0 is the absorbance of the control (without sample), A1 is the absorbance of the sample addition. A2 is the absorbance without liver homogenate.

3 Results

Effect of bile salt and pH on growth of probiotic strains

The initial pH of growth medium were adjusted to various pH values ranging from 2.0 to 4.0 using HCl. Results of probiotic strains growth are recorded in Table (1). From the results, it could be noticed that the growth activity was increased with increasing in pH value. The maximum growth for all investigated strains was recorded at pH 4.

Instead, the tested levels of bile salts did not influence growth or strain survival. There was no difference in cell yield between control cultures and those with bile.

Hydroxyl radical scavenging capacity of probiotic bacterial strains:

The ability of probiotic bacterial strains to scavenge hydroxyl radicals was illustrated in Figure (1) and the results indicated that, there were huge varieties in the scavenging ability among the investigated bacteria. The scavenging capacity could be divided into three groups, weak scavenging ability, obtained with cell free supernatant of *Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* ATCC 7469 recorded inhibition percentages 40, 42 and 45%, respectively. Moderate scavenging ability, obtained with supernatant of *Lactobacillus delbrueckii* subsp. bulgaricus DSMZ 20080, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. bulgaricus, *Lactobacillus fermentum* DSMZ 20049, *Lactobacillus casei*, *Streptococcus lactis* subsp. cremoris and *Bifidobacterium longum* subsp. longum DSMZ 200707 with inhibition percentages 50, 55, 60, 65, 70, 70 and 75% respectively. The excellent scavenging ability was obtained with the cell free supernatant of *Bifidobacterium Bifidum*, *Lactobacillus acidophilus* DSMZ 20079 and *Lactobacillus plantarum* subsp. plantarum DSMZ 2017 recorded inhibition percentages 77, 78 and 90%, respectively.

Table (1): Effect of bile salt and pH on growth of probiotic strains

Bacterial Strains	pH			Bile salt (%)	
	2	3	4	0.3	0.5
<i>Bifidobacterium Bifidum</i>	-	+	+	+	+
<i>Lactobacillus rhamnosus</i> ATCC 7469	-	-	+	+	+
<i>Lactobacillus reuteri</i>	-	-	+	+	+
<i>Lactobacillus plantarum</i> subsp. plantarum DSMZ 20174	-	-	+	+	+
<i>Bifidobacterium longum</i> subsp. longum DSMZ 200707	-	+	+	+	+
<i>Streptococcus lactis</i> subsp. cremoris	-	+	+	±	±
<i>Lactobacillus rhamnosus</i>	-	-	+	+	±
<i>Lactobacillus casei</i>	-	-	+	+	+
<i>Streptococcus thermophilus</i>	-	-	+	+	+
<i>Lactobacillus delbrueckii</i> subsp. bulgaricus	-	-	+	±	±
<i>Lactobacillus delbrueckii</i> subsp. bulgaricus DSMZ 20080	-	-	+	+	+
<i>Lactobacillus acidophilus</i> DSMZ 20079 T	-	-	+	+	+
<i>Lactobacillus fermentum</i> DSMZ 20049	-	-	+	+	±

Note: -, no growth; ± 50-70% and + > 70% of control, respectively.

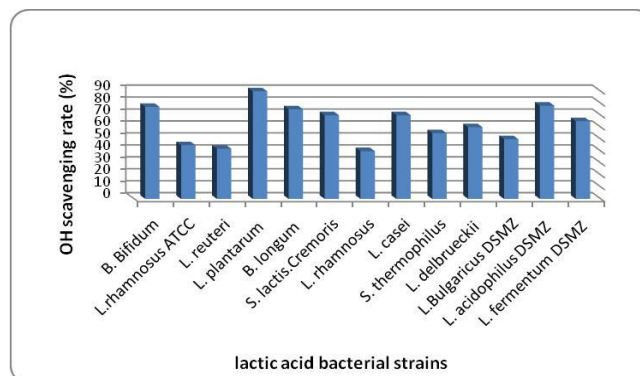


Figure (1): Hydroxyl radical scavenging capacity of probiotic bacterial strains

Inhibition of lipid peroxidation in rat liver homogenate by LAB strains

As shown in Table (2) the concluded data revealed that, among all treatments, the supernatant of *Lactobacillus plantarum* subsp. plantarum DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T were superior as lipid peroxidation inhibitor than the other supernatants. These supernatants reported inhibition percentages reached 78.5 and 77.5, respectively with MDA concentrations 1.67 and 1.65µM/ml, respectively, without significant differences between them.

DPPH radical scavenging:

DPPH radical scavenging activity of lactic acid bacterial supernatant was assayed after 24, 48, 72 and 96 h of incubation periods as shown in Table (3). At the end of incubation, the higher activity was recorded with the supernatants of both *Lactobacillus plantarum* subsp. plantarum DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T with percentages of 90.4% and 92.0%, respectively. Out of all represented data, the supernatant of *lactobacillus rhamnosus* ATCC 7469 showed the lowest scavenging percentage with value 62.35%.

4 Discussion

The production of free radicals was related to many diseases (Devasagayam et al., 2004). Lactic acid bacteria, which have been traditionally used in the production of various fermented foods, are important intestinal microflora and natural antioxidants (Ou et al., 2012). Lactic acid bacteria considered as a bioactive safe microorganisms that normally transit the gastrointestinal tract and colonized intestinal microbial ecosystem promoting host health (Chu-Chynet et al., 2009).

Table (2) Inhibition of lipid peroxidation in rat liver homogenate by the probiotic bacterial strains

Strains	MDA concentration ($\mu\text{M/ml}$)	Inhibition of lipid peroxidation %
Bacterial supernatant		
<i>Lactobacillus reuteri</i>	2.11	62
<i>Lactobacillus rhamnosus</i> ATCC 7469	1.78	70
<i>Bifidobacterium Bifidum</i>	2.30	50
<i>Streptococcus lactis</i> subsp. cremoris	1.74	72.5
<i>Bifidobacterium longum</i> subsp. longum DSMZ 200707	2.10	65.5
<i>Lactobacillus plantarum</i> subsp. plantarum DSMZ 20174	1.67	78.5
<i>Lactobacillus rhamnosus</i>	1.72	75
<i>Lactobacillus casei</i>	1.77	70
<i>Lactobacillus acidophilus</i> DSMZ 20079 T	1.65	77.5
<i>Lactobacillus fermentum</i> DSMZ 20049	1.77	70
<i>Lactobacillus delbrueckii</i> subsp. bulgaricus DSMZ 20080	1.70	76
<i>Streptococcus thermophilus</i>	1.77	70
<i>Lactobacillus delbrueckii</i> subsp. bulgaricus	1.75	71

The aim of the study was to show the possibility of using probiotic bacteria as natural free scavenging radical during many diseases, to achieve that hypothesis; the ability of bacteria to withstand bile salts and acidity were recorded. In the present study the effect of bile salt and pH on growth of probiotic strains showed that the growth activity was increased with increasing in pH value and reached its maximum value at pH 4 with no bile salt it. There was no difference in cell yield between control cultures and those with bile salt. These results indicated that the investigated probiotic strains were tolerant to acidic conditions and provided beneficial aspects as they can be survival during passage to human stomach, digestive system and colonize the human gut. These results were in accordance with Shukla et al., (2010) who observed that some probiotic strains were more tolerant to acidic conditions than others either due to high cytoplasmic buffering capacity (pH 3.72–7.74) or membrane ATPases that in turn may resist changes in cytoplasmic pH and gain stability under acidic conditions (Ritus et al., 1994). Also, they found that all the isolated strains were survived and tolerated bile salts (0.3%) quite effectively.

Table (3) DPPH radical scavenging activity of the probiotic bacterial strains

Strain	DPPH scavenging rate (%)			
	Incubation time (hr.)			
	24	48	72	96
<i>Lactobacillus rhamnosus</i> ATCC 7469	86.6	87.3	62.35	88.9
<i>Bifidobacterium Bifidum</i>	85.6	86.1	83.4	85.6
<i>Bifidobacterium longum</i> subsp. longum DSMZ 200707	88.7	86.5	82.4	85.6
<i>Lactobacillus plantarum</i> subsp. plantarum DSMZ 20174	90.4	87.6	87.6	86.7
<i>Lactobacillus reuteri</i>	88.9	87.1	90	88.4
<i>Streptococcus lactis</i> subsp. cremoris	89.8	89.9	89.9	89.8
<i>Streptococcus thermophilus</i>	87.1	89.3	87.5	86.3
<i>Lactobacillus casei</i>	89.7	86.3	84.1	84.4
<i>Lactobacillus rhamnosus</i>	82.4	89.8	91.5	89.6
<i>Lactobacillus delbrueckii</i> subsp. bulgaricus	84.4	84.9	89.3	84.0
<i>Lactobacillus acidophilus</i> DSMZ 20079 T	83.3	92.0	89.2	82.4
<i>Lactobacillus delbrueckii</i> subsp. Bulgaricus DSMZ 20080	90.8	87.6	90.2	91.1
<i>Lactobacillus fermentum</i> DSMZ 20049	89.1	87.5	86.8	87.9

Note: DPPH Abso B=0.813

On the other hand, the same results were obtained by Cenci et al., (2002) who observed that lactic acid bacteria isolated from commercial dairy products were tolerance to bile salt (0.5%) and acid environment (pH 2.0).

The antioxidative ability might be one of the main mechanisms for probiotic bacteria to prevention or treatment many diseases. Therefore, the hydroxyl radicals, DPPH scavenging and lipid peroxidation inhibition were tested in the present study to investigate the antioxidative properties of the probiotic strains.

In the current study, the ability of probiotic bacterial strains to scavenge hydroxyl radicals was done depending on generation of OH⁻ radicals from the reaction of FeSO₄ and H₂O₂ with sodium salicylate. The excellent scavenging capacity was recorded with the supernatant of *Bifidobacterium Bifidum*, *Lactobacillus acidophilus* DSMZ 20079 and *Lactobacillus plantarum* subsp. *plantarum* DSMZ 2017 with inhibition percentages 77, 78 and 90%, respectively. Also, DPPH radical scavenging activities and percentages of lipid peroxidation inhibition were ranged between 62.35 % - 92 % and 50% to 78.5% respectively. MDA is an end product of lipid peroxidation, and is considered a late biomarker of oxidative stress and cellular damage, and lipid peroxidation is one of the main manifestations of oxidative damage and it has been found to play an important role in the toxicity and carcinogenicity (Carampin et al., 2003). Furthermore, the increase of MDA has been considered a key feature in liver injury (Mateos et al., 2005). Oxidative stress arises when the generation of ROS, by-products of the oxidative metabolism primarily produced in the mitochondria, exceeds the cellular ability to eliminate them and to repair cellular damage, thus leading to oxidation of biomolecules including DNA, lipids and proteins (Hwang and Kim 2007). A similar finding has been reported by Ejtahed et al., (2012) that probiotic yogurt possessed markedly significantly decreased serum malondialdehyde concentration. Zhang and Zhang (2013) showed that many *Lactobacillus* strains with antioxidative effects were not only reducing MDA level, but also enhance the antioxidants production. Based upon, the free radical scavenging ability of lactic acid bacterial strains were significantly differ in scavenging ability according to bacterial strains. *Lactobacillus plantarum* DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T revealed the highest antioxidant activity.

In conclusion, the antioxidative capacity evaluated for thirteen lactic acid bacteria strains in this study to select novel probiotics as antioxidant agent. The results revealed that the investigated lactic acid bacterial strains showed high tolerance against acidity, bile salt and have variable antioxidative activities. *Lactobacillus plantarum* DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T were possessed the most antioxidant properties among other strains. Therefore Lactic acid bacteria especially *Lactobacillus plantarum* DSMZ 20174 and *Lactobacillus acidophilus* DSMZ 20079 T could be used as a supplementary ingredient to provide a dietary strategy in the treatment and prevention of many diseases.

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