PAPER

ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF FERMENTED MEAT PATTY WITH *LACTOBACILLUS* STRAINS

S.M.B. HASHEMI*[a], S. RAEISI[b], E. ZANARDI[c], M. PACIULLI[c] and E. CHIAVARO*[c]

[a]Department of Food Science and Technology, College of Agriculture, Fasa University, Fasa, Iran

[b]Department of Food Science and Technology, College of Agriculture, Urmia University, Urmia, Iran

[c]Dipartimento di Scienze degli Alimenti e del Farmaco, Università di Parma, Parco Area delle Scienze 47/A, 43124 Parma, Italy

*E-mail address: hashemi@fasau.ac.ir; hasshemii@yahoo.com
emma.chiavaro@unipr.it

ABSTRACT

The effect of fermentation by *Lactobacillus fermentum* PTCC 1638, *Lactobacillus plantarum* subsp. *plantarum* PTCC 1745 and *Lactobacillus sakei* subsp. *sakei* PTCC 1712 on antimicrobial activity against Alternaria alternate PTCC 5224, *Aspergillus parasiticus* PTCC 5018, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* O157 H7 ATCC 35150 and *Salmonella Typhimurium* ATCC 14028 as well as antioxidant properties (carbonyl assay, peroxide and anisidine value) in a beef patty during 24 h of fermentation and further storage at 4°C for 8 days were investigated. Results indicated that *L. plantarum* subsp. *plantarum* had the highest radical scavenging activity (54.3±1.7%) before fermentation. During the fermentation process, DPPH and ABTS activities of the meat patty were improved in comparison to the control. The highest antioxidative value was observed for *L. plantarum* subsp. *plantarum*. All of three strains had a strong antimicrobial effect against pathogenic bacteria and fungi. Oxidation products were enhanced in fermented and non-fermented samples. However, the increasing trend of the oxidation process was mitigated in all fermented samples. In particular, the lowest protein and lipid oxidation values were observed in the samples treated by *L. plantarum* subsp. *plantarum*. Generally speaking, fermentation improves the antioxidative and antimicrobial effect of meat patty and lengthens its storage period.

Keywords: antimicrobial activity, antioxidant activity, fermentation, *Lactobacillus* strains, meat product

1. INTRODUCTION

Lactic acid bacteria (LAB) are the primary microorganisms involved in fermentation (SALMINEN *et al*., 2004) by consuming simple sugars such as glucose as the substrate (YADAV, 2017). LAB possess antimicrobial and anticancer activities and play a critical role in the balance of gut microbial flora, synthesis of vitamins, improvement of immunes system, reduction of cholesterol level, prevention of food allergy, improvement of lactose absorption and so forth (MANSOURIPOUR *et al*., 2013). Moreover, as antioxidant and anti-inflammatory agents are used for treatment of various diseases such as diabetes, Alzheimer's, Parkinson's, high blood pressure, liver disorders (WOO *et al*., 2014). Also, application of LAB cultures to promote antibacterial and antioxidant properties of foods has been recommended by many investigations (LI *et al*., 2012; PISANO *et al*., 2014).
Fermentation was a cheap and simple method to conserve meat since ancient time. Production of acid (pH reduction), H_2O_2 and bacteriocins alone or in combination with starter cultures prevents from the growth of meat deteriorating microorganisms. Improving in aroma is another advantage of fermentation. Considering limitation of chemical additives, application of fermentation techniques in meat has been increased (SAKHARE and RAO NARASIMHA, 2003). One of oldest meat products created to increase meat conservation is fermented sausage in which fermenting microorganisms especially LAB are used. The mentioned microorganisms (single species or a mix of different microorganisms) are added into meat paste as starter cultures (YILMAZ and VELIOGLU, 2009). LEROY *et al*. (2005) demonstrated that the application of functional meat starter cultures in fermented sausages promotes product's safety via production of bacteriocins and other antimicrobial compounds (JAFARI *et al*., 2017).
LAB species used in the production of dry fermented sausages possess antibacterial properties against *Listeria monocytogenes*, *Staphylococcus aureus* (PAPAMANOLI *et al*., 2002,2003). Various strains of *Lactobacillus plantarum* show strong antioxidant and antibacterial properties during the fermentation process (HASHEMI *et al*., 2017). Furthermore, use of *Lactobacillus* in fermented pork prevents the growth of different *Clostridium* species (DI GIOIA *et al*., 2016). However, based on the some investigations, other microorganism such as probiotic *Bacillus* could pose similar effects (JAFARI *et al*., 2017).
Considering above mentioned issues, the present study was conducted to investigate the antioxidant and antimicrobial effect of meat patty fermented by *Lactobacillus fermentum* PTCC 1638, *Lactobacillus plantarum* subsp. *plantarum* PTCC 1745, *Lactobacillus sakei* subsp. *sakei* PTCC 1712 during fermentation process and storage period.

2. MATERIALS AND METHODS

2.1. Microbial culture

Lactobacillus fermentum PTCC 1638, *Lactobacillus plantarum* subsp. *plantarum* PTCC 1745, *Lactobacillus sakei* subsp. *sakei* PTCC 1712, *Alternaria alternat*e PTCC 5224, and *Aspergillus parasiticus* PTCC 5018 were purchased from the culture collection at Iran Institute of Industrial and Scientific Research. Reactivation of the *Lactobacillus* strains was done in the MRS broth (Oxoid, UK) at 37°C for 48 h. The mold cultures were cultivated on yeast extract dextrose chloramphenicol agar (Lab M, UK) slants for 9 days at 25°C. *Staphylococcus aureus* ATCC 25923, *Escherichia coli* O157 H7 ATCC 35150 and *Salmonella Typhimurium* ATCC 14028 were obtained from microbial culture stock of Veterinary

School, Shiraz University. The strains were reactivated in defined Mueller Hinton broth (Oxoid, UK) and left for incubation at ~37°C.

2.2. Fermented meat patty preparation and storage

Fresh ground beef was obtained from a local supermarket in Shiraz city (Fars, Iran). Ground beef and irradiated herb spice were pasteurized at 80°C for 15 min. Fermented meat patty was prepared by mixing ground beef (2 kg), herb spice (10 g), pasteurized brine (8 mL, 10% w/v) and each *Lactobacillus* strain culture (~10^5 CFU/g). This preparation was subsequently placed into glass vessels (3 L) and kept in an incubator (Shimazu, SHI1 55 AL, Iran) to ferment at 35°C for 24 h. A time course analysis was carried out prior to fermentation and 4, 8, 16, 20 and 24 h during the fermentation process. A control (non-fermented) sample was also prepared. After fermentation, fermented and non-fermented meat patty samples were kept at 4°C in refrigerator for 8 days. Approximately, 120 samples were prepared and all of the experiments were carried out in triplicate.

2.3. DPPH free radical scavenging activity of *Lactobacillus* strains

The DPPH content was determined using the described method of KAO and CHEN (2006). After centrifugation at 3500 × g (Hettich, EBA21, Germany) for 20 min, the absorbance of cell samples was determined using spectrophotometer (UV/Visible Philips Cambridge, UK) at 517 nm. The blank sample corresponded only to the cells emerged in methanol.

2.4. Meat patty analysis during fermentation

- The pH of fermented and non-fermented samples was determined during the 24 h period of fermentation using a pH-meter (model 520A, Orion Research Inc., MA, USA).
- The enumeration of *L. fermentum*, *L. plantarum* subsp. *plantarum*, and *L. sakei* subsp. *sakei* was done during 24 h period of meat patty fermentation. Enumeration of *Lactobacillus* strains was carried out using MRS agar (Oxoid, UK) after incubation under anaerobic conditions (35°C, 72 h).
- DPPH radical scavenging activity of fermented meat sauce samples was performed according to the method of KATO *et al*. (1988). About 1 mL of the filtrated sample was mixed with 1mL of DPPH reagent (250 mM) and 1mL of 0.1MTris-HCl buffer (pH 7.4) in test tubes. After incubation at room temperature, the absorbance of sample was assessed at 517 nm. Ethanol was used as a blank.
- $ABTS^{\cdot}$ activity was measured according to the method of SHIRWAIKAR *et al*. (2006). About 100 mL of sample extract was blended with 4.9 mL of $ABTS^{\cdot}$ working standard solution and absorbance was determined after 20 min at 734 nm. The $ABTS^{\cdot}$ activity was evaluated by using equation:

$$ABTS^{\cdot} \text{ activity } (\%) = \left[\frac{Absorbance(0) - Absorbance\ (20)}{Absorbance\ (0)}\right] \times 100$$

- The antimicrobial activity of fermented samples against *Alternaria alternata*, *Aspergillus parasiticus*, *Staphylococcus aureus*, *Escherichia coli* O157 H7 and *Salmonella* Typhimurium was measured at the end of fermentation, by means of the well diffusion method. For bacterial cells, the inoculum (10^6 CFU/ mL) was spread on plates containing Mueller-Hinton Agar (Oxoid, UK). For molds, well was created on yeast extract dextrose chloramphenicol agar (Lab M, UK) plates, which had been previously inoculated by 0.1 mL of inoculums

containing indicator molds in the range of 10^4-10^5 spores/mL. Subsequently, aliquot solutions (80 μL) from meat patty samples were forwarded into the wells. The agar plates were incubated at 37°C for 24 h and at 25°C for 48 h for pathogenic bacteria and molds, respectively. Then, the disk diameter of inhibition zones was measured.

2.5. Meat patty analysis during storage

Protein and lipid oxidation of samples were measured during storage.
- Protein carbonyls of fermented and non-fermented meat patty samples were measured according to the described method of LEVINE *et al*. (1994). Carbonyl groups were measured by precipitation of 100 μL of sample with 50 μL of trichloroacetic acid (100%, w/v). After preparation of samples, the absorbance was determined at 280 and 370 nm for measurement of carbonyl content of samples.
- Lipids were extracted according to the described method of BLIGH and DYER (1959) using chloroform/methanol (1:1, v/v). The ferric-thiocyanate technique described by SHANTA and DECKER (1994) was conducted for the measurement of peroxide value (PV). Anisidine value (AnV) of the samples was measured according to the AOCS method (1998).

2.6. Statistical analysis

Statistical analysis was conducted with one-way ANOVA and Duncan's multiple range tests (SPSS package program; v. 20.0 for Windows, SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P<0.05$.

3. RESULTS AND DISCUSSION

3.1. *Lactobacillus* growth and pH changes during fermentation

The reduction of pH, production of lactic acid and antimicrobial compounds including bacteriocins, non-bacteriocins and non-lactic substances can be considered as the major mechanisms of LAB activities include (FAYOL-MESSAOUDI *et al*., 2005). In current study, pH variation of meat patty during 24h fermentation was investigated as depicted in Fig.1A. pH variation trend was similar in the three species *L. fermentum*, *L. plantarum* subsp. *plantarum* and *L. sakei* subsp. *sakei*. Initial pH was 6.1 in all of the three species and decreased to 4.1, 4.3 and 4.4 at the end of the fermentation. Significant variation ($p<0.05$) occurred between 4 and 20 hours of the fermentation process as shown in the Fig. 1A. pH reduction after fermentation is due to the growth of fermenting microorganisms and production of lactic acid from available carbohydrates by these strains (DROSINOS *et al*., 2007). YADAV (2017) reported that application of *L. plantarum* significantly ($p<0.05$) reduced pH during fermentation of chicken sausages. Similar results also have been reported the direct correlation between pH reduction and LAB growth during meat fermentation (COCOLIN *et al*., 2001a, 2001b). According to the United States Department of Agriculture, pH should be lower than 5 in highly stable fermented meat products (LEISTNER and RODEL, 1975).

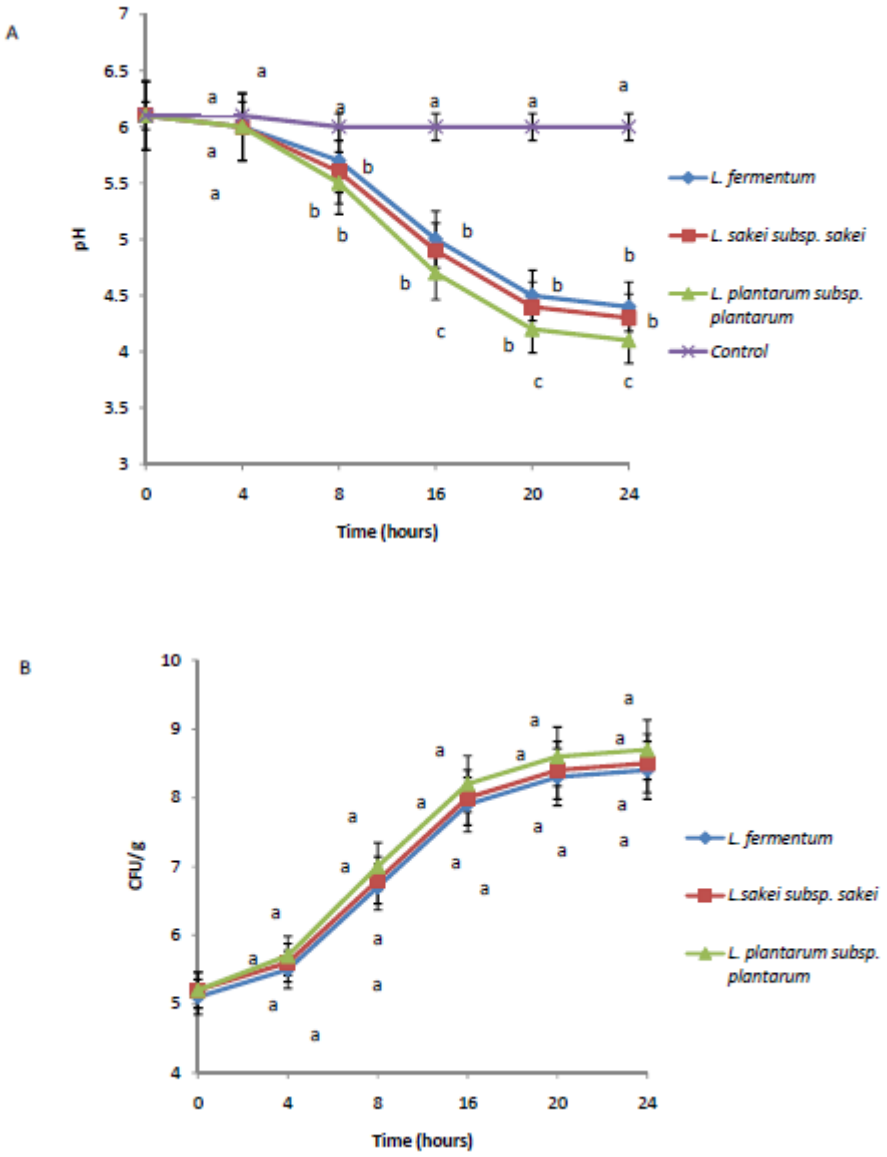

Figure 1. pH changes (A) and changes in cell viability of *Lactobacillus* strains (B)of meat patty samples during fermentation. Means in each hour with the same superscript lowercase letters are not significantly different at $P<0.05$.

The growth of LAB in current investigation was promoted during fermentation (Fig.1B). An initial number of the three strains in fermented meat patty was about 5.2 CFU/g that reached to 8.5 CFU/g at the end of fermentation (24 h). All strains showed similar logarithmic growth during the first 16 hours and a constant and slow growth afterward. Increased LAB growth coinciding with pH reduction suggests resistance and compatibility of them against pH variation (CEBECI and GÜRAKAN, 2003). Indeed, there is a direct correlation between microbial cells population and pH (JOHNSON and STEELE, 2013). According to COMI *et al*. (2005), LAB population increased at early stages of production of dry fermented sausages and then remained at a constant value of 7-9log CFU/g. Moreover, in French fermented sausages, the population of fermenting strains increased and reached the same value at the end of the fermentation process (REBECCHI *et al*.,

1998). Increased LAB growth is attributed to pH reduction during early stages of fermentation (COCOLIN *et al*., 2001a, 2001b).

3.2. Radical scavenging activity of *Lactobacillus* strains and fermented meat patty

Radical scavenging activity was measured, and the corresponding results are presented in Table 1. As seen, there is a significant difference (p<0.05) among the bacterial species, and the highest value was observed in *L. plantarum* subsp. *plantarum* (54.3±1.7%); followed by *L. sakei* subsp. *sakei* and *L. fermentum*.

Table 1. Radical scavenging activity of *Lactobacillus* strains against DPPH radicals.

***Lactobacillus* strains**	**Radical scavenging activity (%)**
L. fermentum	38.2±1.1[c]
L. sakei subsp. *sakei*	44.6±1.5[b]
L. plantarum subsp. *plantarum*	54.3±1.7[a]

[a]Means in the column with different superscript letters differ significantly ($P < 0.05$).

Moreover, radical scavenging activity (RSA) was measured in fermented meat patty during the 24h period (Fig. 2A). The results indicated that RSA was increased through the time. By progress and completion of fermentation, RSA value was elevated. The highest RSA value was observed in the sample treated by *L. plantarum* subsp. *plantarum* that increased from 12.1% to 48.2% at the end of fermentation, followed by *L. sakei* subsp. *sakei* (increase from 12.1% to 41.3%) and *L. fermentum* (from 12.1% to 33.5%). Thus, *L. plantarum* had better antiradical properties. For example, RSA value obtained in fermentation by *L. sakei* subsp. *sakei* can be achieved by fermentation with *L. plantarum* subsp. *plantarum* for 12 hours. LI *et al*. (2012) investigated antioxidant activity of traditional Chinese fermented food and concluded that *L. plantarum* had the highest hydroxyl radical and DPPH scavenging activities (44.31% and 53.05%; respectively). Moreover, it was found out that proteins and polysaccharides residing at the surface of *L. plantarum* provide the species with antioxidant power; hence, degradation of these compounds reduces DPPH free radical scavenging capacity of *L. plantarum*. Evaluation of antioxidant activity of *L. plantarum* isolated from Marcha of Sikkim indicated that this strain possesses high DPPH scavenging activities (DAS and GOYAL, 2015). Similar investigations support our findings regarding application of *L. plantarum* in the fermentation of various foods (KULLISAAR *et al*., 2002; WANG *et al*., 2009; HASHEMI *et al*., 2017).
Evaluation of $ABTS^{\cdot}$ activity in meat sauce during fermentation revealed that $ABTS^{\cdot}$ value was enhanced by an increase in time and the highest value of this parameter was achieved by *L. plantarum* subsp. *plantarum* that increased from 26.3% at the first hours to 64.24% at the 24th hour (Fig. 2B). The last two assays suggest that *Lactobacillus* strains especially *L. plantarum* had high antioxidant power because the results show that the highest value of this parameter obtained by 24h fermentation with *L. sakei* subsp. *sakei* and *L. fermentum* can be achieved by 14 h and 10 h fermentation with *L. plantarum* subsp. *plantarum*. These findings accord with those reported by YADAV (2017) who found out that application of *L. plantarum* in sausage fermentation promotes $ABTS^{\cdot}$ activity and DPPH activity.

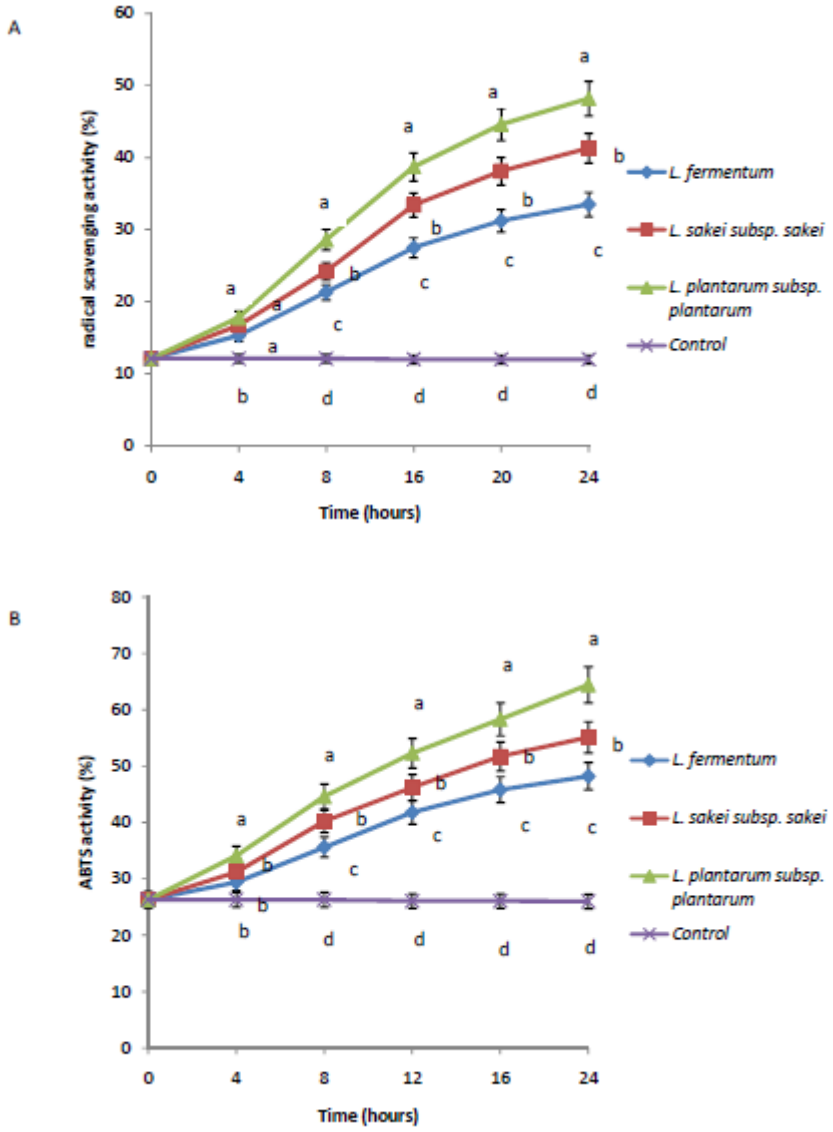

Figure 2. Changes in DPPH activity (A) and ABTS+ activity (B) of meat patty samples during fermentation with Lactobacillus strains. Means in each hour with the same superscript lowercase letters are not significantly different at P<0.05.

3.3. Antimicrobial activity of fermented meat patty

Antimicrobial activity of meat patty during fermentation was investigated against *A. alternat*e, *A. parasiticus*, *S. aureus*, *E. coli* O157 H7 and *S. Typhimurium*, and the result were represented in Table 2. The highest antimicrobial activity was corresponded to sample fermented with *L. plantarum* whose inhibition zone diameters (Table 2). Inhibition zone diameters of *L. plantarum* against *E. coli* O157:H7, *S.* Typhimurium, *S. aureus*, A. *parasiticus* and *A. alternat*e were 22.6±0.3mm, 20.9±0.4mm, 25.9±0.4mm, 28.5±0.5mm, and 27.9±0.8mm; respectively. The highest inhibition zone diameter of *L. plantarum* was 28.5±0.5mm that obtained for *A. parasiticus*; followed by *L. sakei* subsp. *sakei* and *L. fermentum*. Inhibition zone of *L. plantarum* often had significant difference (p<0.05) with that of the two other strains. LABs prevent microbial growth and meat deterioration by acid production (pH reduction), H_2O_2 and bacteriocins production. YADAV (2017)

reported that *L. plantarum* plays a critical role in the prevention of microbial growth during sausages chicken fermentation. YILMAZ and VELIOGLU (2009) found out that LAB was the leading cause of microbial growth inhibition in fermented products. The author observed that the number of *Bacillus* strains was significantly (p<0.05) lower in the samples treated by 24h fermentation, while the number was increased in control group. REA *et al*. (2013) investigated antimicrobial properties of species used in fermented sausages and concluded that the main reason behind the reduction of *Bacillus* in these products was pH reduction and bacteriocins production by the fermenting bacteria. Application of *L. plantarum* in fermentation has antimicrobial activity against *E. coli* O157: H7 and *S. aureus* (HASHEMI *et al*., 2017).

Table 2. Antimicrobial activity of meat patty samples with different *Lactobacillus* strains.

Meat samples	Inhibition zone diameters (mm)				
	***E. coli* O157:H7**	***S. Typhimurium***	***S. aureus***	***A. parasiticus***	***A. alternata***
L. fermentum	18.4 ± 0.5^{cC}	15.7 ± 1.1^{bD}	20.5 ± 0.6^{cB}	23.7 ± 0.6^{bA}	24.1 ± 0.7^{cA}
L. sakei subsp. *sakei*	20.3 ± 0.9^{bD}	16.3 ± 0.8^{bE}	23.1 ± 1^{bC}	27.4 ± 1.2^{aA}	25.5 ± 0.4^{bB}
L. plantarum subsp. *plantarum*	22.6 ± 0.3^{aC}	20.9 ± 0.4^{aD}	25.9 ± 0.4^{aB}	$28.5\pm0.\ 5^{aA}$	$27.9\pm0.\ 8^{aA}$
Control (non-fermented)	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}	0 ± 0^{d}

[a]Values represent means ± standard deviations of inhibition zones. Means within a column with the same superscript lowercase letters are not significantly different at P<0.05 and means within a row with the same superscript uppercase letters are not significantly different at P<0.05.

3.4. Lipid and protein oxidation of meat sauce samples during storage

PV and AnV were measured to evaluate oxidative stability in fermented meat patty at 4°C for 8 days. Lipid oxidation includes continuous formation of hydroperoxide as primary oxidation products that can be degraded to various volatile substances as secondary oxidation products (ADEGOKE *et al*., 1998). PV indicates primary oxidation products that are odorless materials that are degraded during the reaction and converted to a wide range of substances including carbonyl, hydrocarbons, furans and other products creating an unsuitable taste of the foods (YANISHLIEVA and MARINOVA, 2001). PV was measured during 8-day period after fermentation. As depicted in Fig.3A, the samples fermented with three LAB strains had lower PV than the control group (p<0.05). Among fermented samples, the sample fermented with *L. plantarum* subsp. *plantarum* had the lowest PV (p<0.05), suggesting antioxidant properties of the LAB. In this research, the highest antioxidant activity was observed for *L. plantarum* subsp. *plantarum*. PV increased with time, but this increasing trend for *L. plantarum* was at the lowest rate compared to other fermented and control samples (p<0.05).

As an indicator of secondary oxidation, AnV was measured during the 8day period. A similar trend was observed for this parameter (Fig. 3B), meaning that the parameter was increased in all fermented and control samples, but *L. plantarum* was the best species considering resistance against formation of secondary oxidation products. AnV was increased by the time but the lowest value on the first and eighth days was observed in the sample treated by *L. plantarum* subsp. *plantarum* (p<0.05). These results accord with those reported by other authors indicating that food fermentation by LAB especially by *L.*

plantarum improves the oxidative stability of the products (TSENG and ZHAO, 2013; HASHEMI *et al*., 2017; YADAV, 2017).

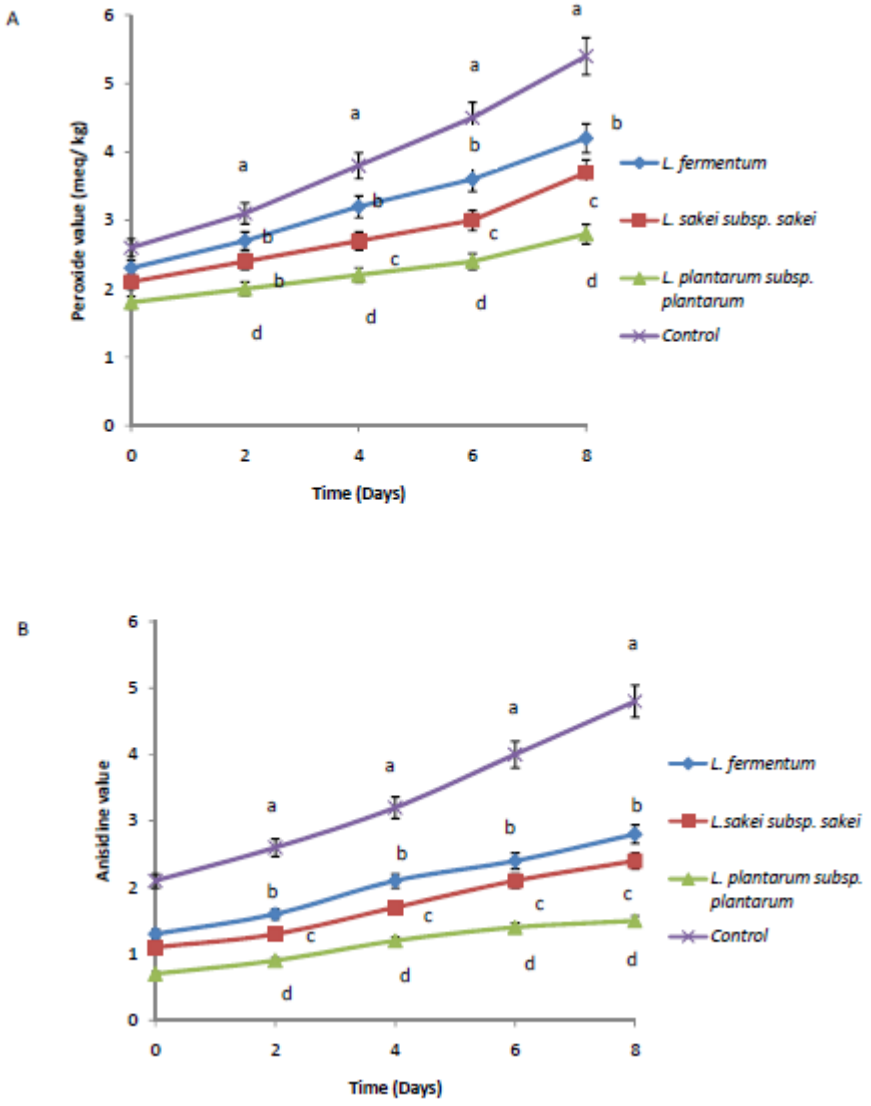

Figure 3. Peroxide value (A) and Anisidine value (B) of meat patty samples during storage at 4°C for 8 days storage. Means in each day with the same superscript lowercase letters are not significantly different at $P<0.05$.

Carbonyl value was measured during 8 days of storage to determine protein oxidation. As depicted in Fig. 4, carbonyl formation was increased by the progress of storage time. The highest value of this parameter was observed in non-fermented (control) sample, and the lowest value was obtained in meat patty fermented with *L. plantarum* subsp. *plantarum* as 0.9 and 2.1nmoL/mg on the first and eighth days, respectively. Indeed, protein oxidation rate was much more decreased in this sample. Protein oxidation may occur naturally during cold storage of foods. Indeed, protein oxidation occurs in side chains of amino acids including thiol and aromatic hydroxyl that results in the formation of carbonyl groups (STADTMAN, 1990). The concentration of carbonyl groups can be considered as

an index of oxidative activities (HASHEMI *et al*., 2015). HASHEMI *et al*. (2017) concluded that application various strains of *L. plantarum* in fermentation reduced protein oxidation rate, which accords with the results obtained in the present study.

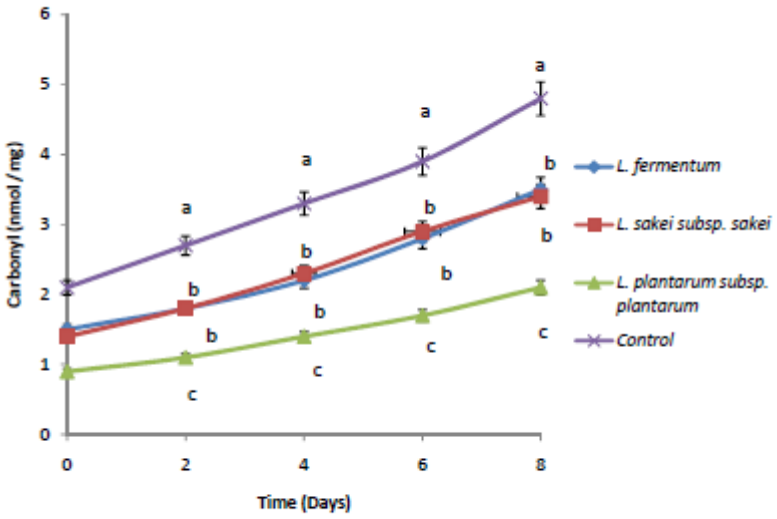

Figure 4. Carbonyl value of meat patty samples during storage at 4°C for 8 days storage. Means in each day with the same superscript lowercase letters are not significantly different at P<0.05.

4. CONCLUSIONS

The results obtained in this research revealed that meat patty fermentation using *Lactobacillus* strains improved its antimicrobial and antioxidative properties. Antiradical activity was enhanced during fermentation, and the fermented product had antimicrobial activity against pathogenic bacteria and fungi. Moreover, during storage of meat patty, lipid and protein oxidation was lower in fermented samples compared to non-fermented ones. Future studies can focus on the antioxidant mechanism of *Lactobacillus* strains.

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