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 antioxidative 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 antioxidative 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
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.

Scavenging activity(%) = \frac{A_{\text{control}} - A_{\text{sample}}}{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 10^8 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:

\[ \frac{\log (\text{CFU})}{\log (\text{CFU})_0} \times 100 \]

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 10^8 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:

TAA = \left(1 - \frac{A_{265 (\text{sample})}}{A_{265 (\text{reference})}}\right) \times 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 10^8 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 mM 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:

Scavenging effect = \frac{([\text{As} - \text{Ap}])/(\text{Ab} - \text{Ap})]}{\times 100

Measurement of the chelating capacity of ferrous ion

A preparation of 0.5 mL of cell pellet (10^7 CFU.mL^-1) was added to a mixture of 0.1 mL of ascorbic acid (1%, w/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 Probiobacterium 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 spp. thermophilus ATCC 19258 and Lb. delbrueckii spp. 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, 14: Lb. helveticus, T5: Lactobacillus sp.](image)

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 Dowarad 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.

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 (Amnor et al., 2006).

**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

<table>
<thead>
<tr>
<th>Assay</th>
<th>activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH scavenging activity of cell pellet</td>
<td>82.65%</td>
</tr>
<tr>
<td>DPPH scavenging activity of the supernatant</td>
<td>75.21%</td>
</tr>
<tr>
<td>The scavenger capacity of hydroxyl radicals</td>
<td>46.15%</td>
</tr>
<tr>
<td>Iron to chelating ability</td>
<td>20.52%</td>
</tr>
<tr>
<td>Total antioxidant activity</td>
<td>21.33%</td>
</tr>
</tbody>
</table>

**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. Afiy et al. (2012) found that probiotic strains *Propionibacterium freudenreichii* and *Lb. reuteri* have antioxidant capacity of 97.75% and 96.74%, respectively.

**Resistence 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).

**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 H2O2 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
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 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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