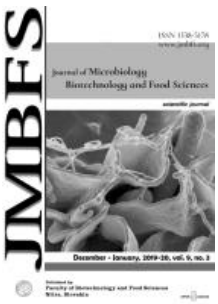

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PROBIOTIC PROPERTIES AND ANTIOXIDANT EFFICIENCY OF *LACTOBACILLUS PLANTARUM* 15 ISOLATED FROM MILK

Karima Riane[1,3]; Mohamed Sifour[1,3]; Houria Ouled-Haddar[1,3];Tayeb Idoui[2,3]; Samira Bounar[3]and Sarra Boussebt[3]*

Address(es):
[1] Laboratory of Molecular Toxicology, Faculty of Nature and Life Sciences, University Mohamed Seddik Benyahia of Jijel, Algeria.
[2]Laboratory of Biotechnology, Health and environment, Faculty of Nature and Life Sciences, University Mohamed Seddik Benyahia of Jijel, Algeria.
[3]Department of Applied Microbiology and Food Sciences, Faculty of Nature and Life Sciences, University Mohamed Seddik Benyahia of Jijel, Algeria.

*Corresponding author: sifourm@yahoo.fr

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ABSTRACT

Many researchers are focusing on the investigation of new molecules and substances to help the body fighting oxidative stress; among them are probiotic bacteria. In this study we investigated the probiotic properties and the antioxidative activity of *Lactobacillus plantarum* 15 isolated from milk. Therefore, to evaluate probiotic potential of the selected strain, the antibacterial activity and resistance to acidic pH and bile salts conditions were evaluated. Moreover, several methods were used to analyze the antioxidant potential of this strain. The evaluation of probiotic properties showed that *Lb. plantarum* 15 resists to acidity and to bile salts (survival rate was 86.40% and 80.58%, respectively). The isolate showed a broad inhibitory activity against several bacterial strains. The antioxidant potential assessed by the DPPH method showed that *Lb. plantarum* 15 cells as well as the cell-free supernatant have important scavenger effect (82.65% and 72.21%, respectively). Furthermore, the strain showed a good resistance to hydrogen peroxide (76.12%), hydroxyl radicals (46.15%) and a considerable iron chelating ability (20.52%). Thus, *Lb. plantarum* 15 could be effectively considered as potent antioxidant probiotic food supplement.

Keywords: Antioxidant activity; DPPH; Lactic acid bacteria; *Lactobacillus plantarum*; Probiotic

INTRODUCTION

The "oxidative stress" is defined as "a disturbance in the pro-oxidant-antioxidant balance" **(Pinchuk *et al.*, 2012; Powers *et al.*, 2011)**. This important factor in the pathophysiology of a variety of pathological conditions is initiated by reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-), the hydroxyl radicals and non-radical species such as hydrogen peroxide (H_2O_2) and oxygen singlet ($^1O_2$) **(Sunil *et al.*, 2013)**. At high concentrations, free radicals and oxygen species can cause oxidation of nucleic acid, lipid, protein and eventually high oxidative stress may result in breaking and coupling of DNA. As a result gene mutation, cell damage and ultimate death can take place **(Islam *et al.*, 2018)**. In addition, atherosclerosis, cancer, chronic kidney disease, neurodegenerative diseases, Alzheimer's disease, cirrhosis, arthritis and Parkinson's disease were correlated with oxidative damage **(Wang *et al.*, 2006; Vitetta *et al.*, 2013)**.
Different mechanisms were used by the body to counteract oxidative stress; they can be classified into endogenous defense system generated naturally *in situ* and exogenous defense system through foods **(Rao *et al.*, 2011)**. To protect cells and organs against damage from free radicals, Humans have developed complex antioxidant systems (enzymatic and non-enzymatic) **(Choudhari *et al.*, 2014).** Many researches are focused on the investigation of new molecules and substances to help the body to fight oxidative stress; among them the use of probiotic bacteria. Various species of *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus lactis* and some species of *Enterococcus* are the most commonly used probiotic bacteria (**Idoui and Sifour, 2016; Jäger *et al.*, 2016)**; they have a role in the improvement of digestion and intestinal transit, maintaining the balance of intestinal flora and the acid-base balance in the colon **(Dib *et al.*, 2012)** , which reduces serum cholesterol levels, inhibition and reduction of the risk of cancer and modulating the immune system **(Temmerman *et al.*, 2003**). Antioxidant activity is an important effect of probiotics which consists in the protection of cells from oxidation problems **(Mahmoudi *et al.*, 2018).** The antioxidant potential of lactic acid bacteria (LAB) has attracted much attention recently such as Lactobacilli that have probiotic effects to human health. Some lactobacilli possess antioxidant activity, and have the ability to reduce the accumulation of ROS after oral ingestion (**Kim *et al.*, 2006; Pieniz *et al.*, 2014)**.The potential antioxidant systems of LABs include intracellular thiols such as glutathione (GSH) and thioredoxin (Trx)), antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) and respiratory metabolism. These systems protect cells by reducing the potential damage of ROS, and help to maintain the redox homeostasis **(Zhang and Li, 2012)**. For example, some lactobacilli have the ability to modulate the enzymes implicated in the metabolism of xenobiotics. *Lb. fermentum* I5007 is capable of increasing the amount of glutathione S-transférase (GST) on the Caco-2 cells **(Valko *et al.*, 2006; Wang and Ho, 2009)**. Carcinogens may cause mutations in the presence of reactive form of oxygen species (ROS). Probiotics can reduce the reactivity of the ROS which reduces the risk of mutations.
The main objective of this work was to screen antioxidant potential of some lactic acid bacteria. The probiotic properties and the antioxidant ability of the selected isolate *Lactobacillus plantarum* 15 isolated from milk was investigate.

MATERIAL AND METHODS

Bacterial isolates and chemicals

The probiotic strains *Lb. viridescens* J13, *Lb. plantarum* G1, *Lb. delbrueckii* ssp *lactis* S3, *Lb. plantarum* J2, *Lb. plantarum* 15, *Lb. helveticus* J14, *Lactobacillus* sp. T5, were kindly provided by Prof. Tayeb Idoui from the laboratory of Biotechnology, Environment and Health, University of Jijel, Algeria. 2,2-diphényl-1-picrylhydrazyl-hydrate (DPPH) and thiobarbituric acid (TBA) were procured from Sigma, USA. All broth and Bile salts were purchased from Pasteur Institute of Algiers. All other reagents were of analytical grade. de Man Rogosa Sharpe (MRS) broth and agar was used for bacterial culture. The MRS broth was inoculated with the bacteria strain then incubated at 37°C for 24 h. The culture was centrifuged at 6000 rpm for 5 min then the cells were washed by phosphate buffer (PBS). The culture was standardized to a cell number of 10^9 CFU.mL^{-1}.

Radical scavenging activity

Strains were cultured in MRS broth, incubated at 37°C for 24 h and centrifuged at 6000 rpm for 10 min at 4°C to obtain the culture supernatant and bacterial pellets. The bacterial pellets were washed twice with sterilized normal saline and resuspended to obtain a concentration of 10^9 CFU.ml^{-1}. Screening of antioxidative bacteria was performed by DPPH method as described by **Mandal**

***et al.* (2013)**. This method was based on the monitoring of DPPH free radicals scavenging activity by intact cells. 1 mL of freshly prepared DPPH in methanol (0.2 mM) were mixed and allowed to react with 0.8 mL of intact cells suspension for 30 min at room temperature. The controls included only sterilized saline water and DPPH solution, the blanks contained methanol and bacterial suspension. The absorbance was measured at 517 nm. The test was performed in triplicate.

$$\text{Scavenging activity}(\%) = \frac{\mathbf{A}\ \text{control} - \mathbf{A}\ \text{sample}}{\mathbf{A}\ \text{control}}$$

Evaluation of probiotic properties

Acid tolerance

This test was performed according to the technique described by **Thirabunyanon *et al.* (2009)**. An overnight culture of *Lb. plantarum* 15 was used to inoculate 20 mL of sterile PBS (pH 2.0) to obtain a number of cells corresponds to 10^9 CFU.ml^{-1} and then incubated for 2, 4 and 6 h at 37°C. The viable count was achieved on MRS agar after incubation of inoculated plates at 37°C for 48 h. The calculation of the bacterial survival rate was carried out as follow:

$$[\text{Log(CFU)T}/\text{log(CFU)T0}]\text{x}100$$

Tolerance to bile salts

The assay was realized according to the method reported by **Mishra and Prasad (2005)**. An overnight culture of *Lb. plantarum* 15 was used to inoculate 20 mL of 0.1 M PBS supplemented with 1 % bile salts. After centrifugation, the obtained pellet, was washed and resuspended in PBS to reach a concentration of 10^9 CFU.mL^{-1}. The culture was then incubated at 37°C. Viability was determined by cell counts on MRS agar media after 2, 4, 6 and 8 h.

Antibacterial activity

Agar well diffusion method was used to determine the antibacterial effect of supernatant according to **Aslim and Kilic (2006)**. Culture of *Lb. plantarum* 15 grown in 100 mL MRS broth for 24 h at 37°C was centrifuged at 6000/30 min the supernatant was sterilized by passage through 0.22µm Millipore filter. The indicator strains were activated on nutrient broth at 37°C for 24 h. Cultures was prepared by adding 10 µL of the tested strain in 20mL of Muller-Hinton agar, well homogenized and then poured in Petri dishes, allowed to cool and then wells of 6 mm diameter were. After that, each well was filled with 100 µL of filtered supernatant, and then the plates were incubated for 24 h at 37°C.
Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms in millimeters.

Evaluation of antioxidant potential

Total antioxidant Activity (TAA)

The method described by **Zanoni *et al.* (2008)** was used to realize this essay. 100 µL ascorbic acid (5 mmol.L^{-1}) was mixed with 4.9 mL of PBS (0.2 mol.L^{-1}) at pH 7.0. A sample of 100 µL of the pellet of the tested strain was added to the mixture. The reference was prepared by replacing the sample by 100 µL of PBS (0.2 mol.L^{-1}). After a reaction of 10 min at 37°C, the absorbance was measured at 265 nm. The TAA expressed as the percentage of inhibition of ascorbic acid auto-oxidation was defined as follows:

$$\text{TAA} = \left[1 - \frac{\text{A265 (sample)}}{\text{A265(reference)}}\right]\text{x}100$$

DPPH method

This method was applied to evaluate the antioxidant activity of the culture supernatant and bacterial pellets of the selected strain. The experiment was performed as described above by the method of **Mandal *et al.* (2013).**

Resistance to hydrogen peroxide

The assay was performed according to **Zhang *et al.* (2011)**. A solution of PBS with 1 mM hydrogen peroxide was prepared; 20 mL of this solution was inoculated by about 10^9 CFU.mL^{-1}, the tubes were incubated for 2, 4 and 6 h at 37°C. The viable count was then performed on MRS agar.

The scavenger capacity of hydroxyl radicals

The generation of hydroxyl radicals was carried out in a solution (Ap) containing 1 mL of 1, 10-Phenanthroline, 1 mL of PBS (0.02 mM) at pH 7.4, 1 mL of distilled water and 1 mL $FeSO_4$ (2.5 mM). The reaction was initiated by the addition of 1 mL of H_2O_2 (20 mM). In parallel, a second mixture (Ab) containing the same solutions except of H_2O_2 which was replaced by 1 mL of distilled water was prepared. The third mixture (As) containing the same solution as Ap except of H_2O_2 which was replaced by 1 mL of the sample (the sample was incubated in MRS broth for 18h, then centrifuged at 3000 rpm for 10 min, then a wash was carried out to remove any traces of metabolites and the solution was standardized at OD from 0.1 to 0.2). The mixtures were incubated at 37°C for 1h and 30 min and the absorbance was measured at 536 nm **(Zhang *et al*., 2011)**. The resistance to hydroxyl radicals was determined as follows:

$$\text{Scavenging effect} = [(\text{As} - \text{Ap})/(\text{Ab} - \text{Ap})]x100$$

Measurement of the chelating capacity of ferrous ion

A preparation of 0.5 mL of cell pellet (10^9 CFU.mL^{-1}) was added to a mixture of 0.1 mL of ascorbic acid (1%, v/v), 0.1 mL of $FeSO_4$ (0.4 g.L^{-1}) and 1 mL of 0.2 M NaOH. 0.2 mL of Trichloroacetic acid (10%) was added to the mixture and incubated in a water bath at 37°C for 20 min. After a centrifugation at 6000 rpm for 20 min; the obtained supernatant was collected and mixed with 0.5 mL of 1, 10-Phenanthroline (0.1%). The absorbance was measured after a reaction of 10 minutes at 510 nm **(Zhang *et al*., 2011)**. The assay was carried in triplicate.

RESULTS AND DISCUSSION

Probiotic may represent an effective strategy to prevent deficiencies of antioxidant; also, it is well known that antioxidant efficiency of probiotic is strain dependent. Thus, searching for new probiotic strain with antioxidative activity is needed.

Radical scavenging activity

All tested bacterial strains were capable to scavenge DPPH radicals. Results summarized in figure 1indicated that the level of free radical scavenging activity varied with the different strains. Among the seventh tested strains, scavenger effect of DPPH radicals showed important level with *Lb. plantarum* 15. Thus, *Lb. plantarum* 15 is the best tested strain since it showed the highest activity with a percentage of about 82%.
Our results are correlated with those obtained by **Afify *et al.* (2012)** where they demonstrated that *Probionibacterium freudenreichii* CFE and *Lb. reuteri* had high antioxidant activity with percentages of 97.75 % and 96.74 %, respectively, scavenging of DPPH radical. Also, **Ou *et al.* (2006)** reported that *S. salivarius* ssp. *thermophilus* ATCC 19258 and *Lb. delbrueckii* ssp. *bulgaricus* ATCC 11842 have specific effect on oxidative stress. Based on this result we selected *Lb. plantarum* 15 for further studies.

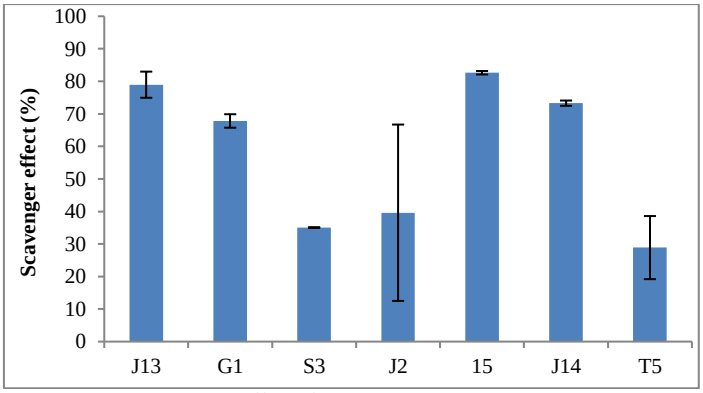

Figure 1 DPPH scavenger effect of probiotic bacteria. J13: *Lb. viridescens*, G1: *Lb. plantarum*, S3: *Lb. delbrueckii* ssp *lactis*, J2: *Lb. plantarum*, 15: *Lb. plantarum*, J14: *Lb. helveticus*, T5: *Lactobacillus* sp.

Probiotic bacteria have antioxidant mechanisms such as the reduction of glutathione and thiol compounds, the ability to chelate metal ions, trapping reactive oxygen species and reducing activity. These protective capabilities result in antioxidant properties of certain lactobacilli and possibly provide additional food sources of antioxidants or probiotic bacteria capable of reducing oxidative stress **(Mahmoudi *et al*. 2018).** Based on the obtained data, the studied LAB strain has a good antiradical activity and can be used to reduce oxidative damage.

Evaluation of probiotic properties of *Lb. plantarum* 15

Resistance to pH and bile salts

To be selected as probiotic, bacterial strain should be able to survive during the passage through the gastrointestinal tract and be capable to tolerate the conditions strongly acid of the gastric juice and the high concentrations of bile salts in the

intestine. Bile salts are one of the barriers to cross by the probiotic bacteria to reach their site **(Mikelsaar and Zilmer, 2009)**. From the results shown in figure 2, it appears that the strain *Lb. plantarum* 15 is resistant to bile salts with a survival rate of 80.58% after 6h of incubation. Similarly, the strain tolerates gastric pH (pH 2.0) with a survival rate of 86.40%. According to **Dowarah *et al.* (2018)** the probiotic microbes have to survive at low pH before reaching in lower tract and must remain viable for 4h or more. Therefore our strain can be considered as a good probiotic bacteria.

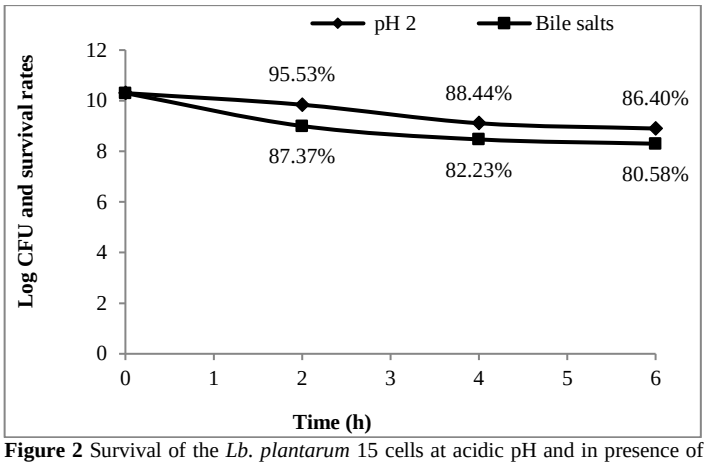

Figure 2 Survival of the *Lb. plantarum* 15 cells at acidic pH and in presence of bile salts.

Our results are in agreement with other studies reported by **Song *et al.* (2015),** which have demonstrated that *Lactobacillus* strains remain viable when exposed to acid pH values of 2.5-4.0, as well as bile salt concentration. In one hand, acid-tolerant strains have an advantage in surviving in the low pH conditions of the stomach (as low as pH 2.0), where hydrochloric and gastric acids are secreted, on the other hand, the physiological concentration of human bile ranges from 0.3% to 0.5%. Therefore, resistance to bile acid is an important characteristic that enables probiotic strains to survive, grow, and remain active in the small intestine (**Song *et al.*, 2015). O'Sullivan and Condon (1997)** reported that the majority of the LAB possess a tolerance mechanism in acidic medium; they are capable of surviving at lethal concentrations of acid. Whereas, the results found by **Burns *et al.* (2008)** showed that most of the strains of *Lb. delbrueckii* ssp. *bulgaricus* and *Lb. delbrueckii* ssp. *lactis* were sensitive to bile salts. Similarly, **Zago *et al.* (2011)** showed a mean survival rate of 40 % in lactobacilli isolated from milk and cheese. Thus, the ability of probiotic bacteria to survive the passage through the stomach was reported variable and strain dependent according to **Kim *et al.* (2006),** these differences might be due to differences in the cell wall structure, because the survival of bacteria was reduced generally by destroying their cell-membranes.

Antibacterial activity

To select any strain as a potential probiotic, it is important to assess the antimicrobial activity **(Fakhruddin *et al.*, 2017).** This test allows highlighting the characteristics possessed by LAB to inhibit the growth of some pathogenic strains. It is clear from the results presented in Table 1 that the studied strain presents a pronounced antagonism against pathogenic bacteria with inhibition zones diameters ranged from 12 to 14.2 mm. These results indicate that the ability of the isolate to produce substances having antibacterial activity such as bacteriocins, organic acids and hydrogen peroxide **(Aslam and Qazi, 2010; Titiek *et al.*, 1996)**. The *in vitro* inhibitory capacity of LAB seems like a good probiotic property, as it can play an important role in maintaining the hygienic quality of food **(Ammor *et al.*, 2006).**

Table 1 inhibitory activity of the supernatant of *Lb. plantarum* 15 against some tested bacteria

Tested strain	*Listeria monocytogenes*	*Escherichia coli* ATCC 25922	*Bacillus subtilis*	*Salmonella* sp.	*Staphylococcus aureus*	Methicillin resistant *Staphylococcus aureus*
Inhibition Zone (mm)	12.5±0.15	12±0.1	14.2±0.05	12.3±0.05	12±0.14	12±0

Evaluation of the antioxidant potential

Total antioxidant activity

The Total antioxidant activity of intact cells was determined by measuring the inhibition of ascorbate autoxidation which is commonly used to evaluate antioxidative activity from microorganisms such as probiotics **(Zanoni *et al.*, 2008).** The results of Table 2 showed that the tested strain *Lb. plantarum* 15 has a total antioxidant activity of about 21.33%. **Lee *et al.* (2005)** showed that probiotic strains *Lb. rhamnosus* GG, *Lb. casei* KCTC 3260, *Lb. casei* 01 and *Lb. casei* KCTC 3109 have a total antioxidant activity of 40.1%, 46.2%, 20.2% and 9.8%, respectively. The importance of the antioxidant activity can be increased the tolerance of the strain to oxygen, extending survival in dairy products and/or pharmaceutical products and to ensure the effectiveness of probiotic strains for a long time. In addition, antioxidants probiotics are beneficial to human health by reducing the risk of accumulation of ROS and could potentially be used to reduce oxidative stress **(Abubakr *et al.*, 2012)**.

Table 2 The different antioxidant activity assays of the strain *Lb. plantarum* 15

Assay	**activity**
DPPH scavenging activity of cell pellet	82.65%
DPPH scavenging activity of the supernatant	75.21%
The scavenger capacity of hydroxyl radicals	46.15%.
Iron to chelating ability	20.52%
Total antioxidant activity	21.33%

DPPH

As shown in Table 2, the strain *Lb. plantarum* 15 present a good antioxidant activity the inhibition percentage of DPPH radical was 82.65% for the pellet and 75.21% for the supernatant. Thus, the scavenger effect of DPPH by intact cells is higher than that of the supernatant. **Afify *et al.* (2012)** found that probiotic strains *Propionibacterium freudenreichii* and *Lb. reuteri* have antioxidant capacity of 97.75% and 96.74%, respectively.

Resistance to hydrogen peroxide

The resistance *Lb. plantarum* 15 intact cells to ROS was evaluated in the presence of 1 mM hydrogen peroxide. The result indicate that *Lb. plantarum* 15 present a survival rate of 76.12% after 6 h of incubation in the presence of 1 mM hydrogen peroxide (Figure 3).

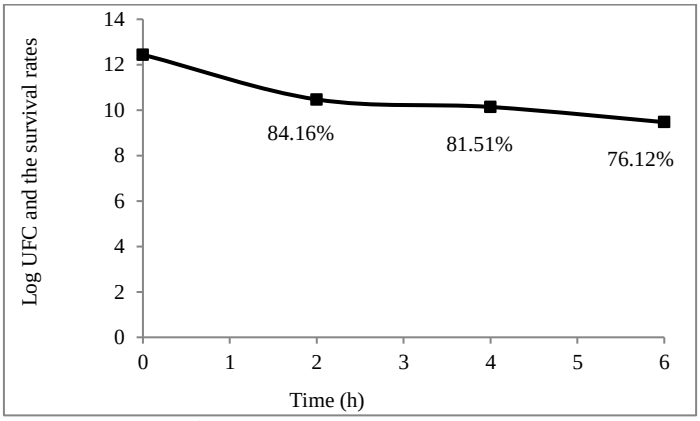

Figure 3 Resistance of *Lb. plantarum* 15 to hydrogen peroxide

The result obtained by **Zhang *et al.* (2011)** indicated that the two tested *Lactobacillus* strains SY13 and LJJ were resistant to 1 mM hydrogen peroxide and remained viable even after 8 h of incubation. Similar results were obtained by **Tang *et al.*, (2016).** In addition they reported that three antioxidant-related genes from *Lb. plantarum* MA2 were supposed to be responsible for resistance to H_2O_2 challenge. Therefore, it is necessary to further identify antioxidant related genes

The scavenger capacity of hydroxyl radicals

The use of the strain *Lb. plantarum* 15 is effective against hydroxyl radicals with a percentage of 46.15%. **Lee *et al.* (2005)** found that *Lb. casei* KCTC 3260 is able to survive until 7h and resist to hydroxyl radicals while *Lb. rhamnosus* GG showed no viability in the presence of hydroxyl radicals. It is reported that the

scavenger ability of the different types of ROS is considered as one of the main mechanisms of antioxidants presented by LAB. Hydroxyl radicals produced in the biological systems involved in the initiation of lipid peroxidation are highly reactive free radicals. They rapidly react with most molecules in living cells, such as amino acids, organic acids, phospholipids, DNA (**Kim *et al.*, 2006)**

Measuring the iron chelating ability

The probiotic bacterium *Lb. plantarum* 15 has a capacity to chelate iron ions with a percentage of 20.52%. **Kim *et al.* (2005)** found that the highest ferrous iron chelating activity was observed for the strain *Lb. casei* 01, reaching 72.06%. The chelating activity of the bacterial strains may be due to chelating agents that can capture metal iron and prevent iron metal from the catalyzing oxidation. Iron participates in hydroxyl radical generation and causes tissue damage by catalyzing the formation of ROS and stimulating lipid peroxidation **(Kim *et al.*, 2005)**.

CONCLUSION

This study showed that *Lb. plantarum* 15 strain isolated from milk resisted to acidity and to bile salts and displayed a broad antimicrobial activity against some tested bacteria. The intact cells and the cell free supernatant of *Lb. plantarum* 15 showed a good scavenger effect and good resistance to hydrogen peroxide. The possible use of this probiotic strain to protect cells and to reduce the possible damage of ROS should be further investigated. *In vivo* studies are needed to confirm the ability of this strain.

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