American Journal of Microbiological Research, 2018, Vol. 6, No. 1, 1-8
Available online at http://pubs.sciepub.com/ajmr/6/1/1

DOI:10.12691/ajmr-6-1-1

Safety and Antioxidant Properties of Five Probiotic *Lactobacillus plantarum* Strains Isolated from the Digestive Tract of Honey Bees

Chancel Hector Momo Kenfack[1], François Zambou Ngoufack[1,*], Pierre Marie Kaktcham[1], Yan Rui Wang[2], Taicheng Zhu[2], Li Yin[2]

[1]Laboratory of Biochemistry, Food Science, and Nutrition (LABPMAN), Department of Biochemistry, Faculty of Science, University of Dschang, P.O. Box: 67 Dschang, Cameroon
[2]China Academy of Science (CAS) Key Laboratory of Microbial, Physiological and Metabolic Engineering, Institute of Microbiology, China
*Corresponding author: fzambou@yahoo.fr

Abstract The objective of this study was the evaluation of safety, adhesion and antioxidant properties of five *L. plantarum* strains isolated from the digestive tract of honey bees. The set of variables generated from this study was submitted to a normalized Pearson (n) Principal Component Analysis (PCA). The five *L. plantarum* strains showed no gelatinase activity and were checked to be non-hemolytic. They were susceptible to chloramphenicol, amoxicillin, penicillin G and tetracycline. The MICs were ranged between 1-4 µg/ml for erythromycin and resistance was observed among 80% of strains (*L. plantarum* H15, H21, H24, and H28). A significantly high percentage of hydrophobicity in n-hexane was observed with *L. plantarum* H47 (71.99±1.39) followed by *L. plantarum* H28 (65.68±1.49) while the highest value in the presence of chloroform, belonging *L. plantarum* H24 and *L. plantarum* H28 (28.39±0.88 and 23.58±0.68 respectively). Both the Intact Cells (ICs) and Cell-Free Supernatants (CFSs) of *L. plantarum* H24 strain displayed the higher percentage (p 0.05) of DPPH radical scavenging activity (76.58±0.55% and 59.13±4.01% respectively). With the HRS activity, ICs of *L. plantarum* H24 strain exhibited the highest ($p >$ 0.05) activity (73.37±0.62%) whereas, the CFS of *L. plantarum* H47 was the best (29.49±1.28%, $p > 0.05$). In all-purpose, based on PCA, *L. plantarum* H28, and *L. plantarum* H24 seem to be quite promising as they possessed the best properties tested. Accordingly, they can be chosen as representative of the potential probiotic strains.

Keywords: *honeybee, lactic acid bacteria, safety, hydrophobicity, antioxidant*

Cite This Article: Chancel Hector Momo Kenfack, François Zambou Ngoufack, Pierre Marie Kaktcham, Yan Rui Wang, Taicheng Zhu, and, Li Yin, "Safety and Antioxidant Properties of Five Probiotic *Lactobacillus plantarum* Strains Isolated from the Digestive Tract of Honey Bees." *Journal Name*, vol. 6, no. 1 (2018): 1-8. doi: 10.12691/ajmr-6-1-1.

1. Introduction

Lately, the use of probiotics has become gradually popular. Lactic acid bacteria (LAB) are known as microorganisms that have probiotic attributes and have been classified as GRAS (generally recognized as safe). New strains of probiotics LAB with novel functional properties are of interest. The documented potential benefits comprised prevention of intestinal disorders, modulation of the immune system, increase resistance to infection, promotion of good digestion, inhibit the growth of harmful and pathogen bacteria, lowering of serum cholesterol level [1,2,3]. LABs, especially lactobacilli, are the genus commonly used as probiotics, of their harmless character and their frequent use in food production [4].

It has been recognized that high concentration of cholesterol in the blood of humans constitute a risk factor in cardiovascular diseases (CVDs), a major cause of death in many countries [5,6]. The prevalence of CVD in developed and developing countries remains high and is increasing [7]. According to WHO, in 2020, over 40% deaths should be attributed to cardiovascular disease [8]. Long-standing high levels of blood cholesterol could lead to atherosclerosis and may consequently cause a major threat to the establishment of CVDs. As reported by WHO, 10% diminution in serum cholesterol in men aged 40, could within 5 years, reduce the prevalence of heart disease by 50 % [9]. Right now, there is a growing awareness of the use of bile salt hydrolase activity of lactobacilli since they might potentially perform the reduction of serum cholesterol level in humans [10].

The oxidation and oxidative processes of low-density lipoproteins (LDL) are assumed to be a significant factor in atherosclerosis genesis [11]. The high rate of this oxidation may be due to the dissimilarity between elimination and production of free radicals reactive and oxygen species (ROS); which are above all removed by the endogenous antioxidant protection system, resulting to oxidative stress [12]. To date, assessment and selection of natural molecules or substances that have or contain

antioxidant power is the novel research tendency of medicine, biology and also in food science. Lately, some studies described the performance of selected probiotic strains of their antioxidant capacity [13,14].

Bile salt hydrolytic activity is well recognized as the contributing factor that may permit resistance of the LABs and to the toxicity of conjugated salts in the duodenum, and consequently, is an essential colonization factor [15]. Adhesion to intestinal mucus is a wanted attribute for probiotic bacteria, as it increases persistence in the GIT (Gastrointestinal Tract) and the aptitude to colonize intestine efficiently [16,17]. In vitro assay facilitates a preliminary selection of strains with probiotic potential, focusing on significant features as adhesion and safety properties, antibiotics susceptibility.

The promising probiotic properties of *L. plantarum* strains isolated from honeybee tract in Menoua division (Cameroon) have been done in our previous study. They exhibited high resistance to low pH, resist to bile salt and have the good hydrolytic activities on the oxgall bile salts. The present study was aimed to evaluate safety properties, antibiotics susceptibility, and adhesion property by determination of their cell surface characteristics, of the5 *L. plantarum* strains isolated from honeybee's tracts. Also, determine their antioxidant activity using DPPH free radical and Hydroxyl radicals scavenging assays.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

This study was performed using five (5) LAB strains isolated from honeybee tract (Table 1). They can survive to low pH and are resistant to 0.3% bile salts. Their BSH activity may contribute to lower the cholesterol levels. The strains were earlier identified by 16S rRNA gene sequencing and were deposited in the NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to acquire their nucleotide sequences accession numbers. *L. plantarum* strains were grown in MRS broth (Difco) [18] at 37°C for 18-24h. *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* DSM 20523 were cultivated in Muller Hinton (Tulip Diagnostics, India) broth at 37°C for 18 h. All these strains were maintained at -80 °C in the suitable cultivation broth (MRS or Muller Hinton) containing 20% (v/v) glycerol.

Table 1. Lactic Acid Bacteria Strains Used in this Study

Strains	Origin	Accession Number
Lactobacillus plantarum H15	Penka-Michel centre	KU886171
Lactobacillus plantarum H21	Penka-Michel centre	KU886168
Lactobacillus plantarum H24	Penka-Michel centre	KU886174
Lactobacillus plantarum H28	Penka-Michel centre	KU886169
Lactobacillus plantarum H47	Bamendou, *QtNguim*	KU886166

Penka-Michel centre (5°27'N; 10°18'E), Bamendou "Qt Nguim" (5°26'N; 10°12'E).

2.2. Safety Assessment of the Strains

2.2.1. Production of Gelatinase

The assessment of gelatinase activity of the strains was carried out as described by Harrigan and McCance [19]. Two microliters of a 6 h old culture of the strain was spotted onto the surface of nutrient agar containing 7% (w/v) gelatin (Oxoïd). The plates were incubated in anaerobic conditions at 37°C (Incubator, Techmel TT9052, USA) for 48 h after which they were swamped with saturated ammonium sulfate solution and observed for clear zones surrounding colonies. *Staphylococcus aureus* ATCC 25923 was used as positive control. The assay was performed in triplicate

2.2.2. Hemolytic Activity

The hemolytic character of each strain was investigated using the method described by Gerhardt et al. [20] with slight modifications. Two µl of a 6 h old culture was spotted into sterile blood agar which was prepared by adding 7% sheep-blood (freshly collected in a sterilized EDTA-containing tube) sheep blood agar base (Oxoïd) at 25°C. Plates were anaerobically incubated for 48 h at 37°C after which they were observed for the recording characteristics as follow: α-hemolysis (a green zone around colonies), β-hemolysis (clear zones around colonies) and γ-hemolysis (no halo around colonies). A strain of *Streptococcus mutans* DSM 20523 was used as positive control. The assay was carried out in triplicate

2.2.3. Antibiotic Susceptibility Assay

Minimal inhibitory concentration (MIC) was assessed using microdilution method according to the national committee for clinical laboratory standards (NCCLS) guidelines. The antibiotics used were streptomycin, tetracycline, erythromycin, chloramphenicol, penicillin G, and amoxicillin. The range of concentrations for each tested antibiotic varied according to the breakpoints defined for *Lactobacillus* by EFSA [21]. Overnight-cultured colonies of each strain were suspended into MRS broth to approximately 0.5 Mc Farland standards. A volume of 100 µl of the obtained suspension was inoculated into each MRS broth containing each antibiotic. The microplates containing the mixture were incubated at 37°C for 24 h. The tests were conducted in triplicate for each strain.

2.3. Determination of Cell Surface Characteristics

2.3.1. Cell Surface Hydrophobicity

The cell surface hydrophobicity of the LAB was assessed by measuring adhesion to hydrocarbons (MATH) as described by Ji et al. [22]. Bacteria strains cultured at 30°C for 18 h in MRS broth were collected by centrifugation (5000g/10 min/4°C) was washed twice with physiological solution and resuspended in the same preparation. The optical density of the suspension was measured at 580 nm (reading 1) (UV/VIS spectrophotometer, Perkin Elmer Instruments). Then, this bacterial suspension was added to either n-hexane or chloroform at a ratio 1:1 correspondingly. That mixture was mixed by vortexing for 2 min and left at room temperature for 30 min to split the layer. After this period, the water-soluble layer was also measured at the same wave length as the bacterial suspension (reading 2). This assay was performed in three replicates. Hydrophobicity (H) was calculated using equation (1).

$$H(\%) = \left[\frac{(\text{OD reading1} - \text{OD reading2})}{\text{OD reading1}}\right] \text{x100.} \quad (1)$$

2.3.2. Auto-Aggregation

The auto-aggregation test was done according Solieri et al. **[23]**.One ml of 4 Mc Farland cell suspensions in PBS buffer (pH 7.4; 0.02 mM) was well mixed by vortexing for 10 s and incubated at room temperature for 5 h. Then, 0.1 ml aliquot of this suspension was taken and mixed with 500 µl of PBS buffer and the absorbance at 600 nm was measured. Auto-aggregation (Auto-A%) was done as percent decrease in the absorbance after 5 h (ΔAbs x 100) relative to that of original suspension (Abs_{t0}) as shown by equation (2)

$$\text{Auto} - \text{A}(\%) = \frac{\Delta\text{Abs}}{\text{Abs}_{t0}} \text{x100.} \quad (2)$$

2.4. Determination of LAB Strains Antioxidant Activity

2.4.1. Preparation of Intact Cells (ICs) and Cell-Free Supernatants (CFSs)

An overnight grown culture (37°C for 18 h) of the LAB strains was centrifugated (5000 g/10 min at 4°C) to obtain CFSs. Then, the resulting pellet of each strain was adjusted to approximately 10^9 CFU/ml in PBS (pH7.4, 0.02 mM) before used as ICs. Both ICs and CFSs of the strains were submitted to different antioxidant assays, specifically DPPH free radical scavenging and hydroxyl radical scavenging ability assays.

2.4.2. Determination of 2,2-Diphenyl-1-Picryl-Hydrazyl (DPPH) Free Radical Scavenging Activity

The DPPH free radical scavenging activity was evaluated as described by Zhang et al. [24] with slight modification. Briefly, ICs or CFSs (0.8 ml) was mixed with 1 ml of freshly prepared DPPH solution (0.2 mM in ethanol) and allowed to react for 30 min. Either deionized water or PBS constitutes blank samples. The scavenged DPPH activity was at that moment monitored, by measuring the decrease in absorbance at 517 nm. This activity was calculated by equation (3).

$$\text{Scavenging effect}(\%) = \frac{A_{517\text{nm(sample)}}}{A_{517\text{nm(blank)}}} \text{x100.} \quad (3)$$

2.4.3. Hydroxyl Radical Scavenging Ability (HRS)

The HRS activity of ICs and CFSs was analyzed as described by Xing et al. [25]. A sample (ICs or CFSs, MRS broth, 1 ml), 1,10-phenanthroline (2.5 mM, 1 ml; Sigma) , $FeSO_4$ (2.5 mM, 1 ml), and PBS (pH 7.4, 0.02 mM, 1 ml) was mixed. The reaction was started by adding H_2O_2 (20 mM, 1 ml). This mixture was incubated at 37°C for 90 min. HRS activity was monitoring by identifying the increase in absorbance at 536 nm by using a Spectrophotometer. The percentage of resistance to hydroxyl radicals was calculated using the following equation (4).

$$\text{HRS activity}(\%) = \frac{A_s - A_c}{A_b - A_c} \text{x100} \quad (4)$$

Where, AS and AC represent the absorbance of the sample and the control solution (deionized water was used at the same amount in place of the sample) respectively, and Ab is the absorbance of the solution with no samples and H_2O_2.

2.5. Data Processing and Statistical Analysis

The assays were repeated on three independent times. The mean values, as well as standard deviation were calculated from the obtained data. All these data were compared by Duncan's multiple range method at the significance level of $p < 0.05$. Statistical analysis was done using the software GraphPad Instat (GraphPad Software Inc, V3). The set of variables was reduced by a factor extraction method using principal component analysis (PCA). The Kaiser's rule was applied to the selection of the number of principal components. The cases introduced in the analysis were the 5 LAB strains whereas the discriminating variables were the cell surface characteristic (auto-aggregation, hydrophobicity) and antioxidative ability (DPPH, HRS). The XLSTAT2007.8.04 software (Addinsoft, Paris, France, http://www.slstat.com) was used, and a normalized Pearson (n) PCA applied.

3. Results

3.1 Safety Properties of the Strains

LAB strains have been assessed for their gelatinase and hemolytic activities. The results reveal that all the five strains showed no gelatinase activity, and were checked to be non-hemolytic as pathogenicity factors.

The MICs of the antibiotics are available in Table 2. This table shows the MICs values of the 5 LAB strains to cell wall inhibitors (amoxicillin and penicillin G) and protein synthesis inhibitors (erythromycin, tetracycline, streptomycin, and chloramphenicol). All the LAB strains assayed (100%) were susceptible to chloramphenicol (MICs < 8µg/ml) and tetracycline (MICs ≤ 32 µg/ml). For erythromycin, the MICs of our strains were ranged between 1-4 µg/ml. In the case of this antimicrobial substance tested, resistance was observed among 4 of our strains (80%); i.e., *L. plantarum* H15, H21, H24 and H28 when comparing their MICs with the cut-off value of EFSA. Since the cut-off values of some antibiotics (penicillin G, amoxicillin) are not accounted in the European Food Safety Authority (EFSA) guidelines for testing lactobacilli, the breakpoint values suggested by authors took into account. For these antibiotics, results were appreciated according to the cut-off values indicated by Charteris et al. [26]. Thus, the breakpoint of penicillin was chosen as16 µg/ml and that of amoxicillin as 32 µg/ml. Our results showed that all the five *L. plantarum* strains tested (100%) were susceptible to amoxicillin and penicillin. For aminoglycoside streptomycin, the strains showed MICs distribution between < 0.5 and 4 µg/ml.

Table 2. Minimal Inhibitory Concentration (MIC) Against the LAB Strains

Strains	MICs (µg/ml)					
	S	E	T	C	P	A
***L. plantarum* H15**	< 0.5	4	8	8	4	16
***L. plantarum* H21**	2	4	8	8	2	16
***L. plantarum* H24**	< 0.5	2	4	4	2	16
***L. plantarum* H28**	< 0.5	4	8	8	2	16
***L. plantarum* H47**	4	1	4	4	1	16
MIC C-O*	n.r	1	32	8	ND	ND

S: streptomycin, E: erythromycin, T: tetracycline, C; chloramphenicol, P: penicillin G, A: amoxicillin. *MIC C-O: Minimal inhibitory concentration Cut-Off values by EFSA [21] (µg/ml) for *L. plantarum* strain. ND: not defined. n.r : not required

Table 3. Cell Surface Properties of the Five Tested LAB Strains

Strains	Auto-A (%)	H (%)	
		n-Hexane	Chloroform
***L. plantarum* H15**	43.66 ± 0.45^{a}	45.18 ± 1.39^{a}	20.02 ± 0.72^{a}
***L. plantarum* H21**	38.57 ± 3.07^{a}	31.66 ± 0.69^{b}	14.94 ± 0.86^{b}
***L. plantarum* H24**	62.66 ± 3.31^{bc}	60.86 ± 1.43^{c}	28.39 ± 0.88^{c}
***L. plantarum* H28**	63.29 ± 0.22^{b}	65.68 ± 1.49^{d}	23.58 ± 0.68^{d}
***L. plantarum* H47**	50.67 ± 1.20^{c}	71.99 ± 1.39^{e}	20.72 ± 0.64^{a}

In the same column, the value presenting the different letters differ significantly ($p < 0.05$). Auto-A: auto-aggregation, H: hydrophobicity.

3.2. Cell Surface Characteristics

In the aim to *in vitro* evaluate the cell adherence capacity; strains were checked for their cell surface properties (Table 3). The results obtained from the experiments show that *L. plantarum* H24 and *L. plantarum* H28 displayed upmost auto-aggregation percentage after 5 h of incubation time (62.66 ± 3.31 and 63.29 ± 0.22 respectively), whereas *L. plantarum* H21 showed the lower value (38.57 ± 3.07). The result also reveals that all the strains displayed more cell hydrophobicity in n-hexane than chloroform. The significantly highest percentage of hydrophobicity in n-hexane was exhibited by *L. plantarum* H47 (71.99 ± 1.39), followed by *L. plantarum* H28 (65.68 ± 1.49). It results from the hydrophobicity test in the presence of chloroform that all the strains showed percentage between 14% and 28%. The highest value was belonging to *L. plantarum* H24 and *L. plantarum* H28 (28.39 ± 0.88 and 23.58 ± 0.68 respectively) while the percentage belongs to the strain *L. plantarum* H21 (14.94 ± 0.86).

3.3. Antioxidant Activity of the Strains

3.3.1. DPPH Free Radical Scavenging Activity

DPPH is a relatively stable organic radical; it has been broadly used to evaluate antioxidant activities. The results showed that ICs exhibited the highest DPPH radical scavenging rate than that of CFSs (Figure 1). The DPPH radical scavenging activity was comprised between 65% (*L. plantarum* H47) and 76% (*L. plantarum* H15, *L. plantarum* H24, *L. plantarum* H28). Whereas, with CFSs, the lower value was observed with *L. plantarum* H28 (20.16 ± 0.36%) and the maximum with *L. plantarum* H24 (59.13 ± 4.01%) ($p > 0.05$). Both the ICs and CFSs of *L. plantarum* H24 strain indicated the higher percentage ($p > 0.05$) of DPPH radical scavenging activity (76.58 ± 0.55% and 59.13 ± 4.01% respectively).

3.3.2. HRS Activity

Hydroxyl radicals are estimated to be the mainly reactive oxygen radicals. They can bring on harsh damage in the living cell when reacting with every one biomacromolecule. Consequently, scavenging of hydroxyl radical plays a unique function in dropping oxidative injury. Fenton reaction was used in this work, to evaluate the hydroxyl radicals scavenging activity of the ICs and CFSs of the strains.

As shown in Figure 1 above, among the five strains, tested, the ICs of *L. plantarum* H24 strain displays the highest ($p > 0.05$) hydroxyl radicals scavenging activity (73.37 ± 0.62%) while the lower was 49.04 ± 2.15% with *L. plantarum* H47. However, the CFS of *L. plantarum* H47 was the best to display the higher hydroxyl radicals scavenging activity than other strains (29.49 ± 1.28%, $p > 0.05$). *L. plantarum* H15 showed the lower value with CFS (8.31 ± 1.51%) ($p < 0.05$).

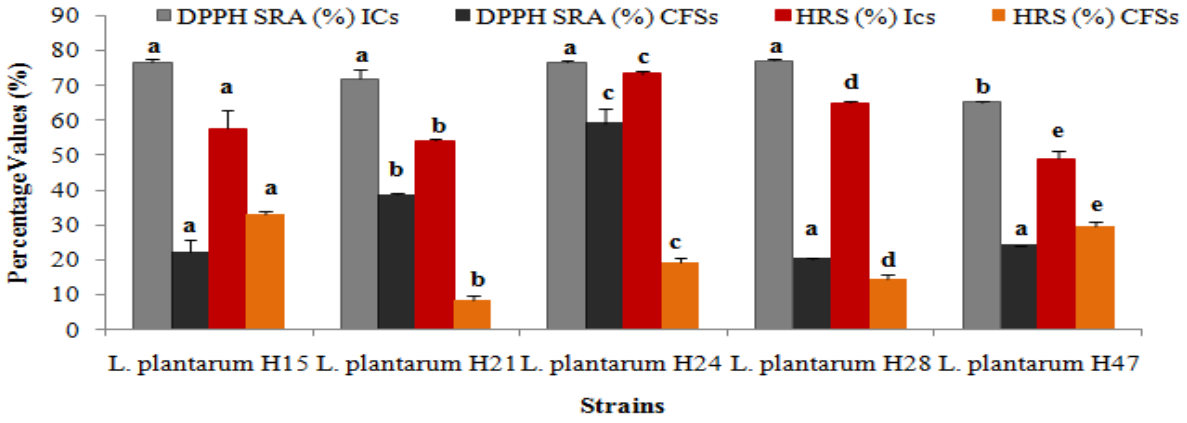

Figure 1. Scavenging activity of the strain against DPPH free radical and Hydroxyl radicals both with their ICs and CFSs. HRS_{ICs} (%) :Hydroxyl radical scavenging ability of the intact cells; HRS_{CFSs} (%): Hydroxyl radical scavenging ability of the Cell-Free Supernatants; DPPH RSA_{ICs}: 2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the intact cells; DPPH RSA_{CFSs}(%): 2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the Cell-Free Supernatants. ^{a,b,c,d,e} In the same cross hatch /color, the values presenting the different letters differs significantly ($p < 0.05$). Error bars represent the standard deviation

Table 4. Correlation Matrix between Variables (Pearson (n))

Variables	Auto-A (%)	$H_{chloroform}$ (%)	$H_{n\text{-}hexane}$ (%)	DPPH RSA_{ICs} (%)	DPPH RSA_{CFSs} (%)	HRS_{ICs} (%)	HRS_{CFSs} (%)
Auto-A (%)	1						
$H_{chloroform}$ (%)	0.763	1					
$H_{n\text{-}hexane}$ (%)	0.905*	0.669	1				
DPPH SRA_{ICs} (%)	0.356	-0.234	0.412	1			
DPPH SRA_{CFSs} (%)	0.211	-0.276	0.409	0.411	1		
HRS_{ICs} (%)	0.735	0.168	0.813*	0.788*	0.698	1	
HRS_{CFSs} (%)	-0.083	0.351	0.155	-0.208	-0.432	-0.266	1

H chloroform (%) : Hydrophobicity on chloroform; $H_{n\text{-}hexane}$ (%) : Hydrophobicity on hexane; Auto-A (%) : Auto-aggregation; HRS_{ICs} (%) : Hydroxyl radical scavenging ability of the intact cells; HRS_{CFSs} (%) : Hydroxyl radical scavenging ability of the Cell-Free Supernatants; DPPH RSA_{ICs}: 2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the intact cells; DPPH RSA_{CFSs} (%) :2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the Cell-Free Supernatants. *Correlation is significant at the 0.05 level.

3.4. Principal Component Analysis (PCA)

PCA was achieved at the final step to investigate the correlation amongst the probiotic properties of the LAB strains (cell surface characteristic and antioxidative ability), and select the promising probiotic. Table 4 presented the Pearson's r coefficient or correlation matrix of variables that were studied. Auto-aggregation was significantly and positively correlated to hydrophobicity on n-hexane (0.905). The other important positively correlations were $H_{n\text{-}hexane}$ -HRS_{ICS} (0.813); DPPH RSA_{CFSs}-HRS_{ICS} (0.788). The HRS_{CFSs} was negatively correlated to DPPH RSA_{ICs} (-0.208), DPPH RSA_{CFSs} (-0.432) and HRS_{ICs} (-0.266).

Two components can be extracted, which together accounted for 80.75% of the variability as unveiled by the analysis (Figure 2). The PC1 which took account 50.39% of the total variance separated the strain with a high antioxidative ability and cell surface characteristic from those with a relative low of these properties. The PC2 that accounted for 30.35% of the total variance separated between cell surface characteristic and antioxidative ability.

Based on the factorial space of PCA, four major groups could be pointed out. The first group is composed of strains *L. plantarum*H21 and *L. plantarum* H15 (negative side of both F1 and F2), characterized by low attributes for the variables considered in the analysis. The second group consists of *L.plantarum*H47 which exhibited the high value of its hydroxyl radical scavenging ability of its cell-free supernatants. It falls on the negative side of F1 and the positive side of F2. The third group contains *L. plantarum* H24 which expressed the high value of antioxidative capacity (DPPH and HRS). The fourth group consists of *L. plantarum* H28 (positive side of both F1 and F2) combined the highest activities for all the variables, and it seems like the best one for the probiotics traits. In general, *L. plantarum* H28 and *L. plantarum* H24 were quite promising as they possessed the tested properties at their highest levels. Thus, they can be selected as representative of the promising probiotic strains.

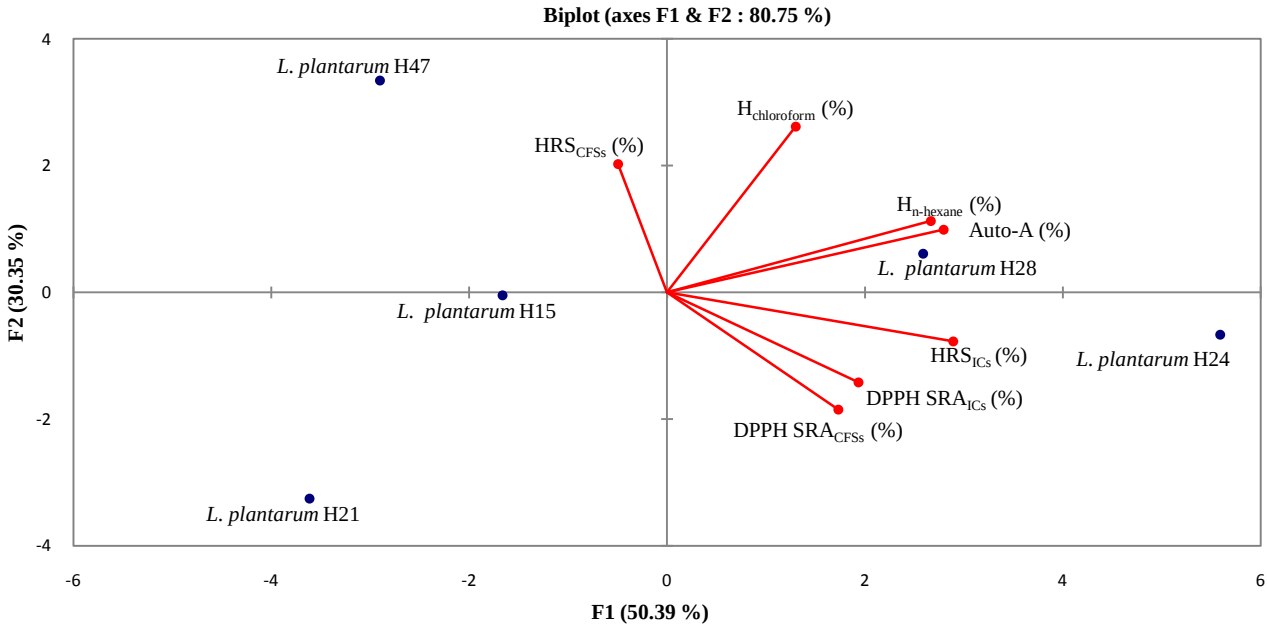

Figure 2. Factorial biplot defined by the principal components 1 and 2 (PC 1: 50.39%; PC 2: 30.35%) resulting from the PCA performed on cell surface characteristic and antioxidative ability of the LAB strains. $H_{chloroform}$ (%) : Hydrophobicity on chloroform; $H_{n\text{-}hexane}$ (%) : Hydrophobicity on n-hexane; Auto-A (%) : Auto-aggregation; HRS_{ICs} (%): Hydroxyl radical scavenging ability of the intact cells; HRS_{CFSs} (%) : Hydroxyl radical scavenging ability of the Cell-Free Supernatants; DPPH RSA_{ICs} : 2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the intact cells; DPPH RSA_{CFSs} (%) : 2,2-Diphenyl-1-Picryl-Hydrazyl radical scavenging activity of the Cell-Free Supernatants

4. Discussion

Every strain which would be assumed as a probiotic candidate should be appraised regarding safety aspects. To ensure their safe use in humans the five *L. plantarum* strains were screened for the antibiotic resistance, the hemolytic and the gelatinase activities. Gelatinase activity could disturb the mucoid lining even as hemolysis activity would destroy the epithelial layer [27], and these injuries would be the pathways for infections. In the present study, none of the tested LAB strains presented β-hemolytic and gelatinase activity. The absence of these activities is a selection criterion for probiotic strains, suggesting that these bacteria are non virulent [28]. Similar results were obtained by Sieladie et al. [3] with *L. plantarum* strains isolated from raw cow milk in the highlands of Cameroon and by Anas et al. [29], from raw coat's milk in the region of western Algeria.

Probiotic should not carry transferable antibiotic resistance genes. This is one of a key requirement. The resistance to antibiotics varying inside the *Lactobacillus* genus as an intrinsic or acquisition characteristic, it's a pertinent attribute. A high hazard of horizontal dissemination of resistance genes exist, when the resistance is contracted from an exogenous DNA material [21]. Our result showed that 80% of the tested strains (*L. plantarum* H15, H21, H24, and H28) were resistant to erythromycin. In the study performed by Cauwerts et al. [30] on the isolated *Lactobacillus* strains from broiler chickens in Belgian farms, it was reported that 78% revealed resistance to erythromycin. Chromosomally encoded resistance to this antimicrobial is regular in *lactobacillus* species [31]. For *L. plantarum* strains, there are no approved breakpoints with streptomycin. In fact, as commonly known, lactobacilli have a high natural resistance to streptomycin [32]. The resistance to aminoglycosides such as streptomycin is judged to be intrinsic, principally due to the membrane impermeability [33]. All the strains were sensitive to the other remaining antibiotics used. Danielsen and Wind [32] showed that *Lactobacillus* sp. had been known to be susceptible to several cell wall synthesis inhibitors, such as penicillins. Kaktcham et al. [34] reported that all of the 9 tested *L. plantarum* strains isolated from Cameroonian traditional fermented foods were sensitive to penicillin G, amoxicillin, chloramphenicol, and tetracycline. Through consideration of common concern on the safety of probiotics, i.e., potential transferability of antibiotic-resistance gene, hemolytic and gelatinase activities, *L. plantarum* strains of this study appear safe for use.

One of the essential criteria for the choice of putative probiotic lactobacillus stains is their adhesion and their ability to colonize the gastrointestinal tract. These requirements may improve their residence time in the intestine and therefore permit to achieve their probiotic effects [35]. Hydrophobicity assessment provides information on the structural properties of the cell surface which is accountable for aggregation and adhesion of the LAB strains [36]. LAB strains with hydrophobicity cell surface and aggregation potential could be more able to adhere to intestinal cells. In this study, hydrophobicity was done by using chloroform as a polar solvent and n-hexane as a non-polar solvent. The results indicated that all the strains had more affinity for n-hexane than chloroform, indicating more hydrophobic cell surface hydrophilic than. Indeed, the presence of hydrophobic components may justify the high affinity to n-hexane. Former studies like those of Zavaglia et al. [37] on the physico-chemistry of microbial have revealed that hydrophilic surfaces are related with the existence of polysaccharides at the cell surface while the occurrence of (glyco-) proteinaceous material results in the higher hydrophobicity. It has been established that bacterial cells with hydrophobicity possess a high affinity for epithelial or mucus adhesion [38]. Base on the studies of Lee and Yii [39], it was acknowledged that bacterial cell is hydrophobic when its adhesion percentage is at least 50%, hydrophile when this percentage is less than 20% and moderately hydrophobic if the rate is included between these two values. Consequently, we can say that *L. plantarum* H24, *L. plantarum* H28, and *L. plantarum* H47 are hydrophobic while *L. plantarum* H15 and *L. plantarum* H21 hydrophilic.

Auto-aggregation of the same probiotic strains appeared to be essential for their adhesion to intestinal epithelial cells and is correlated with the colonization of gastrointestinal tract via adhesion to this tissue [36,40]. The results of this study have shown that all the strains exhibited variables auto-aggregation percentage varying between 38.57% and 63.29% after 5 h. The detected auto-aggregation can be linked to cell surface component. Ramos et al. [41] also analyzed the autoaggregation percentage of *L. plantarum* strains after 5 h, from the Brazilian food products. They reported that these percentages were varied between 27.55% and 62.92%. Auto-aggregation was significantly and positively correlated to hydrophobicity on n-hexane in our study. Correlation between hydrophobicity and adhesion ability of several lactobacilli was previously indicated in the studies [42,43]. Mostly cell surface-associated proteins are involved in aggregation and adhesion even though numerous additional factors existed like polysaccharide, lipoteichoic and teichoic acids [44].

Reactive oxygen species (ROS) are always produced *in vivo*. When these ROS are generated in excess, or cellular neutralization process is defective, they can cause lipid, protein and nucleic acids injury [13]. Thus, these harmful molecules can lead to cellular oxidative damage, which is related with the biological and pathological process such as carcinogenesis, cirrhosis, inflammation (e.g., arthritis) and atherosclerosis (which can also lead to cardiovascular diseases) [45]. To control reactive radicals, the selection of probiotics and their usage according to their efficiency are essential in the preservation of human health and avoidance of some disease [46,47]. We have attempted with this study to evaluate the antioxidative activity of LAB strains.

The DPPH-RSA assay is commonly used to assess antioxidant activity for the reason of its simplicity, simplicity, sensitivity, rapidity and reproductively compared with other methods [24]. While, hydroxyl radical which is mostly generated from Fenton reaction (through transition metals such as copper (Cu^{2+}) and iron (Fe^{2+})), has been acknowledged to be the principally dangerous ROS that is accountable for the oxidative damage of biomolecules. Chelating ions may do inhibition of the formation of these compounds by antioxidants [48]. The result of these two

radical scavenging assays (DPPH and HRS) reveals that all the tested strains presented variable percentage activity and could inhibit the risk of ROS accumulation in vitro. So, some level of inter-specific variation in radical scavenging ability could exist among the five probiotic strains tested. Varying activities in DPPH radical scavenging was also pointed out by Liu and Pan [49] on 12 Lactobacillus strains. Li et al. [50] reported that the DPPH free radical scavenging rate by the intact cell of the *L. plantarum* C88 strain was 53.05% at the dose10^{10} UFC/ml. In a study analogous to us, Kaur et al. [51] documented about more than 50% of this scavenging capacity of theYbis, L5 and Yb LAB strain with their ICs. Furthermore, our results showed that ICs exhibited higher DPPH radical scavenging rate than that of CFSs. And the CFSs of our strains also demonstrated comparatively weaker HRS activity than DPPH free radical scavenging activity. This result is consistent with that of Shen et al. [52] and even with the report of Xing et al. [25]. Both the DPPH and HRS methods showed that the ICs of *L. plantarum* H24 and *L. plantarum* H24 displayed high antioxidant activities. Many studies reveal that antioxidant activity of LAB strains might be linked to their production of cell-surface compounds, e.g., lipoteichoic acid from the cell surface of bifidobacteria [53], extracellular polysaccharides by *Bifidobacterium animalis* RH [54] and *Lactococcus lactis* subsp . Lactis [55].

5. Conclusion

Finally, the five *L. plantarum* strains isolated from the digestive tract of honey bees present some important probiotics abilities with varying levels. In this study, we attempted to demonstrate that *L. plantraum* strains from the digestive tract of honey bees have antioxidative activity and can be good candidates for epithelial cell adhesion. From the above results and the PCA analysis, we suggested that *L. plantarum* H24 and *L. plantarum* H28 have required *in vitro* probiotics properties comparable or higher to those that are already known. They are good candidates for complementary analysis with *in vivo* assays to investigate their possible technological characteristics and their health promotion.

Acknowledgements

Chinese Academy of Sciences (CAS), Visiting President's International Fellowship Initiative (PIFI) supported this research. Grant N° 2015VBB051.

Conflict of Interest

The authors declare no competing interests

References

[1] Guo, X.H., Kim, J.M., Nam, H.M., Park, S.Y., and Kim, J.M., "Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties", *Anaerobe*, 16: 321-326, 2010.

[2] Dhanasekaran, D., Saha, S., Thajuddin, N., and Panneerselvam, A., "Probiotic effect of Lactobacillus isolates against bacterial pathogens in Claris orientalis", *Facta Universitatis Series: Medicine and Biology*, 15 (3): 97-102, 2008.

[3] Sieladie, D.V., Zambou, N.F., Kaktcham, P.M., Cresci, A., and Fonteh, F., "Probiotic properties of lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon", *Innov Rom. Food Biotechnol*., 9:12-28, 2011.

[4] Whitehead, K., Versalovic, J., Roos, S., and Britton, R.A., "Genomic and Genetic Characterization of the Bile Stress Response of Probiotic *Lactobacillus reuteri* ATCC 55730", *Appl. Environ. Microbiol.*, 74 (6): 1812-1819, 2008.

[5] Kuzawa, C.W., and Sweet, E., "Epigenetics and the embodiment of race: developmental origins of US racial disparities in cardiovascular health", *Am. J. Hum. Biol.*, 21:2-15, 2009.

[6] Ahn, Y.T., Kim, G.B., Lim, K.S., Baek, Y.J., and Kim, H.U., "Deconjugation of bile salts by Lactobacillus acidophilus isolates", *Int. Dairy J.*, 13: 303-311, 2003.

[7] Sudha, M.R., Chauhan, P., Dixit, K., Babu, S., and Jamil, K., "Probiotics as complementary therapy for hypercholesterolemia", *Biol. Med*., 1 (4):1-13, 2009.

[8] Kumar, R., Grover, S., and Batish, K.V., "Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing *Lactobacillus plantarum* strains in Sprague-Dawley rats", *Br. J. Nutr*., 105: 561-573, 2011.

[9] World Health Organization (WHO). Cardiovascular Disease fact sheet: 2013. [online] Available: http://www.who.int/mediacentre/factsheets/fs317/en/index.html. [Accessed Nov. 19, 2015].

[10] Pereira, D.I., and Gibson, G.R., "Effects of consumption of probiotics and prebiotics on serum lipid levels in humans", *Crit. Rev. Biochem. Mol. Biol*., 37: 259-281, 2002.

[11] Westhuyzen, J., "The oxidation hypothesis of atherosclerosis: An update", *Ann. Clin. Lab. Sci.*, 27:1-10, 1997.

[12] Halliwell, B., and Gutteridge, J., "Free radicals in biology and medicine." *Oxford Clarendon Press*, 23-30, 1985.

[13] Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairaneand C., Kilk, A., "Two antioxidative lactobacilli strains as promising probiotics", *Int. Food Microbiol*., 72:215-224, 2002.

[14] Annuk, H., Shchepetova, J., Kullisaar, T., Songisepp, E., Zilmer, M., and Mikelsaar, M., "Characterization of intestinal lactobacilli as putative probiotic candidates", *J. Appl. Microbiol.*, 94: 403-412, 2003.

[15] Shigwedha, N., and Jia, L., "*Bifidobacterium in Human GI Tract: Screening. Isolation. Survival and Growth Kinetics in Simulated Gastrointestinal Conditions*". In Kongo J. M. (Eds.) Lactic acid bacteria- R & D for food health and livestock purposes. InTech press. JanezaTrdine 9. 51000 Rijeka. Croatia. 2013, 281-308.

[16] Lavilla-Lerma, L., Pérez-Pulido, R., Martínez-Bueno, M., Maqueda, M., and Valdivia, E., "Characterization of functional, safety and gut survival related characteristics of Lactobacillus strains isolated from farm house goat's milk cheeses", *Int. J. FoodMicrobiol*., 163: 136-145, 2013.

[17] Etzold, S., Kober, O.I., MacKenzie, D.A., Tailford, L.E., Gunning, P., Walshaw, J., Hemmings, A.M., and Juge, N., "Structural basis for adaptation of lactobacilli to gastrointestinal mucus", *Environ. Microbiol*., 16:888-903, 2014

[18] De Man, J.C., Rogosa, M., and Sharpe, M.E., "A medium for the cultivation of lactobacilli", *J. Appl. Bacteriol.*, 23: 130-135, 1960

[19] Harrigan, W.F., and McCance, M.E., *Laboratory Methods in Food and Dairy Microbiology*. Academic Press. London. 1990, 8thedition.

[20] Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R., and Phillips, G.B., *Manual of methods for general bacteriology. American Society for Microbiology*. NW Washington, DC 20006, 1981.

[21] European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Journal, 2012, 10.

[22] Ji, K., Jang, Y.N., and Kim, T.Y., "Isolation of Lactic Acid Bacteria Showing Antioxidative and Probiotic Activities from Kimchi and Infant Feces", *J. Microbiol. Biotechnol*., 25 (9):1568-1577, 2015

[23] Solieri, L., Bianchi, A., Mottolese, G., Lemmetti, F., and Giudici, P., "Tailoring the probiotic potential of non-starter *Lactobacillus*

strains from ripened Parmigiano Reggiano cheese by *in vitro* screening and principal component analysis", *Food Microbiol.*, 38:240-249, 2014.

[24] Zhang, S., Liu, L., Su, Y., Li, H., Sun, Q., Liang, X., and Lv, J., ''Antioxidative activity of lactic acid bacteria in yogurt'', *Afr. J. Microbiol. Res*., 5 (29): 5194-5201. 2011.

[25] Xing, J., Wang, G., Zhang, Q., Liu, X., Gu, Z., Zhang, H., Chen, Y.Q., and Chen, W., "Determining Antioxidant Activities of Lactobacilli Cell-Free Supernatants by Cellular Antioxidant Assay: A Comparison with Traditional Methods", PLoS ONE 10: e0119058, 2015.

[26] Charteris, W.P, Kelly, P.M., Morelli, L., and Collins, J.K, "Antibiotic susceptibility of potentially probiotic *Lactobacillus* species", *J. Food Prot*., 61:1636-1643. 1998

[27] Thakkar, P., Modi, H.A., and Prajapati, J.B. "Isolation, characterization and safety assessment of lactic acid bacterial isolates from fermented food products", *Int. J. Curr. Microbiol. App. Sci.*, 4 (4): 713-725, 2015.

[28] De Vuyst, L., Foulquie, M.R., and Revets, H., "The role and application of enterococci in food and health", *Int. J. Food Microbiol*., 84:299-318, 2003.

[29] Anas, M., Ahmed, K., and Mebrouk, K., "Study of the antimicrobial and probiotic effect of *Lactobacillus plantarum* (P6) isolated from Raw Goat's Milk from the region of Western Algeria", *World Appl. Sci. J*., 32 (7): 1304-1310. 2014.

[30] Cauwerts, C.K., Asmans, P.F., Evriese, D.L.A., Aesebrouck, H.F., and. Ecostere, D.A., "Cloacal *Lactobacillus* isolates from broilers often display resistance toward tetracycline antibiotics", *Microb. Drug Resist*., 12:284-288, 2006.

[31] Ashraf, R., and. Shah, N.P., "Antibiotic resistance of probiotic organisms and safety of probiotic dairy products", *Int. Food Res. J*., 18 (3):837-853, 2011.

[32] Danielsen, M., and. Wind, A.A., "Susceptibility of *Lactobacillus* ssp. to antimicrobial agents", *Int. J. Food Microbiol*., 82:1-11, 2003.

[33] Charteris, W.P., Kelly, P.M., Morelli, L., and Collins, J.K., "Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli", *J. Food Prot.*, 64: 2007-2014, 2001.

[34] Kaktcham, P.M., Zambou, N.F., Tchouanguep, F.M., El-Soda, M., and Choudhary, M.I., "Antimicrobial and Safety Properties of Lactobacilli Isolated from two Cameroonian Traditional Fermented Foods", *Sci. Pharm*., 80:189-203, 2012.

[35] Von Ossowski, I., Reunanen, J., Satokari, R., Vesterlund, S., Kankainen, M., Huhtinen, H., Tynkkynen, S., Salminen, S., de Vos, V.M., and Palva, A., "Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits", *Appl. Environ. Microbiol.*, 76:2049-2057, 2010.

[36] Raghavan, K.T., Jacob, A.A., and Chandran, H., "Honey bee Gut flora as a Source of LAB (Lactic Acid Bacteria) with Probiotic Capabilities", *J. Food Technol.*, 105:126-134, 2013.

[37] Zavaglia, A., Kociubinski, G., Pérez, P., and De Antoni., A.G.G., "Isolation and characterization of *Bifidobacterium* strains for probiotic formulation", *J. Food Prot*., 61:865-873, 1998.

[38] Tuo, Y., Hanli, Y., Ai, L., Wu, Z., Guo, B., and Chen, W.," Aggregation and adhesion properties of 22 *Lactobacillus* strains", *J. Dairy. Sci*., 96:4252-4257. 2013.

[39] Lee, K.K., and Yii, K.C., "A comparison of three methods for assaying hydrophobicity of pathogenic vibrios", *Lett. Appl. Microbiol*., 23: 343-346, 1996.

[40] Kaktcham, P.M., Temgoua, J-B., Zambou, N.F., Diaz-Ruiz, G., Wacher, C., and Pérez-Chabela, M.L., "*In Vitro* Evaluation of the Probiotic and Safety Properties of Bacteriocinogenic and Non-Bacteriocinogenic Lactic Acid Bacteria from the Intestines of Nile Tilapia and Common Carp for Their Use as Probiotics in Aquaculture", *Probiotics Antimicro. Prot*., 2017.

[41] Ramos, C.L., Thorsen, L., Schwan, R.F., Jespersen, L., "Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products", *Food Microbiol*., 36: 22-29, 2013.

[42] Wadstroum, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S., and Gullmar, B., "Surface properties of lactobacilli isolated from the small intestine of pigs", *J. Appl. Microbiol*., 62: 513-520, 1987

[43] Xu, H., Jeong, H.S., Lee, H.Y, and Ahn, J., "Assessment of cell surface properties and adhesion potential of selected probiotic strains", *Lett. Appl. Microbiol*., 49:434-442, 2009.

[44] Goh, Y.J., and Klaenhammer, T.R., "Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM". *Appl. Environ. Microbiol*., 76:5005-5012, 2010.

[45] Lin, M.Y., and Yen, C.L., "Antioxidative ability of lactic acid bacteria", *J. Agric. Food. Chem.*, 47:1460-1466, 1999.

[46] Songisepp, E., Kals, J., Kullisaar, T., Mändar, R., Hütt, P., Zilmer, M., and Mikelsaar, M., "Evaluation of the functional efficacy of an antioxidative probiotic in healthy volunteers", *Nutr J*., 4:22, 2005.

[47] Foligné, B., Dewulf, J., Breton, J., Claisse, O., Lonvaud-Funel, A., and Pot, B., "Probiotic properties of non-conventional lactic acid bacteria: Immunomodulation by Oenococcusoeni", *Int. J. Food Microbiol*., 140:136-145, 2010.

[48] Kao, T., and Chen, B., "Functional components in soybean cake and their effects on antioxidant activity", *J. Agricult. Food Chem.*, 54:7544-7555, 2006.

[49] Liu, C., and Pan, T., "*In Vitro* Effects of Lactic Acid Bacteria on Cancer Cell Viability and Antioxidant Activity", *J. Food Drug Anal*., 18:77- 86, 2010.

[50] Li, S., Zhao, Y., Zhang, L., Zhang, X., Huang, L., Li, D., Niu, C., Yang, Z, and Wang, Q., "Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods", *Food Chem*., 135:1914-1919, 2012.

[51] Kaur, M., Singh, H., Jangra, M., Kaur, L., Jaswal, P., Dureja, C., Nandanwar, H., Chaudhuri S.R., Raje, M., Mishra, S., and Pinnaka, A.K,. "Lactic acid bacteria isolated from yak milk show probiotic potential", *Appl. Microbiol. Biotechnol*., 10:7635-7652, 2017

[52] Shen, Q., Shang, N., and Li, P., "In vitro and in vivo antioxidant activity of Bifidobacterium animalis 01 isolated from centenarians", *Curr. Microbiol*., 62: 1097-1103, 2011.

[53] Yi, Z., Fu, Y., Li, M., Gao, K., and Zhang, X., "Effect of LTA isolated from bifidobacteria on D -galactose-induced aging", *Exp. Geron*., 44: 760-765. 2009.

[54] Xu, R., Q. Shen, X. Ding, W. Gao, and P. Li., "Chemical characterization and antioxidant activity of an exopolysaccharide fraction isolated from *Bifidobacterium animalis* RH", *Eur. Food Res. Technol*., 232: 231-240, 2011.

[55] Pan, D., and Mei, X., "Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. Lactis 12". *Carbohydr. Polymers*, 80: 908-914, 2010.