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# Antibacterial and Antioxidant Activity of Extracts from Selected Probiotic Bacteria

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**Abstract**

Probiotic extracts can potentially be used as bio-preservatives and in reduction of oxidative stress. The study investigated the antibacterial and antioxidant activity of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains identified using molecular techniques. The quantitative microplate method, which employed *p*-iodonitrotetrazolium (INT) and the method by Brand-Williams et al. (1995) were employed to investigate quantitatively the antibacterial and the antioxidant activity, respectively, of probiotic extracts. The MIC values extracts from most probiotic strains, tested against indicator bacterial pathogens, were in the range of 1.25 – 5 mg/mL while that of *Lb. casei* strain B and *Lc. lactis* subsp *lactis* strain X was at least 20 mg/mL after 24 h of incubation at 37°C. At the highest extract concentration of 20 mg/mL used in the study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities of 77.9 - 86.1%, 45.7 - 86.4% and 36.9 – 45.8% respectively. Quantitative antibacterial and antioxidant activities of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains was determined for the first time.

**Keywords:** antibacterial, antioxidant, *Lactobacillus*, methanol extract, probiotic

## 1. Introduction

Probiotics are defined as 'live microorganisms of human intestinal origin, which, in adequate quantities, impart health benefits to the consumer beyond basic nutrition' (FAO/WHO, 2002). Several of the probiotic species belong to the *Lactobacillus* and *Bifidobacterium* genera and have been used extensively in foods, beverages and therapeutic formulations as probiotic strains due to their GRAS (generally recognized as safe) status (Franz et al., 2003; Nyanzi & Jooste, 2012). The potential health benefits of probiotics include improvement in gut health and lactose intolerance, prevention of diarrhoea, constipation alleviation, reduction in serum cholesterol, prevention of vaginitis and intestinal infections, management of diabetes mellitus, prevention of hepatic diseases and inflammatory bowel disease, risk reduction in colon tumours and cancer, oxidative stress reduction and host immune system modulation (Marchand & Vandenplas, 2000; Holzapfel & Schillinger, 2002; Blandino et al., 2003; Franz et al., 2003; Rousseau et al., 2005; Shen et al., 2011; Ejtahed et al., 2012; Li et al., 2012; Sharma et al., 2014; Nyanzi et al., 2014). A number of probiotic strains antagonize enteropathogens and the proposed mechanisms (although not always clear) include competition for nutrients and receptor sites, aggregation with bacterial pathogens and hydrogen peroxide production, production of bacteriocins (protein-like substances) and, stimulation of the immune system (Shalev et al., 1996; Atanassova et al., 2003; Strus et al., 2005; Guéniche et al., 2010; Bendali et al., 2011; Nyanzi et al., 2014).

Diarrhoea is a global public health challenge and annually as many as 2 million people, mainly under the age of 5 years, die in the developing world (Bendali et al., 2011). Enteropathogenic *E. coli* is the major cause of diarrhoea while *Salmonella enterica* Typhimurium is linked to human gastroenteritis (Bendali et al., 2011). Antibiotics can reduce infections, however, antibiotic resistance as in the case of methicillin-resistant *Staphylococcus aureus* implies that there is a need to have alternative pathogen inhibitors (Yasunaka et al., 2005).

Plant extracts have been shown to have antimicrobial activity, however, cytotoxicity can be a concern while, on the other hand, probiotics have not been shown to be toxic.

Antibacterial effects of lactic acid bacteria (LAB) have been reported (Ström et al., 2002). The antibacterial activity of the probiotic strains is largely attributed to low pH in their growth environments and the production of organic acids and bacteriocins (Atanassova et al., 2003; De Muynck et al., 2004; Strus et al., 2005). There are reports of the demonstrated antimicrobial activity of organic acids (Ström et al., 2002; De Muynck et al., 2004), probiotic culture supernatants and their ethyl acetate extracts (Lavermicocca et al., 2000). Besides antimicrobial activity, probiotics are also reported to have antioxidant and anti-ageing activities (Ejtahed et al., 2012; Li et al., 2012). The presence of excessive reactive oxygen species (ROS) such as hydroxyl radicals (OH), superoxide anions ($^{\cdot}O_2^-$) and hydrogen peroxide ($H_2O_2$), released during cellular metabolism, in the absence of adequate antioxidants can cause *in vivo* oxidative damage to biomolecules such as proteins, lipids and chromosomes (Choi et al., 2002; Kullisaar et al., 2002; Wang et al., 2009; Li et al., 2012). *Lactobacillus* strains with proven or demonstrated antioxidant activities *in vitro*, may be applied in the prevention or reduction of oxidative stress. However, information relating to the antibacterial and antioxidant activity of extracts from freeze-dried probiotic bacterial cells is scanty. Consequently the aim of the present study was to determine possible antibacterial and antioxidant activities of methanol extracts from freeze-dried preparations of selected *Lactobacillus* strains.

## 2. Materials and Methods

### *2.1 Probiotic Cultures*

A range of *Lactobacillus* strains were isolated from probiotic dairy products, pharmaceutical probiotic supplements while others were obtained from supplier companies. Thirteen *Lactobacillus* strains examined in this study were selected from the original number of 32 isolates on the basis of their bile tolerance, acid tolerance and antibiotic resistance profiles (Nyanzi, 2013). Phenotypic identification of the probiotic isolates was done using API 50 CHL galleries as well as the API WEB (V3.2 and V5.1) software (API system, Biomerieux, France). The probiotic strains were preserved as frozen cultures according to the procedure described by Nyanzi (2007).

### *2.2 Confirmation of Identity of Presumptive Probiotic Isolates*

The identities of the isolates were confirmed by employing molecular typing techniques (Nyanzi et al., 2013) and the MEGA 6.06 software (Tamura et al., 2013) for phylogenetic analysis using sequences of the 16S rRNA gene [amplified using 27F and 1492R universal primers (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3' respectively; Guo et al., 2010)], and *rpoA* and *pheS* protein-coding genes [amplified using primer pairs, rpoA-21-F / rpoA-23-R (5'-ATGATYGARTTTGAAAAACC-3' / 5'-ACHGTRTTRATDCCDGCRCG-3') and pheS-21-F / pheS-23-R (5'-CAYCCNGCHCGYGAYATGC-3' / 5'-GGRTGRACCATVCCNGCHCC-3') respectively; Naser et al., 2005].

The PCR products were subjected to gel electrophoresis and sequenced in a Genetic analyser ABI PRISM$^{TM}$ 3100 (Applied Biosystems, USA). The generated sequences were edited using softwares, Chromas Lite 2.0 and BioEdit v. 7.0.9 (http://en.bio-soft.net/format.html/BioEdit). The isolates were identified by comparing the sequences obtained to those in the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/blastn/) using the *blastn* search option.

### *2.3 Indicator Bacterial Pathogens*

The indicator bacterial pathogens used were from the American Type Culture Collection (ATCC) and included the following: *Escherichia coli* ATCC 8739, *E. coli* ATCC 11775, *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 12600 and *Salmonella typhimurium* ATCC 14028. The cultures were maintained in nutrient broth and were preserved as frozen cultures as described by Nyanzi (2007).

### *2.4 Probiotic Bacterial Cell Mass Growth and Extraction Procedures*

The procedure followed was as described by Nyanzi et al. (2014). In brief, two litres of De Man, Rogosa and Sharpe broth (MRS broth), in a 2L Duran Schott bottle, were inoculated with 4% of an overnight probiotic broth culture and incubated in a water bath at 37°C for 18 to 20 h. The MRS broth culture was centrifuged (Sorvall RC 6 centrifuge, USA) at 7000 rpm at 4°C for 10 minutes (min) to separate the cell pellet from the supernatant. The cell pellet was washed twice with phosphate buffer saline (PBS) and then suspended in sterilized, distilled water. The suspension was frozen at -80°C for at least 4 h and then freeze-dried at -85°C for 24 h. The freeze-dried bacterial cell mass was then subjected to particle reduction using a mortar and pestle. The powdered bacterial cell mass was stored in sterile bottles ready for particle size analysis and extraction as described by Nyanzi et al. (2014).

The powdered cell pellet was extracted using methanol. Acetone was not used because in a previous study (Nyanzi et al., 2014), such extracts were found to be ineffective. The cell powder (2 g) and the methanol extractant (10 mL) were mixed, vigorously shaken and then sonicated (Bandelin electronic sonicator, Pro-Nr 780.06021340.008, Berlin, Germany) at 100% power (120/240 W, 1.2 A, 230 V, 35 KHz frequency) for 15 min prior to filtering using Whatman No. 1 filter paper. The filtrate was dried in a fume cupboard at room temperature and the dried extract was weighed before storage.

*2.5 Determination of the Minimum Inhibitory Concentrations (MICs) of Extracts*

The two-fold serial-dilution microtitre plate method described by Eloff (1998), which employs *p*-iodonitrotetrazolium (INT) violet as an indicator of microbial growth, was used to determine the MICs of the extracts obtained in section 2.4. The MIC value was recorded as the lowest concentration of extract that inhibits growth of test organisms (Shai et al., 2009). A 100 µL volume of sterile distilled water was pipetted into each of the wells of the microtitre plate.

2.5.1 Antibacterial Assay

One hundred microliters (100 µL) of the extracts (80 mg/mL) dissolved in 25% acetone in water was added to separate microtitre plate wells in row A in duplicate. After thorough mixing of the well contents in row A, 100 µL was withdrawn and added to the next well in row B and thoroughly mixed. The procedure was continued until all the wells were mixed with extract except for row H. Some of the columns were used for sterility control and growth control in which cases test bacteria were not added or extracts were replaced with a standard antibiotic agent (0.1 mg/mL gentamicin). Test bacterial pathogen cultures were prepared by adjusting the turbidity of the bacterial suspension to a McFarland standard 0.5 that was equivalent to a concentration of 1-5 x $10^7$ cfu/mL and was further diluted (1:100) in nutrient broth to obtain a final concentration of 1-5 x $10^5$ cfu/mL. A volume of 100 µL of the broth culture of each of the test pathogens was distributed into separate wells with the exception of the wells reserved for the sterility controls. The microtitre plates were then sealed in a plastic bag and incubated at 37°C for 16 -18 hours (hr) at 100% humidity. To demonstrate the presence or absence of growth, 40 µL of 0.2 mg/mL of INT violet, (dissolved in sterile distilled water) was added to each of the wells and the plates were incubated for a further 2 hours to promote visualization. Bacterial growth was shown by the development of a red colour arising from the formation of the red/purple formazan (Eloff, 1998).

2.5.2 Antioxidant Activity of Extracts

A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Cat. No.: D913-2, Sigma-Aldrich, Germany) was prepared at a concentration of 3.7 mg DPPH/ 100 mL of absolute methanol (analytical grade). The initial concentration of probiotic extracts was 80 mg/mL in 25% acetone (Sigma-Aldrich). This concentration was subjected to a two-fold serial dilution using 25% acetone until a final concentration of 1.25 mg/mL was obtained. The initial concentration of ascorbic acid (control) was also two-fold serially diluted until a final concentration of 0.03125 mg/mL was obtained. The dilutions of the extracts and ascorbic acid were performed in a microplate deep well (Cat. No.: 219012, 96-well 1 mL round well, Porvair Sciences, UK). The antioxidant assay was performed in a 96 well microtitre plate with each well filled with 200 µL of DPPH solution. The extracts and ascorbic acid along with their sequential dilutions, in 50 µL volumes, were introduced to the microtitre plate using a multi-channel pipette. The assay was replicated 5 times and 25% acetone was used as the negative control. The method was based on the description by Shikanga et al. (2010). A spectrophotometric reading was taken in an ELX 800 Universal Microplate Reader (Serial No. 185050, BioTek Instruments Inc., USA). Corrected antiradical activity was calculated using the following formula:

Total DPPH antiradical activity (%) = [1-(A570 nm sample / A570 nm control)] X 100] (Ng et al., 2011).

## 3. Results and Discussion

*3.1 Confirmation of Identity of Bacterial Isolates*

The studied isolates were identified based on their 16S rDNA sequences. The identity of the study isolates was confirmed using *rpoA* and *pheS* gene sequences (Table 1). The 16S rRNA gene is the most frequently used but not very discriminative for sole use between closely related strains (Rajendhran & Gunasekaran, 2011), while the *rpoA* and *pheS* genes are reported to be more discriminative than the former (Naser et al., 2005; Naser et al., 2007). The measurable superiority of *rpoA* and *pheS* gene sequencing over the 16S rRNA gene sequencing in discriminating and identifying the LAB isolates was largely illustrated by Nyanzi et al. (2013). The strains studied were regarded as probiotic since most species or strains used as such belong to the genera *Lactobacillus* and *Bifidobacterium* (Blandino et al., 2003; Manning & Gibson, 2004; Commane et al., 2005; Picard et al., 2005) and are generally regarded as safe (Prado et al., 2008; Liu et al., 2009; Lannitti & Palmieri, 2010).

Table 1. Confirmation of the identity of the probiotic isolates used in the present study

| Isolate code | 16S rDNA sequence identification | *pheS* gene sequence identification | *rpoA* gene sequence identification |
|---|---|---|---|
| M | *Lb. rhamnosus* | *Lb. rhamnosus* | *Lb. rhamnosus* |
| N | *Lb. casei* | *Lb. casei* | *Lb. casei* |
| O | *Lb. rhamnosus* | *Lb. rhamnosus* | *Lb. rhamnosus* |
| P | *Lb. casei* | *Lb. casei* | *Lb. casei* |
| C | *Lb. rhamnosus* | *Lb. rhamnosus* | *Lb. rhamnosus* |
| D | *Lb. rhamnosus* | *Lb. rhamnosus* | *Lb. rhamnosus* |
| Y | *Lb. rhamnosus* | *Lb. rhamnosus* | *Lb. rhamnosus* |
| B | *Lb. casei* | *Lb. casei* | *Lb. casei* |
| X | *Lc. lactis* subsp. *lactis* | *Lc. lactis* subsp. *lactis* | *Lc. lactis* subsp. l*actis* |
| U | *Lb. acidophilus* | *Lb. acidophilus* | *Lb. acidophilus* |
| Z | *Lb. helveticus* | *Lb. helveticus* | *Lb. helveticus* |
| V | *Lb. acidophilus* | *Lb. acidophilus* | *Lb. acidophilus* |
| W | *Lb. acidophilus* | *Lb. acidophilus* | *Lb. acidophilus* |

### *3.2 Particle Size of Extracted Powder from Freeze-Dried Cells of LAB*

The particle size of powdered freeze-dried cells of the LAB strains was in the range 44.85 – 70.22 μM (Nyanzi, 2013). This meant that the differences were generally marginal and the powder samples would be expected to have relatively similar extractability. However, the yield of the extracts ranged between 4.14% and 42.08% (Nyanzi, 2013) and the differences may be attributed to species and strain differences of the probiotic organisms in Table 1.

### *3.3 The Minimum Inhibitory Concentrations (MIC) of Extracts*

#### 3.3.1 Antibacterial Activity of extracts from Freeze-Dried Probiotic Bacteria

The probiotic *Lactobacillus* methanol extracts had a dose related inhibition of the indicator bacterial pathogens *E. coli* ATCC 8739, *S. aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028. Table 2 presents a summary of the MIC values of the extracts from selected strains of *Lactobacillus* species against the indicator pathogens after different periods of incubation at 37°C.

Table 2. The MICs of probiotic extracts of the *lactobacillus* test strains against the selected bacterial pathogens after different time intervals

| Probiotic strain extract | MICs (mg/mL) against indicator organism | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* ATCC 8739 | | | *S. aureus* ATCC 6538 | | | *Sa. typhi* ATCC 14028 | | |
| | 24 h* | 48 h | 60 h | 24 h | 48 h | 60 h | 24 h | 48 h | 60 h |
| U | 5 | 5 | 7.5 | 2.5 | 5 | 10 | 5 | 5 | 5 |
| V | 5 | 5 | 10 | 2.5 | 5 | 10 | 5 | 5 | 10 |
| W | 10 | 10 | 20 | 2.5 | 10 | 10 | 5 | 10 | 20 |
| Z | 5 | 5 | 10 | 2.5 | 5 | 5 | 5 | 5 | 5 |
| M | 5 | 10 | 10 | 5 | 5 | 10 | 5 | 10 | 10 |
| Y | 2.5 | 5 | 5 | 2.5 | 5 | 5 | 2.5 | 5 | 10 |
| O | 5 | 5 | 10 | 5 | 5 | 10 | 5 | 10 | 10 |
| D | 2.5 | 5 | 10 | 2.5 | 5 | 5 | 2.5 | 5 | 5 |
| C | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 10 | 10 |
| X | 20 | >20 | >20 | 10 | 20 | >20 | 20 | >20 | >20 |
| P | 5 | 10 | 10 | 5 | 10 | 10 | 5 | 10 | 10 |
| B | 20 | >20 | >20 | 10 | >20 | >20 | 20 | >20 | >20 |
| N | 2.5 | 5 | 10 | 1.25 | 5 | 5 | 2.5 | 5 | 10 |

* h= hours.

The MIC value of extracts from *Lb. acidophilus* strain U and V, and *Lb. helveticus* strain Z was 2.5 mg/mL against *S. aureus* ATCC 6538 while against *E. coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028, it was 5 mg/mL after 24 h of incubation at 37°C. This implied that inhibition was not only specific to the probiotic strain, but also to the pathogen strain.

The MIC value of extracts from *Lb. rhamnosus* strain Y and D was 2.5 mg/mL while that of *Lb. rhamnosus* strains M, C and O was 5 mg/mL against indicator pathogens after 24 h of incubation at 37°C. *Lactobacillus casei* strain B and *Lactococcus lactis* subsp. *lactis* strain X had a MIC value of 20 mg/mL, or more, against indicator bacterial pathogens. However, *Lb. casei* strain N and P had values of 1.25 - 2.5 mg/mL and 5mg/mL as MIC values against the indicator pathogens after 24 h of incubation at 37°C.

Overall, extracts from *Lb. rhamnosus* strain Y and D and *Lb. casei* strain N had the highest antimicrobial activity since their MIC values, in the range 1.25 – 2.5 mg/mL after 24 h, were lower than extracts from the other *Lactobacillus* strains tested. Since extracts from *Lb. rhamnosus* strains (M, C and O) and *Lb. casei* strain P had an MIC of 5 mg/mL, they could also be considered along with extracts from strains Y, D and N for further investigation.

From the results in Table 2, it was clear that some extracts were bacteriostatic because the MICs increased after an extended incubation period. However, when 100 µL from row 'A' wells of the microtitre plate with a concentration of 20 mg/mL, was aseptically spread-plated on nutrient agar (Merck, Germany) plates, no growth was observed after incubating aerobically for 24 h at 37°C. In contrast contents from corresponding dilutions in rows 'F' and 'H' of the microtitre plate with concentrations of 0.625 mg/mL and 0 mg/mL respectively, led to confluent growth on the nutrient agar petri-plates. This indicates that at higher concentrations the extracts were bactericidal.

With the exception of recent results of Nyanzi et al. (2014), there has been hardly any report on determining the MICs of methanol extracts from probiotic cells. Other scientists have found evidence for the anti-pathogenic activity of probiotics using supernatants in agar disc diffusion methods (Voulgari et al., 2010; Bendali et al., 2011) or a spot-on lawn method (Bendali et al., 2011). These authors found that *S. aureus, L. monocytogenes* and other bacterial pathogens were inhibited by by-products of LAB and that *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. faecalis*, *B. cereus* and *S. aureus* were inhibited by *Lb. paracasei* subsp. *paracasei* (Bendali et al., 2011). Using the spot-on lawn method, pathogen inhibition by LAB was attributed to bacteriocins (De Muynck et al., 2004) or to fermentation by-products such as organic acids, hydrogen peroxide and antibiotics (Ström et al., 2002; Atanassova et al., 2003; De Muynck et al., 2004).

A major problem with these methods is that agar diffusion and spot-on lawn methods do not provide results that can be compared between different laboratories. Furthermore, if plant extracts contain non-polar antimicrobial compounds, agar diffusion methods under estimate the activity because the non-polar compounds would not readily diffuse in an aqueous agar matrix (Eloff & McGaw, 2014). By determining the MICs, the potential that these preparations could be active *in vivo* can be evaluated.

There have been many viewpoints on what should be considered as an interesting MIC for plant extracts. These values varied from 8 mg/mL (Fabry et al., 1998) to 0.1 mg/mL (Eloff, 2004; Rios & Recio, 2005; Cos et al., 2006). The lowest MIC found in this study 1.25 mg/mL. In evaluating the probability of *in vivo* efficacy, the aim of using the extract should be kept in mind. For an oral dosage of a plant extract parental concentrations of higher than 0.1 mg/mL could be too high. In the case of probiotics, which are not known to be toxic, a concentration of probiotic extracts greater than 0.1 mg/mL may arguably not be considered to be too high.

Other mechanisms such as pathogen adhesion prevention against infections may also play a role (Johnson-Henry et al., 2007). Surface-layer protein (SLP) extracts from the paracrystalline layer on the outside of the cell wall of probiotic strain *Lb. helveticus* R0052, inhibited adhesion of *E. coli* 0157:H7 to epithelial cells (Johnson-Henry et al., 2007). Pathogen antagonism against adhesion to epithelial surfaces (Johnson-Henry et al., 2007) and inhibition of their growth, as observed in the present study, is indicative of potential benefits that can be derived from non-viable probiotic cell extracts. This implies that probiotic bacterial extracts may have the potential for application as biopreservatives. In the present study, lower extract concentrations of some LABs were bacteriostatic and higher extract concentrations were bactericidal.

3.3.2 Antioxidant Activity of Probiotic Cell Extracts

The 2,2-Diphenyl-picrylhydrazyl (DPPH) free radical reduction spectrophotometric assay has been used to determine the antioxidant activity of extracts and compounds from different sources (Armatu et al., 2010). The assay depends on the stability of DPPH free radicals, which get decolourised by an effective antioxidant (Armatu

et al., 2010). The antioxidant activity of methanol extracts of probiotics has been determined by reaction with 2,2-Diphenyl-picrylhydrazyl (DPPH), a stable free radical, in a solution of methanol (Brand-Williams et al., 1995).

Table 3 shows the antioxidant activity of the methanol extracts from freeze-dried probiotic bacteria at concentrations in the range of 1.25 – 20 mg/mL. The anti-radical activity was presented in the form of percentages (%) by which different concentrations of the probiotic extracts reduced the absorbance of control DPPH in methanol at a wave length of 570 nm. Different wavelengths have been used in the past, Brand-Williams et al. (1995) and Shikanga et al. (2010) used 515 nm and 492 nm respectively while Armatu et al. (2010) and Ng et al. (2011) used 517 nm in determining antiradical activities.

Among the *Lb. acidophilus* strains tested in the present study, extracts from strains U and V reduced the DPPH free radicals by 19% to 86.7% while extract from strain W, with a slightly weaker antiradical capacity, had a DPPH scavenging activity of 11.4% to 82%. Among the *Lb. rhamnosus* strains, the extract from strain Y (10.2% – 86.4%) had the highest free radical scavenging activity while the extract from strain M (4.9% - 45.7%) had the least DPPH absorbance reducing activity. *Lactobacillus rhamnosus* strain D (11.3% - 66.9%) was relatively moderate in its antiradical activity. The antioxidant activity of the extract from *Lc. lactis* subsp. *lactis* strain X in the range 15.6% to 81.2% was almost double the free-radical scavenging activity (2.6% to 45.8%) of the extracts from *Lb. casei* strains (P, B, N).

It is worth noting that while the extract from *Lb. rhamnosus* strain Y, which had the highest antibacterial activity, also had the highest antioxidant activity as already stated. On the other hand, the extract from *Lb. casei* strain N had a low antioxidant activity despite depicting the highest antibacterial activity (MIC value of 1.25 mg/mL against *S. aureus*).

Table 3. Antioxidant activity (%) of selected probiotic bacteria methanol extracts

| | Concentration of extracts (mg/mL) | | | | | | $IC_{50}$ of extracts |
|---|---|---|---|---|---|---|---|
| Source of extracts | 20 | 10 | 5 | 2.5 | 1.25 | Linear equation | (mg/mL) |
| *Lb. acidophilus* group | | | | | | | |
| U | 84.50 ± 1.13 | 82.8 7 ± 1.37 | 56.62 ± 3.62 | 33.11 ± 2.48 | 18.99 ± 3.56 | Y = 9.9446X + 7.235 | 4.3 |
| V | 86.07 ± 0.7 | 80.11 ± 1.52 | 57.02 ± 0.95 | 33.88 ± 2.71 | 21.91 ± 0.63 | Y = 9.3474X + 10.34 | 4.24 |
| W | 81.98 ± 1.77 | 52.20 ± 1.53 | 30.32 ± 2.21 | 17.13 ± 0.43 | 11.40 ± 1.42 | Y = 5.0783X + 4.805 | 8.9 |
| Z | 77.93 ± 1.81 | 71.92 ± 1.26 | 55.08 ± 3.75 | 36.96 ± 1.42 | 24.11 ± 3.99 | Y = 8.114X + 15.05 | 4.31 |
| *Lb. rhamnosus* group | | | | | | | |
| M | 45.71 ± 3.97 | 44.35 ± 7.32 | 26.80 ± 2.99 | 10.65 ± 3.14 | 4.91 ± 1.63 | Y = 5.9263X -3.165 | 8.97 |
| Y | 86.38 ± 0.54 | 63.07 ± 0.81 | 39.23 ± 2.3 | 21.32 ± 2.66 | 10.23 ± 1.1 | Y = -7.652X+ 98.725 | 6.37 |
| O | 73.07 ± 1.4 | 47.76 ± 1.99 | 29.11 ± 1.28 | 13.13 ± 0.76 | 7.80 ± 3.25 | Y = -5.784X + 100.19 | 8.7 |
| D | 66.87 ± 1.67 | 55.87 ± 0.77 | 38.36 ± 0.79 | 19.57 ± 0.47 | 11.29 ± 1.82 | Y = -7.2611X + 98.11 | 6.63 |
| C | 79.86 ± 1.07 | 62.85 ± 1.11 | 37.51 ± 1.82 | 21.26 ± 1.71 | 14.84 ± 2.24 | Y = -2.663X + 70.995 | 7.88 |
| *Lb. casei* group | | | | | | | |
| N | 45.8 ± 1.19 | 39.76 ± 2.87 | 26.64 ± 2.24 | 11.94 ± 1.23 | 6.91 ± 2.33 | Y = -5.350X + 100.44 | 9.43 |
| P | 45.24 ± 0.82 | 41.64 ± 2.33 | 29.86 ± 1.22 | 15.84 ± 1.12 | 12.68 ± 0.98 | Y = -4.72X + 94.33 | 9.39 |
| B | 36.92 ± 0.44 | 22.27 ± 1.04 | 10.49 ± 1.42 | 5.77 ± 1.51 | 2.63 ± 0.64 | Y = -2.066X + 99.73 | 24.07 |
| X | 81.22 ± 2.25 | 70.47 ± 0.86 | 48.42 ± 1.01 | 28.25 ± 1.69 | 15.62 ± 2.95 | Y = 8.6497X + 5.535 | 5.14 |
| Control (Ascorbic acid) | 90.99 ± 1.14 | 82.8 ± 0.55 | 73.46 ± 0.94 | 69.22 ± 0.61 | 50.13 ± 1.52 | Y = 62.515X + 40.913 | 0.15 |

To the best of our knowledge, this is the first time that methanol extracts from freeze-dried cells of selected *Lactobacillus* strains were shown to possess free radical DPPH scavenging activity. Cell-free supernatant extracts from *Lb. plantarum* C88 and *Lb. fermentum* had DPPH inhibition activities of 53.1% and 87.9% respectively (Wang et al., 2009; Li et al., 2012). In a study of *Bf. animalis* 01, Shen et al. (2011) observed that MRS broth, culture supernatant, intact cells and intracellular cell free extracts had a DPPH free radical scavenging activity of 6.6%, 73.1%, 11.1% and 27.7% respectively. In other studies, *Lactobacillus* strains such as *Lb. brevis* BJ20 (Lee et al., 2010; Li et al., 2012), *Lb. plantarum* 7FM10 (Kanno et al., 2012; Li et al., 2012) have also been shown to have DPPH anti-radical activity.

At the highest extract concentration of 20 mg/mL used in this study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had DPPH scavenging activities of 77.9 - 86.1%, 45.71 - 86.4% and 36.92 – 45.80% respectively.

To compare the efficacy of different extracts, the concentration scavenging 50% of DPPH free radicals was calculated using a linear equation, derived for each extract, from a graph of percentage scavenging activity and probiotic extract concentration ($IC_{50}$). Extracts from *Lb. acidophilus* strains U, V and *Lb. helveticus* strain Z had the highest antioxidant activity since they each had the lowest $IC_{50}$ of 4.24 - 4.31 mg/mL. All extracts from the *Lb. rhamnosus* strains studied had an $IC_{50}$ in the range 6.37 – 8.97 mg/mL. Extracts from *Lb. casei* strains were the least active in reducing DPPH free radicals as they had higher $IC_{50}$ values in the range 9.35 – 24.07 mg/mL. The antioxidant activity of extracts from non-probiotic *Lc. lactis* subsp. *lactis* strain X (5.14 mg/mL) was only second to that of *Lb. acidophilus* strains U and V (4.27 – 4.3 mg/mL). This augments the findings of Li *et al*. (2012) that *Lc. lactis* subsp *lactis* strain 12 had free-radical scavenging activity.

Oxidative damage and/or stress *in vivo* can negatively affect the health status of patients suffering from ailments that include cancer, allergies, cardiovascular diseases, atherosclerosis, inflammation, progression of HIV status to AIDS and senescence-related diseases such as rheumatoid arthritis, Parkinson's disease and Alzheimer's disease (Choi et al., 2002; Shen et al., 2011; Li et al., 2012). Unhindered oxidative stress may deteriorate type 2 diabetes and subsequently weaken the antioxidant defenses (Ejtahed et al., 2012). The present study showed that extracts from freeze-dried probiotic cells contain antioxidant components at varying levels. This suggests that the consumer of selected probiotic strains may benefit through oxidative stress reduction. This has been observed in reported nutritional intervention studies involving *Lb. acidophilus* LA5; *Bf. animalis* subsp. *lactis* Bb12; and *Lb. fermentum* ME-3 (Kullisaar et al., 2003; Ejtahed et al., 2012) and in vivo studies involving Lb. plantarum C88 (Li et al., 2012) and Lb. casei Zhang (Zhang et al., 2010). The antioxidant activity of LAB has been attributed to cell-surface proteins, exopolysaccharides and lipoteichoic acids which are capable of chelating iron metal and scavenging superoxide anions (Kullisaar et al., 2002; Li et al., 2012). These attributes were corroborated by Choi *et al*. (2006). The heat-killed cells ($10^8$ cfu/mL) and soluble polysaccharides (10 mg/mL) from *Lb. acidophilus* 606 exhibited a potent antioxidant activity of 52.06 ± 2.06% and 36.05 ± 3.65% respectively in terms of DPPH free radical scavenging ability (Choi et al., 2006). According to Choi et al. (2006), it was the soluble polysaccharide fraction, and not the enzymatic fraction, from *Lb. acidophilus* 606 that was responsible for the antioxidant activity. The findings of the present study, augmented by other workers' reports, imply that cells or components of certain *Lactobacillus* strains can be the source of effective antioxidant activity.

## 4. Conclusions

This study investigated possible antibacterial activity and antioxidant activity of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains. The methanol extracts from certain *Lactobacillus* strains demonstrated measurable antibacterial activity. Extracts from *Lb. rhamnosus* strains, Y and D, and *Lb. casei* strain N had the highest antibacterial activity and their MIC values were in the range 1.25 – 2.5 mg/mL after 24 h of incubation at 37°C. Extracts from *Lactobacillus* strains M, C, O and P had a MIC value of 5 mg/mL, which, in a two-fold serial dilution, may arguably be relatively close to 2.5 mg/mL. Hence, they should also be considered for further investigation along with extracts from above strains Y, D and N.

This investigation illustrated that methanol extracts from freeze-dried probiotic cells of *Lactobacillus* strains tested had antioxidant components at varying levels. At the highest extract concentration of 20 mg/mL used in the study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had DPPH scavenging activities of 77.9 - 86.1%, 45.7 - 86.4% and 36.9 – 45.8% respectively. This suggests that the consumer of selected probiotic strains may benefit through oxidative stress reduction. The extracts need to be investigated further for the constituent active compounds to be elucidated. Also, further investigation is required for possible use as biopreservatives in food and beverages in addition to the need for their non-cytotoxicity to be confirmed. The limitation to the present study was the need to have a considerably costly (but necessary) continuous centrifuge, which would facilitate faster collection of probiotic biomass.

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