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ANTIOXIDANT ACTIVITY AND EVALUATION OF LYSING CELL EFFICIENCY FROM DIFFERENT FRACTIONS OF *LACTOBACILLUS ACIDOPHILUS* NCFM®

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ABSTRACT

Several investigations have shown that *Lactobacillus acidophilus* has antioxidant systems, both in its whole cell and in extract of cell lysate. Thus, this study aimed at evaluating the efficiency of lysing cell to obtain extract of cell lysate, its antioxidant activityas well as intact cells, obtained from *Lactobacillus acidophilus* NCFM® cultures. Cell lysis was carried out using sonication and centrifugation processes, whose efficiency was measured by plating in depth after obtaining the extract process from cell lysate. The antioxidant activity was measured based on the method of reducing 2.2-diphenyl-1-picryl-hidrazylfree radical (DPPH) with different concentrations of sample and reducing power of ferric-ferrocyanide complex (Prussian Blue). The result was compared to the positive standardButylhydroxytoluene. The results indicated that there was no lysis in extract of cell lysate, and the applied processes only separated the colonies. During the DPPH and Prussian Blue trials, the antioxidant activity increased proportionally to the sample concentration and it was superior in the sample obtained by sonication and centrifugation when compared to intact cells. The non-occurrence of cell lysis may be explained by the fact that *Lactobacillus acidophilus* has thick cell wall, which may have interfered on ultrasonic forces action.

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INTRODUCTION

Acid-lactic bacteria consists in a group formed by different genera of Gram-positive bacteria, classified according to their morphological characteristics, fermentation, configuration of lactic acid produced, growth in different temperatures, besides their tolerance to acid or alkaline environments (Leroy, Vuyst, 2004). Some species can be used as food additives due to their contribution on foodstuff texture, increase in both digestibility and nutritional values (Hugenholtz, 2008). Besides, they can be added as starters to control the fermentation process, reliability, standardization and product quality (Leroy, Vuyst, 2004; O'Driscoll *et al*., 1997).So, their resistance is essential during the industrialization process, up from fermentation, drying and during their storage (Guchte *et al*., 2002). Among

contact with oxygen is considered a significant factor in cell

death (Dave, Shah, 1997). Furthermore, for the survival of lactic acid bacteria, as well as for oxygen and free radical tolerance, some species of *Lactobacilli*reduce the intracellular environment by expressing genes encoding proteins such as thioredoxin and glutathione, or increasing L-cysteine uptake that is disrupted to form the same molecules, major contributors in redox balance during bacterial stress conditions (Serrazanetti *et al*., 2009; Serrano *et al*., 2007). Lactic acid bacteria can eliminate free radicals formed by enzymes activity such as superoxide dismutase, thioredoxinreductase, glutathione peroxidase (GPx)/glutathione oxidase (GSH oxidase) and NADH oxidase/NADH peroxidase (Pophaly *et al*., 2002; Yu, 1995) and in some cases by the expression of a hemi or pseudo-catalase (Guchte *et al*., 2002). They can also develop systems with chelating ability of transition metals in

order to unfeasible oxidative reactions (Amanatidou *et al*., 2001). Bacteria can also prevent free radicals formation and change fatty acids composition of their cell membrane, by increasing oxygen consumption based on desaturase system (Guerzoni *et al*., 2001). The exopolysaccharides present in the cell wall also protect cells, since polymers with different physical properties, able to reduce reactive species of oxygen (Serrazanetti *et al*., 2009), form them. Besides these systems, it is possible to find high concentration of intracellular manganese in some strains of *Lactobacilli*that acts out as a non-enzymatic complex by removing superoxide anion, whose role is similar to superoxide dismutase (Archibald, 1986; Barnese *et al*., 2012). In this sense, the industry is highly interested in lactic acid bacteria since they can reduce toxicity of free radicals, consequently replace synthetic antioxidants, while they keep the stability of pharmaceuticals, cosmetics and foodstuffs. They also minimize oxidative effect caused by free radicals in human body (Dave, Shah, 1997; Hugenholts, 2008). Several investigations that were carried out both in vivo and in vitro (Kaizu *et al*., 1993; Kuda *et al*., 2010) have already shown that lactic acid bacteria have antioxidant systems, especially Lactobacillus acidophilus, since its activity is extensively researched (Amaretti *et al*., 2012; Lin, Yen, 1999), in its intact cells, cell lysate extracts and by different methods (Lin, Yen, 1999; Uugantsetseg, Batjargal, 2014).Thus, there is a great diversity of protocols even for the same bacterial strain, which makes it difficult to standardize and compare studies among researchers (Sharma, Bhat, 2015). Although cell lysate extract has been frequently evaluated, methodologies that are available for disruption of a cell wall from Lactobacillus spp do not establish the comparative efficiency of lysis processes (Afify *et al*., 2012; Choi *et al*., 2006; El Ghany *et al*., 2014; Lin, Chang, 2000; Ou *et al*., 2009; Pieniz *et al*., 2014; Saadatzadeh *et al*., 2013; Sharma, Bhat, 2015).

One of the applied methods in order to determine the antioxidant activity of lactic acid bacteria in intact cells and cell lysate extracts is the sequestering activity of 2.2-diphenyl-1-picryl-hydrazyl (DPPH) free radical. It is a simple, quick and inexpensive method in which DPPH is reduced by the action of an antioxidant (AH) or a radical species (R•), which causes a color change that can be monitored by decreasing absorbance (Brand-Williams *et al*., 1995). During this process, there is a yellow colored diphenyl-picryl-hydrazine (DPPH) generation. The antioxidant activity percentage (AA%) corresponds to the amount of DPPH that has been consumed by an antioxidant into the reaction medium (Brand-Williams *et al*., 1995). Another method applied in this study concerns about the reducing power, a technique that relies on the antioxidant ability to reduce ferricyanide ion to the ferrous form. In this test, the yellow color of the solution changes to green or blue, depending on the reducing power of the sample. This ability is evaluated by the formation of a complex between ferrous form and ferric chloride (Prussian Blue), which generates an increase in the solution absorbance (Santos *et al*., 2007). Such activity is measured by the change in absorbance at 700nm wavelength (Oyaizu, 1986). In the present study, those tests were applied based on the same principle of electron or hydrogen transfer because, according to some authors, the DPPH method does not consider some parameters in complex cellular environments, such as bioavailability and membrane permeability, although it is affected by light intensity, oxygen concentration and type of solvent (Xing *et al*., 2015).So ,this study aims at evaluating the efficiency of cell lysis to obtain lysed cell extract and its antioxidant activity as well as the intact cell obtained from cultures of *Lactobacillus acidophilus* NCFM®, isolated from a strain of the pharmaceutical industry. Methods to reduce 2.2-diphenyl-1-picryl-hydrazyl free radical (DPPH) and the ferrocyanide-ferric reductant-complex complex (Prussian Blue) were also used and compared to the positive standard BHT.

MATERIALS AND METHODS

Reagents, Instruments and General Experimental Procedures: The reactive 2.2-diphenyl-1-picryl-hydrazila (DPPH) (Sigma-AldrichCo.), Potassium ferricyanide, trichloroacetic acid, BHT, ferric chloride, anhydrous monobasic sodium phosphate and anhydrous bibasic sodium phosphate (VetecQuímicaFina Ltda.) and methanol (Merck) used in this study were analytically pure. The *Lactobacillus acidophilus* ATCC700.396 strain was from pharmaceutical origin and purchased at Labsynth. Agar and MRS from Man Rogosa and Sharpe broth (Oxoid) and TSB-Tryptic Soy Broth broth (Acummedia) also took part of this trial. The equipment was: vortex (Phoenix Luferco - AP56), Excelsa centrifuge model 280 R (Fanem-Brazil), ultrasonic sonicator USC750 (Ultra Sonic Cleaner-Unique). The absorption measurements were obtained by a UV-Vis spectrophotometer model 1600 (Nova) using glass cuvettes with 1.0 cm optical path.

Activation of lyophilized strains of *Lactobacillus acidophilus* NCFM®: In order to activate and prepare the cell suspension, 1 gram of *Lactobacillus acidophilus* NCFM® lyophilize ate was aseptically weighed and transferred into tubes containing 10 ml of 10% (w/v) Tryptic soy broth (TSB) that were subsequently incubated for a 24-hour period in an anaerobic jar at 37 ° C ± 1 (Redondo, 2008).

Cell Viability Assessment of *Lactobacillus acidophilus* NCFM®: After 24 hours of incubation, the culture was centrifuged for 5 minutes at 5000 rotation per minute (rpm) in a cooled centrifuge at 10 °C. The supernatant was discarded and the pellet was resuspended in a 10-mL buffered phosphate saline solution. Then, this suspension was subsequently homogenized in vortex and centrifuged for three consecutive times. The supernatant was discarded again and the pellet resuspended with a 10-mL buffered phosphate saline solution. Eight successive dilutions were carried out in saline solution from this bacterial suspension. Cell viability was determined by the depth seeding technique using the MRS agar medium. For counting, plaques with colonies that varied from 30 to 300 were used, than the individual result was multiplied by the inverse of its respective dilution (Zayed, 1995). Then, the analyses were carried out in duplicate with two replications and the average dilution results were expressed in CFUg-1 of product.

Preparation of Cell Lysate and Intact Cell Extract: The methodology was applied as described in literature, but with some changes (Bezkorovainy, Kot, 1998). *Lactobacillus acidophilus* NCFM® was inoculated in MRS broth and incubated at 37 °C for 24 hours in anaerobiosis jar. The procedure was repeated twice with 1 ml of the above inoculum for complete activation of this culture. After some bacterial growth, cells were centrifuged for 30 minutes at 4 °C and 5000 rpm. The precipitate was washed twice with buffered phosphate saline solution, and then resuspended in deionized water to prepare a free-cell extract and in saline solution to prepare the intact cell. The total number of cells was adjusted

to 10^9 CFU mL-1. Cells' disruption demanded a 25-kHz-frequency ultrasonic sonicator during five minutes at one-minute intervals in ice bath. Cell debris was removed by centrifugation (10,000 rpm for 10 minutes at 4 °C) and the supernatant was the cell free extract. At the end of the process, the cell lysate extract was plated by depth in order to prove the bacterial cell disruption. While for counting, plaques ranging from 30 to 300 colonies were used, multiplying the individual result by the inverse of the respective dilution (Zayed, 1995).This procedure was conducted in duplicate with one replication and the average dilution results were expressed in CFU mL-1.

Inoculum Cell Adjustment: The studied bacterium was analyzed using aliquots at different concentrations. So, it was necessary to adjust the amount of microbial cells and standardize them to compare the results in different fractions. Inoculum standardization was performed taking into account the absorbance equivalent to a 0.5 standard solution on McFarland scale at 625 nm wavelength, whose aimed optical density was 0.10, and this was equivalent to 12.30 x 109 CFU mL-1 (National Committee for Clinical Laboratory Standards, 2003).

Antioxidant Activity Evaluation by DPPH Method: This method was carried out according to Arasu *et al.* (Arasu *et al.*, 2014), with some changes. Aliquots (1.0, 1.5 and 2.0 mL) of a freshly prepared sample (109 CFU mL-1) were added to 1.0 mL of DPPH methanolic solution (50 mgL-1) (Abs.sample). The mixture was vigorously vortexed and incubated at room temperature in the dark for 30 minutes. The total analysis time did not exceed 2 hours. The negative control included water for samples from the cell-free extract and saline solution for samples containing intact cells, added with DPPH methanolic solution (Abs.control). Blank was made with added sample of methanol (Abs.blank).The absorbance of samples was measured in triplicate with a replication at wavelength of maximum absorption and antioxidant activity percentage (AA%), calculated according to Equation 1 (Arasu *et al.*, 2014):

$$\%AA = [1 - (Abs._{sample} - Abs._{blank}/Abs._{control})] \times 100$$

The obtained values were compared to the positive standard BHT, prepared in alcohol-acetone solution in µg mL-1.

Antioxidant Activity by Reducing Power Evaluation: The reducing power of samples was determined by the author (Oyaizu, 1986), with some changes. Samples (1.0, 1.5 and 2.0 mL) were mixed with 1.0 mL phosphate buffer (0.2 mol.L-1, 6.6pH) and 1.5 mL potassium ferricyanide at 1%aqueous solution (w/v). After 30 minutes of incubation at 50 °C, 1.5 ml of 10% trichloroacetic acid (m/v) were added to the mixture and vortexed. A 2-mL aliquot was taken from the supernatant, then 2.5mL distilled water and 0.5mL ferric chloride at 0.1% (m/v) were added. From the supernatant, a 2mL aliquot was prepared, then2.5mL of distilled water and 0.5mL of ferric chloride 0.1% (m / v) were added. The blank was prepared under the same conditions, using all reagents, but potassium ferricyanide.After 15 minutes, the sample was transferred to a cuvette and absorbance was measured at 700- nm wavelength. The readings were obtained in triplicate with one replication and compared to the positive standard BHT.

Statistical Analysis: The data had been obtained in antioxidant activity tests, they were put in tables, and average of triplicates was calculated with one replication (n = 6). It should be pointed out that the standard deviation of average (± S) was also calculated. Values for triplicated samples were statistically verified by variance (ANOVA) and Tukey tests using SISVAR software version 5.3. Differences that presented probability levels less than and equal to 5% ($p \leq 0.05$) were considered statistically significant.

RESULTS

Cell Viability Evaluation: *Lactobacillus acidophilus* culture was practicable, with a $2.0x10^9$ CFU g-1 microbial-cell counting. As the viability declared by the manufacturer was 10^9 CFU g-1, the obtained results showed that the product was in accordance with the information presented (Schmitt, 2014).

Preparation of Lysate Extract of Cells and Intact Cells: The numbers of viable cells before and after total sonication time (15 minutes) are shown in Table 1 and were obtained after plating, concerning depth to check the lysis process occurrence in *Lactobacillus acidophilus* NCFM® cell lysate extract.

Table 1. Viable cells, total viability and lysis in the intact cell*

Before sonication	After sonication
$12.3x10^9$CFU mL^{-1}	$15.0x10^9$CFUmL^{-1}

Antioxidant Activity Evaluation - DPPH Method: The Antioxidant activity (AA%) versus concentration for each sample was drawn according to the average of calculated percentages from antioxidant activity (Lee *et al.*, 1998; Sies, 1993). The results concerning quantitative evaluation of antioxidant activity (AA%) of different fractions from NCFM®*Lactobacillus acidophilus* (intact cell and sonicated sample) were determined by the DPPH method and are shown in Figure 1.

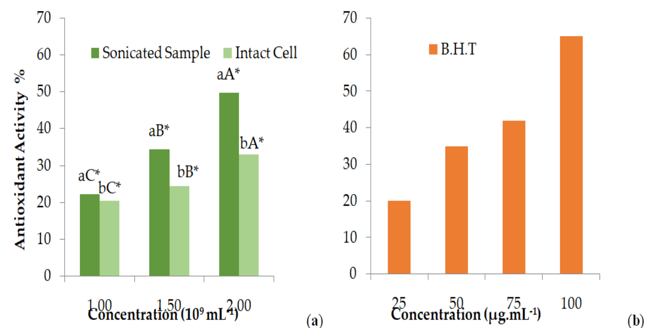

Figure 1.(a) Antioxidant activity (AA%) in different fractions and concentrations of *Lactobacillus acidophilus* standard NCFM®. Significant differences (p <0.05) among fractions (lowercase letters) and concentrations (uppercase letters) as sources of free radical scavengers. (b) Antioxidant activity (AA%) of positive standard BHT in different concentrations. Significant differences (p <0.05) when compared to samples (a) with positive standard BHT*(b)

Antioxidant Activity Evaluation - Reducing Power Method: The results were expressed in an absorbance table versus sample concentration and compared to the positive standard BHT. The highest absorbance value of a reaction mixture (average of replications) indicated higher reducing power of the sample and the obtained values are shown in Table 2.

Table 2. Absorbance obtained in different fractions of *Lactobacillus acidophilus* NCFM® by the Reducing Power method the positive standard BHT

Sample Concentration (mL)	Absorbance Intact Cell	Absorbance Sonicated Sample	BHT Concentration (μg mL^{-1})	Absorbance BHT
1.0	0.23 ^{bC*}	0.24 ^{aC*}	50	0.30*
1.5	0.24 ^{bB*}	0.25 ^{aB*}	75	0.40*
2.0	0.25 ^{bA*}	0.26 ^{aA*}	100	0.50*

Averages of triplicates with two replications. Averages followed by the same lowercase letter (fractions) and uppercase letters (concentrations) differ among themselves at 5% significance level (p<0.05) and among the positive standard BHT. The intact cell concentration was 10^9 CFU mL^{-1}.

DISCUSSION

As the cell viability declared by the manufacturer was 10^9 CFU g-1, the counting obtained showed that the culture was in accordance with the information presented for the manufacturer and expected to that indicated by the literature (Schmitt, 2014). Regarding the preparation of lysate extract of cells and intact cells, the results obtained after plating indicated that there was no lysis in to the evaluated cell culture, even using a methodology similar to several authors for the same purpose (Afify *et al*., 2012; Choi *et al*., 2006; El Ghany *et al*., 2014; Lin, Chang, 2000; Ou *et al*., 2009; Pieniz *et al*., 2014; Saadatzadeh *et al*., 2013; Sharma, Bhat, 2015). As observed in Table 1, the number of cells increased indicating that the methodology was able to separate the colonies by increasing the sample cells number. The inefficiency of lysis process can be explained based on the fact that *Lactobacillus acidophilus* presents thick cell wall formed by multilamellar layers of peptideoglycans (90%), in addition to neutral polysaccharides, teichoic and lipoteichoic acids (Delcour *et al*., 1999). The whole structure is also surrounded by an outer envelope of proteins, called S-layer (Jafarei, Ebrahimi, 2011), which may have hampered the action of ultrasonic power. Thus, the sample called cell lysate extract was pointed out as sonicated sample in this research. The cell wall structure of *Lactobacillus acidophilus* is shown in Figure 2.

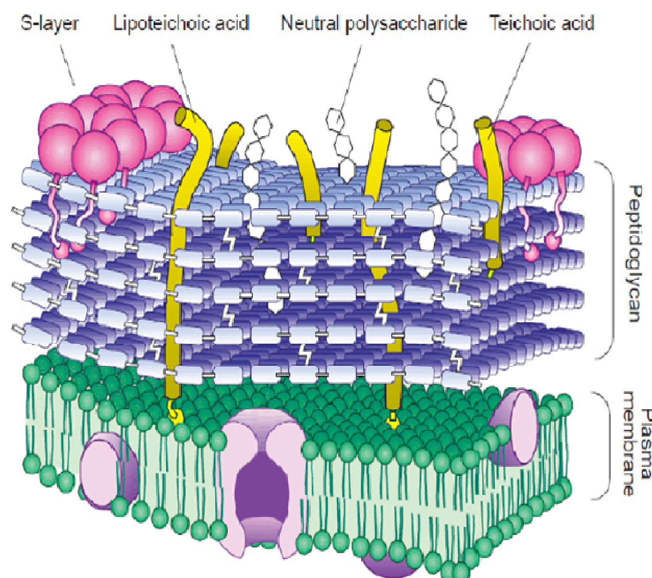

Figure 2: Cell wall structure of *L. acidophilus*.Bilipidic plasma membrane with embedded proteins is covered by a multilayered peptidoglycan shell, decorated with neutral polysaccharides, lipoteichoic acids and teichoic acids, surrounded by an outer envelope of S-layer proteins; And, in order to make things clear, cell wall-associated proteins are not discarded (Delcour et al., 1999)

It was observed in the evaluation of the antioxidant activity by DPPH method that both fractions showed antioxidant activity and proportional to its concentration, as they presented significant differences (p <0.05) in the potential as sources of free radical scavenging substances under different concentrations for them both. This behavior was in agreement with those ones described in previous studies, where the authors determined dose-dependent activity by the same method (Arasu *et al*., 2014; El Ghany *et al*., 2014; Jain *et al*., 2009; Kuda *et al*., 2010; Wang *et al*., 2008). The antioxidant activity had its highest value in the sonicated sample (49.68%) when compared to the intact cell answer (32.95%), a result evidenced in 2.0mL concentration, who has observed difference in relation to the intact cell was 16.73%. This suggested that cells that are more available have contributed particularly to the sequestering action of free radicals, mainly and more effectively, after sonication and separation of colonies. Other researchers (Afify *et al*., 2012; El Ghany *et al*., 2014; Ou *et al*., 2009) observed similar results when they analyzed different *Lactobacilli*and recorded that cell lysate extract showed a markedly greater antioxidant activity than into an intact cell. However, as previously mentioned, the same authors did not determine lysis efficiency. Thus, it was not possible to establish whether the antioxidant activity was due to lysate extract or the greater number of intact cells.

When these analyses were compared to the positive standard BHT, there was a great difference (15.32%) in sonicated sample, but this answer was 32.05% in intact cell under 2.0-mL concentration. Sharmaand and Bhat (2015), evaluated four species of *Lactobacilli* regarding inhibitory capacity against DPPH radical and compared with BHT at the same concentrations of the samples, so, they recorded the best activity (56.84%) with the highest concentration, whereas for BHT this response was 69.29%. According to that study, there was a 12.45% difference, which was much lower than the one recorded in this study (Lee *et al*., 2006). This fact can be explained by the strain used as well as the number of cells evaluated. However, it was possible to determine that the higher antioxidant activity was presented by positive standard, even in low concentrations. The reducing ability showed by reducing power method has been observed according to the analysis on Table 2, which was proportional to the concentration analyzed and, therefore, dose dependent. It was also observed that the sonicated sample showed a higher reducing power (0.26) than the intact cell (0.25) and, although the difference in absorbance value was lower, it was statistically significant (p<0.05). The small difference between the samples in this analysis corroborates the finding previously on the cellular lysis and indicates that there was only a separation of the colonies and not the disruption of the bacterial cells. Then the value found for the sonicated sample was higher since more colonies were present in the sample.

The sonicatedsample also presented an absorbance answer close to the positive standard BHT at the lowest concentration (0.30). Thus, the reductive activity of *Lactobacillus acidophilus* NCFM® under study may be due to the presence

of cysteine-containing molecules, such as glutathione and thioredoxin (Serrano *et al*., 2007). Glutathione is a tripeptide reducer that can be found in cytoplasm, which is able of defending cells against oxidative radicals. Its potential is mainly due to the sulfhydryl (SH) group involved in most of its reactions, which makes it a compound able to donate electrons, both for elimination of reactive oxygen species produced, for example, in cellular respiration, as well as for metabolic reactions and reduce iron ions (Kim *et al*., 2005; Mrvčić *et al*., 2012; Serrazanetti *et al*., 2009; Yu, 1995). Reduced glutathione (GSH) transfers electrons to hydrogen peroxide in order to change it into water, and this process is catalyzed by the enzyme glutathione peroxidase (GPx). When glutathione is oxidized, it becomes glutathione disulphide (GSSG) by the enzyme glutathione oxidase action. Glutathione reductase (GSR) is the main responsible for reduced glutathione disulphide (GSSG) into a reduced glutathione (GSH), its active form. Glutathione reductase uses hydrogen electrons of Nicotinamide adenine dinucleotide phosphate (NADPH) from the pentose phosphate pathway (Jansch *et al*., 2007). Glutathione reductase can also use Fe+3 electrons formed during Fenton's reaction, in order to reduce iron, help keeping thiol levels in the environment, and ensure a reduced microenvironment. Glutathione reductase activity has been demonstrated for *Lactobacillus acidophilus* NCFM® the clear role of such enzyme as a mechanism of protection against oxidative stress (Girgis *et al*., 2000).

Thioredoxin (TRX) is a class of small proteins that have two cysteine residues in their active site, which reversibly oxidizes when present in a redox reaction. Trx is then reduced by Trxreductase, taking electrons from NADPH. While reduced thioredoxin contributes to keep redox balance into the cell (Serrano *et al*., 2007). The antioxidant activity of an intact cell can also be attributed to the composition of its cell wall, especially the exopolysaccharides. Among them, several polymers can be found from different chemical compositions and physical properties like sugars, proteins, peptides and microelements (Kuda *et al*., 2010; Leroy, Vuyst, 2004; Li *et al*., 2014). Pan and Mei (2010) have already reported that exopolysaccharides of *Lactococcuslactis* subsp. *lactis* 12 have antioxidant ability both in vitro and in vivo. Other authors have demonstrated that these polysaccharides, isolated from *Bifidobacteriumanimalis* RH can inhibit lipid peroxidation and have some activity against superoxide and hydroxyl radicals similar to ascorbic acid (Xu *et al*., 2010). Li *et al*. (2012), evaluated isolated *Lactobacillus plantarum* C88 from Chinese fermented foods, observed that this bacterium also inhibits lipid peroxidation, and can eliminate reactive oxygen species by enzymatic and non-enzymatic components regulation. In another study, the authors attributed the antioxidant capacity of exopolysaccharides to their ability on increasing the activity of intracellular antioxidant enzymes, such as superoxide dismutase and non-enzymatic levels of defense (Li *et al*., 2013; Zhang *et al*., 2011). Seo *et al*. (2015),analyzed exopolysaccharide of a *Lactobacillus plantarum* YML 009 culture, they observed that exopolysaccharides had marked ferric ions (Fe3+) reducing ability along with electron donor properties for neutralizing free radicals by forming stable products. Liu and Pan (2010), also observed the reducing ability of exopolysaccharides extracted from *Lactobacillus paracasei* NTU 101 and *Lactobacillus plantarum* NTU 102 when they used K3Fe (CN)6 as a reducing method . These studies would explain the reduction of both ferricyanide and DPPH in such study. Another mechanism for the intact cell action is the chelating capacity of this bacterium. According to Bezkorovainy and Kot (1998), *Lactobacillus acidophilus* can accumulate Fe+2 in its inner part, but it should be in millimolar concentrations, both by biosorption and bioaccumulation. Firstly, there is an internalization of Fe+2 followed by intracellular oxidation, so that Fe+2 become sun available for Fenton's reaction. It is believed that this reaction is made from ferroxidases, as it was demonstrated in *Streptococcus thermophilus*, *Lactobacillus plantarum*and *Bifidobacteriumthermophilum* (Bezkorovainy, Kot, 1998; Kot *et al*., 1991; Kot *et al*., 1994; Kot *et al*., 1995). In addition, Fe+2 can be oxidized extracellularly by Hydrogen peroxide, produced by bacterial cells during their growth in O2 presence, generating Fe (OH) 3, which binds itself to the bacterial surface at 37 ºC (Mrvčić*et al*., 2012). According to a study carried out by Li *et al*. (2014), exopolysaccharide of *Lactobacillus helveticus* MB2-1 was able to minimize Fe+2concentration. This study could also record that polysaccharides with the highest percentage of uronic acid were negatively charged, whose result was a better chelating activity of this bacterium. NADH oxidase/NADH peroxidase is a common enzymatic system to lactic acid bacteria and it may have contributed to the results [57-58]. Microaerophilic lactic acid bacteria, such as *Lactobacillus acidophilus*, use molecular oxygen to regenerate NAD+ from NADH during the fermentation process by NADH oxidase activity (Talwalkar, Kailasapathy, 2004).This reaction produces hydrogen peroxide or water that depends on the enzyme ability to transfer 2 or 4 electrons. When the final product is hydrogen peroxide, it is used by NADH peroxidase as the final electron acceptor, generating a water molecule (De Angelis, Gobbetti, 2004; Guchte *et al*., 2002; Miyoshi *et al*., 2003). Thus, it is possible that these enzymes have transferred electrons to the radical DPPH and caused their reduction. However, in the trial concerning the reducing power, in NAD+ regeneration as well as in human organism, electrons can be transferred to groups containing iron, which would explain the reduction of ferricyanide ion (Arasu *et al*., 2014; Bezkorovainy, Kot, 1998; Zayed, 1995).

Conclusion

The results express that *Lactobacillus acidophilus* NCFM® has a thick cell wall, which made it difficult to disrupt a cell, therefore, other methods are necessary for its lysis. However, intact cells in the sample represents a potential source of natural antioxidants, since it has significant antioxidant activity at different concentrations, especially when there was a larger number of intact cells in this sample (sonication). The data obtained along the reducing power trial confirmed the ones recorded for the DPPH trial in this research. This has shown that, despite the differences in methodology, samples were efficient on reducing both DPPH radical and ferricyanide ion. These data suggest that *Lactobacillus acidophilus* NCFM® is a promising antioxidant component that can be used to keep stability of pharmaceuticals, cosmetics and foodstuffs as well as minimize the oxidative effect caused by free radicals in human body.

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