

Research Article

Proteolytic Characterization of Trimmed Beef Fermented Sausages Inoculated by Indonesian Probiotics: *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4

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Abstract: Proteolysis is one of the most important biochemical changes affecting proteins during the ripening and preservation of fermented beef sausages. In this study, proteolytic activities of two Indonesian probiotics, *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4 used as starters in trimmed beef are investigated. *L. plantarum* IIA-2C12 and *L. acidophilus* displayed remarkable proteolytic activities against milk casein substrate, in which the activity of *L. plantarum* IIA-2C12 is higher than that of *L. acidophilus* IIA-2B4. Similar evidences were observed when proteolytic activities of both strains were visualized by using SDS-Page against meat sarcoplasmic proteins. The differences in the number of proteases encoded by the genomes of both starters might account for these differences. The activities of both strains were slightly reduced upon storage at room temperature for 28 days due to decreasing of the amount of substrate and or stability of proteases. In addition, we found also that the sausage inoculated by *L. acidophilus* IIA-2B4 tends to produce more aromatic amino acids than that of *L. plantarum* IIA-2C12. This might differently contribute to flavor (especially aroma) of both sausages. Altogether, this is, to our knowledge, first evidences for the proteolytic activity of *L. acidophilus* strain towards muscle proteins during sausage fermentation.

Keywords: *Lactobacillus acidophilus* IIA-2B4, *Lactobacillus plantarum* IIA-2C12, meat sarcoplasmic protein, proteolysis, trimmed beef sausage

INTRODUCTION

Sausage is not only known as the oldest and most enduring form of processed meat, but also the most popular meat product in the world (Morgan and Tallard, 2006). In Indonesia, sausage is getting famous as one of meat product consumed to meet protein requirements. In 2012, the production of sausage in Indonesia is keeping increasing up to 30% with market value about USD 250 million.

Introduction fermentation in sausage manufacturing is aimed not only to increase shelf life of the sausages, but also taste and flavor development (Esmaeilzadeh *et al.*, 2013). Fermented sausages are traditionally prepared from seasoned, raw meat that is stuffed in casings and allow fermentation process for ripening and maturation. Lactic acid bacteria (LAB) are commonly used as starters for the fermentation as it may improve safety and quality of the final product (Leroy *et al.*, 2006). Beef commercial cuts are mainly used for fermented sausages, however non-commercial

parts also promising to be used as raw material for the sausages. Trimmed beef, the non-commercial part obtained from remaining meat attached in the bone after deboning or cutting to have desired commercial parts, nutritionally is similar to beef commercial cuts (Mastuti, 2008). Trimmed beef can be easily obtained from slaughtering house and considered as waste product. Given these advantages of trimmed beef, however, the usage of trimmed beef remains limited.

Previously, two Indonesian probiotics, *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus plantarum* IIA-2B4, were isolated from beef obtained from Indonesian cattle, Peranakan Ongole (Arief *et al.*, 2015). As local probiotic, both lactobacilli strains are sought to display some advantages including adaptability to local environment so that the application of these strains does not need any additional modifications (Astawan *et al.*, 2012). These bacteria had met requirements to be classified as probiotic (Arief *et al.*, 2014). In addition, these bacteria also had displayed remarkable ability to prevent EPEC-causing

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diarrhea (Arief *et al.*, 2010) and repair the hematology condition of diarrheal suspected rats (Astawan *et al.*, 2011).

During fermentation, proteolysis is considerably the most important reaction (Candogan and Acton, 2001). It influenced both texture and flavor development that contribute to the overall quality of fermented sausages. The reaction involves fragmentation of protein by internal proteases in the raw material (indigenous proteases) and external proteases secreted out by bacteria used as starter (Fadda *et al.*, 1999). It yields low molecular weight of protein (peptides) and their derivatives that contribute to formation of specific aroma, taste characteristic and meat tenderization.

Despite LAB is considered to exhibit weak proteolytic activity; however some of LAB have been reported to exhibit strong protease activity in fermented sausages (Nie *et al.*, 2014). The proteolytic activities of LAB during fermentation is part of their attempts to obtain the energy from protein breakdown and also to utilize free amino acids for their growth. Unfortunately, comprehensive study on characterization of proteolytic activity of some LAB members in fermented sausages is limited. *L. plantarum* strains have been reported to exhibit strong proteolytic activity towards meat proteins (Fadda *et al.*, 2002), however there is no report, to our knowledge, for the activity of *L. acidophilus* strains.

In this research, we use trimmed beef as raw material for the sausage fermentation, with the addition of two Indonesian probiotics, *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 as starters. The objective of this study was to determine proteolysis activity of both strains towards muscle sarcoplasmic proteins. For the first time, we found that proteolytic activity of *L. plantarum* IIA-2C12 toward muscle sarcoplasmic proteins is significantly higher than that of *L. acidophilus* IIA-2B4. Some possibilities account for this difference are discussed in this study.

MATERIALS AND METHODS

Bacterial strains: LAB used in this study were *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4 isolated from Indonesian local beef, Peranakan Ongole breeds (Arief *et al.*, 2015). To be used as a starter for sausage fermentation, the population of these strains should be more than 10^8 cfu/mL. To confirm, initial population of these strains were checked based on Arief *et al.* (2008). Briefly these strains were cultured in MRS broth (Man Ragosa Sharpe) at 37°C for 24 h. This culture then plated on MRS agar at 37°C for overnight. The population was determined based on number of colonies appear after overnight incubation.

Preparation of beef fermented sausage: Beef fermented sausage was prepared following the recipe of

Arief *et al.* (2014). The sausage formulation consisted of 3 kg of meat with addition of 2.0% sugar, 2.0% common salt, 0.5% white pepper, 0.5% ginger, 0.5% nutmeg and 0.5% lime leaves. These materials were mixed in meat cutter and 2% of *L. plantarum* IIA-2C12 and *L. acidophilus* 2B4 (with population more than 10^8 cfu/mL) was added. The cocktail was then filled in 12.5 cm-diameter casing prior to 24 h conditioning to allow initial fermentation. Ripening was then performed through cold smoking at 27-30°C for 3 days, 4 h each. The sausages were prepared for two treatments and three replications each.

Total lactic acid bacteria analysis: Population of LAB on the sausages was determined according to AOAC (2005). Briefly, 25 g of sausages was aseptically transferred into 225 mL of buffered peptone water. The mixture was poured in MRS agar (Oxoid) and LAB colonies were calculated after 48 h incubation at 37°C.

pH and titratable acid measurement: To measure pH values of the sausages, pH meter (Hanna Instrument, USA) was used. For this purpose, 50 g fermented beef sausages was as for titratable acid, 5 g of sausages were homogenized in 250 mL of distilled water. The initial pH was measured by pH meter (Hanna Instrument, USA) and titration was performed by drop-wise of 0.1 N NaOH until the solution reaches neutral pH. Titratable acid was then calculated based on the following formula:

$$\% \text{ titratable acid} = \frac{\text{ml NaOH} \times \text{N NaOH} \times 0.01 \text{ N} \times 90}{\text{sample (g)}} \times 100\%$$

Extraction of sarcoplasmic proteins: Sarcoplasmic protein were extracted from the sausages based on Mauriello *et al.* (2002) with slight modifications. Briefly, 1 g of sausages were diluted 1:10 (w/v) with 20 mM phosphate buffer pH 6.5 and homogenized for 3 min followed by a centrifugation at 13 000 g for 20 min at 4°C. The supernatant (sarcoplasmic fraction) was sterilized by filtration 0.22 µm and stored at 4°C for further analysis.

Detection of proteolytic activity by agar plate method (Mauriello *et al.*, 2002): Proteolytic activity of the strains used for the fermentation was visually determined by agar plate method according to Mauriello *et al.* (2002). Sarcoplasmic extracts standardized at 5 mg protein/mL, were added at concentration of 1 mg/mL to an agar medium consisting of tryptone 0.5%, yeast extract 0.25%, glucose 0.1%, agar 1.5%, pH 6.9 and then poured into petri dishes. After solidification of the medium, 70 µL of a 24 h culture broth of each strain were pipette in to wells (6 mm) borer in agar plate. Plates were incubated at 37°C

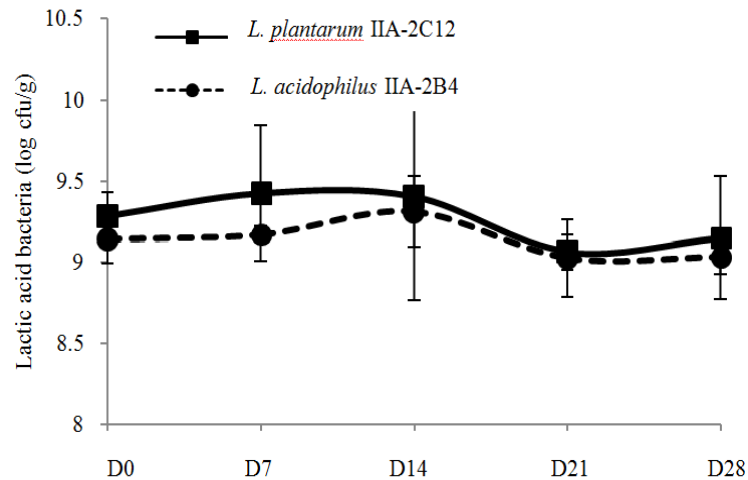


Fig. 1: Lactic Acid Bacteria (LAB) in fermented beef sausages with *L. plantarum* IIA-2C12 (—) and *L. acidophilus* IIA-2B4 (---) during storage

for 48 h. After incubation, the agar layers stained for 5 min in 0.05% (w/v) Brilliant Blue R in methanol: acetic acid: distilled water (50: 5: 40). After destaining in methanol: acetic acid: water (25: 5: 70), the presence of a clear zone surrounding the inoculated wells indicated proteolytic activity.

Analysis of proteolysis by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

To investigate proteolytic activity of both strains during the fermentation 12% SDS-PAGE was employed. For this purpose, sarcoplasmic proteins were extracted from the sausages as described by Mauriello *et al.* (2002). Each sample was mixed with an equal volume of a sample buffer containing 2% of SDS, 5% of β -mercaptoethanol, 10% of glycerol, 0.05% of bromophenol blue (as front marker), in 0.5% mmol l-1 Tris-HCl pH 6.8 and heated for 5 min at 90°C.

Electrophoresis was carried out in a Mini Protean II (Biorad) at 220 V until bromophenol blue marker reached the bottom of the gel. Gels were subsequently stained for 1 h in a 0.1% (w/v) Brilliant Blue R in methanol: acetic acid: water (40: 10: 50) and then destained for 2 h.

Protein composition analysis: Amino acids composition were determined following the high-performance liquid chromatography. Sample preparation and extraction were performed according to Osthoff *et al.* (2002). HPLC analysis was performed by using liquid chromatography (Agilent Technologies). Five micro liters of samples were injected into the HPLC column and wait until complete separation of all amino acids, it takes run about 30 min. Work on stage OPA reagent addition until complete separation of amino acids is done automatically. The nitrogen content of total protein was determined by the Kjeldahl method (AOAC, 2005).

Statistical analysis: Factorial-based complete randomized design was used as experimental design. Data was then analyzed by Analysis of Variance (ANOVA) with Tukey test as post-hoc test (Steel and Torrie, 1995).

RESULTS AND DISCUSSION

Survival of lactic acid bacteria in the sausages:

Population of LAB of the sausages 28 days room temperature storage is relatively stable (Fig. 1). Despite the population has tendency to increase until D14 storage and then slightly reduces up to D28, statistical analysis showed that these differences are not significant ($p > 0.05$). In this study, we believe that the population of LAB are mostly dominated, if not all, by the strains used as fermentation starter: *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4. To be considered as a viable probiotic in food, the minimum LAB population is 10^8 cfu/mL (Arief *et al.*, 2010). Figure 1 remarkably shown that the populations of LAB in both sausages during storage are higher than 10^9 cfu/mL indicating LAB in both sausages are acceptable to be considered as probiotic, in term of population.

Despite the population of LAB in both sausages are not significantly changed upon storage, population of LAB in *L. plantarum* IIA-2C12 fermented sausage was significantly higher than that of *Lactobacillus acidophilus* IIA-2B4. Johansson *et al.* (1993) reported that *L. plantarum* frequently predominant in lactic acid fermented foods. It might be due to adaptability of *L. plantarum* to many different conditions (Kleerebezem and Quadri, 2001).

The survival of LAB in both sausages can be monitored by the production of organic acids (mainly lactic acid) as final product of their fermentation activities. Figure 2 showed that pH and titratable acid of

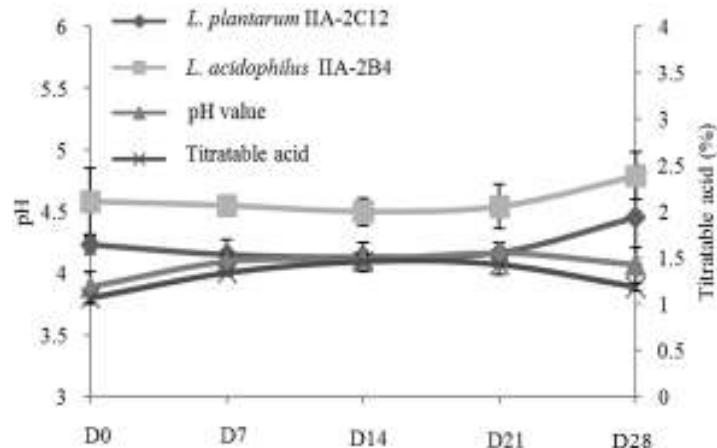


Fig. 2: Changes of pH and titratable acid content of the sausages with *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 during storage

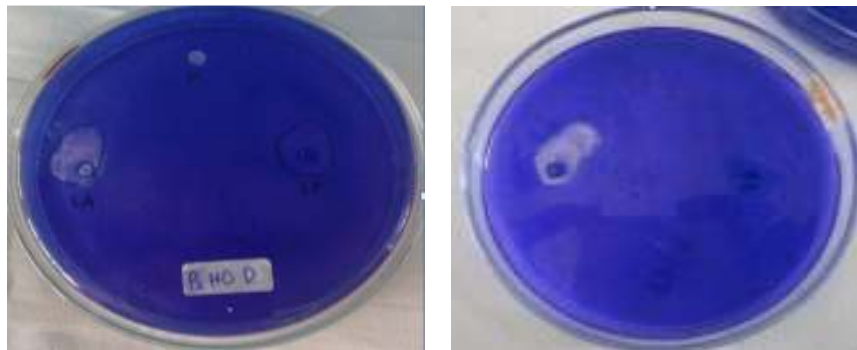


Fig. 3: Proteolytic activity of *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 on the agar medium containing sarcoplasmic proteins

both sausages are not significantly different during 28 days storage at room temperature. This result is consistent with growth rate of LAB in the sausage (Fig. 1). Aro Aro *et al.* (2010) reported that high acidification rates usually accompanied by fast lactic acid bacteria growth rates in fermented beef sausages.

pH and titratable acid are associated with the organic acid produced by LAB during fermentation and/or storage. Therefore, these parameters might indicate metabolism activity, especially in term of ability to produce lactic acid, of both strains used in the sausages. The pH values of both sausages are considerably low (less than 5) which provides uncomfortable environment for growing of pathogenic bacteria. Low pH is an important factor in the control of pathogenic and spoilage bacteria (Riebroy *et al.*, 2008). Noteworthy, titratable acid of both sausages were higher than that of reported by Nie *et al.* (2014) that using *L. plantarum* ZY40 in grass carp sausages. It is plausible, as differences in the strain and raw materials used for sausage production might affect acidification rate.

Consistent with the population of LAB in plantarum-fermented sausages, pH values on this

sausage also lower than that of acidophilus-fermented sausages. The higher population of LAB in plantarum-fermented sausage might promote production of more organic acid rather than in acidophilus-fermented sausage. In addition, *L. plantarum* IIA-2C12 had a better acidification capacity than fermented beef sausages with *L. acidophilus* IIA-2B4.

Proteolytic activity: By using agar plate method, *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 demonstrated remarkable proteolytic activity towards casein as a substrate (Fig. 3). Proteolysis activity of both strains represents hydrolysis of casein which is indicated by a zone of clearing around the growing colony. Casein, milk, or gelatin are used to test the proteolytic activity of microorganism isolated from food (Mauriello *et al.*, 2002). The clear zone formed by *L. plantarum* IIA-2C12 is significantly wider than that of *L. acidophilus* IIA-2B4 indicating that *L. plantarum* IIA-2C12 exhibited higher protease activity than *L. acidophilus* IIA-2B4 against casein.

When proteolytic activities of both strains were detected against sarcoplasmic proteins from meat, the clear zone of both strains were not significantly different (Table 1). Substrate specificity between protease

Table 1: Proteolysis activity of *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 (mm) on fermented beef sausages during room storage

	Day of storage ^b					Mean
	H0	H7	H14	H21	H28	
<i>L. plantarum</i> IIA-2C12	17.70±1.34	14.21±4.01	12.85±0.14	13.67±2.50	11.23±0.24	14.39±1.65
<i>L. acidophilus</i> IIA-2B4	15.75±0.30	13.34±0.51	17.12±0.23	14.46±0.30	11.29±0.59	13.93±0.74
Mean	16.73±0.82 ^a	13.78±2.26 ^{ab}	14.99±0.19 ^{ab}	14.07±1.40 ^{ab}	11.26±0.42 ^b	

^{a,b,c}: Value with different superscript letter in the same line are significantly different

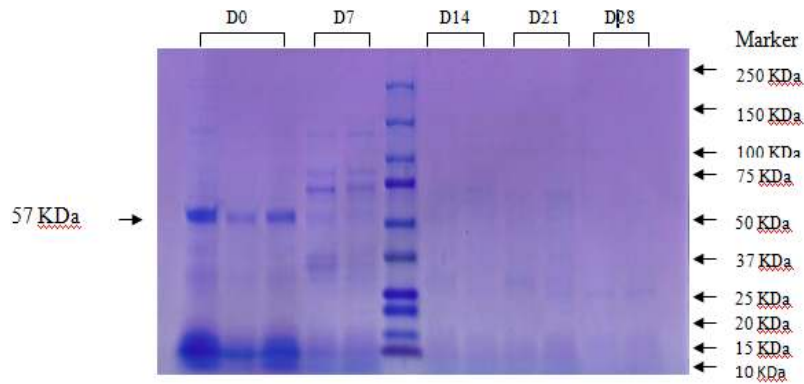


Fig. 4: SDS-PAGE profile of sarcoplasmic proteins throughout the preservation of fermented beef sausages with *L. plantarum* IIA-2C12

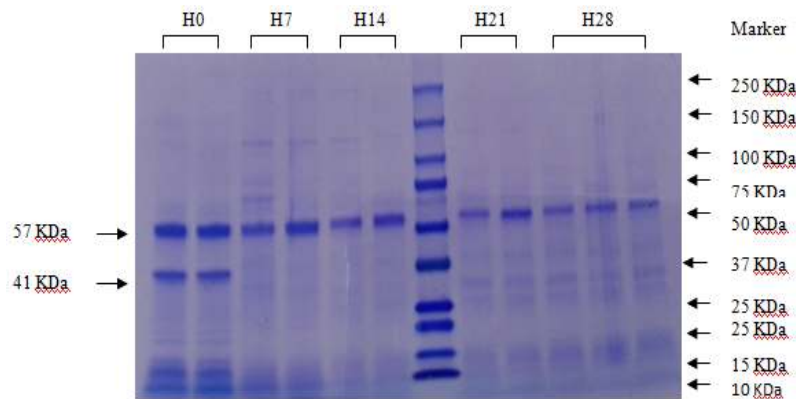


Fig. 5: SDS-PAGE profile of sarcoplasmic proteins throughout the preservation of fermented beef sausages with *L. acidophilus* IIA-2B4

produced by *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 might account for the differences on the proteolytic activity against casein and meat sarcoplasmic proteins. However, SDS-PAGE analysis (Fig. 4) clearly indicated that *L. plantarum* IIA-2C12 exhibited higher proteolytic activity than *L. acidophilus* IIA-2B4 (Fig. 5). Remarkable evidence is shown by disappearing 41-kDa band in *L. plantarum* IIA-2C12 gel indicating that this protein is completely proteolyzed even at D0. In addition, the 57-kDa protein seems to be stable in *L. acidophilus* IIA-2B4 gel, meanwhile this protein started to be completely degraded in *L. plantarum* IIA-2C12's gel at D7. The differences on proteolytic activity of these two strains shown in sarcoplasmic proteins containing-agar plate and SDS-PAGE methods might be caused by the differences in the system. Agar plate provides different

environment than meat. SDS-PAGE method reflects proteolytic activity of both strains in native environment (meat). In this method, both strains exhibited proteolytic activity against sarcoplasmic proteins in meat environment, as raw material. SDS-PAGE only displays proteolytic products from both strains. Meanwhile, in agar plate method, proteolytic activities of both strains occurred in agar environment. Despite the agar is mixed with sarcoplasmic proteins, the final condition might be different compared to the environment in meat. Indeed, environment (pH, temperature and so on) might seriously affect activity of proteases.

Given the results of casein-containing agar plate and SDS-PAGE profiles, it is apparently *L. plantarum* IIA-2C12 exhibited higher proteolytic activity than *L. acidophilus* IIA-2B4. Strong proteolytic activity of

L. plantarum IIA-2C12 towards sarcoplasmic proteins is in agreement with previous reports showing that *L. plantarum* strain, together with *L. sake*, *L. casei*, *L. curvatus* and *L. pentusus*, exhibited strong proteolytic activity towards muscle proteins (Castellano *et al.*, 2012; Fadda *et al.*, 1999; Sanz *et al.*, 1999; Sriphochanart and Skolpap, 2010). The difference of proteolytic activities of *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 is might be due to differences in the number of proteases enzyme encoded by their genomes. Mostly, among all strains of *L. plantarum* and

L. plantarum that have been genomically sequenced and stored at gene bank, *L. plantarum* strains have more proteases (19-24 proteases) proteins compared to that of *L. acidophilus* (10-17).

Sarcoplasmic proteins-containing agar plate indicated that proteolytic activity of *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4 slightly reduced upon storage time at room temperature. These phenomena might be caused by the substrate availability during the storage. As an example, SDS-PAGE showed that 41- and 57-kDa proteins, as substrates, are disappeared in

Table 2: Amino acid composition (mg/100 g of samples) fermented beef sausages with *L. plantarum* IIA-2C12 during room storage

Amino acid		Day of storage ^b					Mean
		H0	H7	H14	H21	H28	
Non polar							
Alanine	P1	100±4.00 ^{ab}	87.10±2.00 ^{ab}	96.30±3.00 ^{ab}	146.15±38.00 ^{ab}	72.37±2.00 ^{ab}	100.38±10.00
	P2	100±3.00 ^b	126.19±6.00 ^{ab}	103.77±7.00 ^{ab}	90.91±5.00 ^{ab}	238.00±4.00 ^a	131.77±5.00
	Mean	100±4.00	102.88±4.00	100.00±5.00	117.76±22.00	138.10±3.00	
Valine	P1	100±1.00 ^b	64.29±1.00 ^b	144.44±5.00 ^b	84.62±2.00 ^b	145.45±5.00 ^b	107.76±3.00
	P2	100±19.00 ^a	108.85±10.00 ^a	99.19±27.00 ^a	103.01±0.00 ^a	101.06±0.00 ^a	102.42±11.00
	Mean	100±10.00	107.08±6.00	100.26±16.00	102.37±1.00	102.32±3.00	
Leucine	P1	100±758.00	101.53±359.00	107.89±858.00	92.92±438.00	97.80±303.00	97.80±543.00
	P2	100±176.00	164.28±22.00	73.74±23.00	152.99±534.00	115.54±249.00	115.54±201.00
	Mean	100±467.00	124.14±191.00	91.60±441.00	115.98±486.00	103.68±276.00	
Isoleucine	P1	100±1.00 ^{bcd}	112.17±0.00 ^{bcd}	107.75±25.00 ^{bc}	120.86±38.00 ^{ab}	112.44±0.00 ^a	112.44±13.00
	P2	100±7.00 ^{cd}	117.58±7.00 ^{bcd}	80.37±30.00 ^{cd}	87.21±9.00 ^d	98.63±17.00 ^{cd}	98.63±14.00
	Mean	100±4.00	114.56±4.00	95.34±28.00	108.00±24.00	107.04±9.00	
Methionine	P1	100±153.00	126.45±45.00	98.04±38.00	110.67±17.00	108.36±3.00	108.36±51.00
	P2	100±10.00	107.53±21.00	110.83±47.00	79.31±7.00	99.53±12.00	99.53±19.00
	Mean	100±82.00	116.10±33.00	104.52±43.00	93.83±12.00	103.61±8.00	
Phenylalanine	P1	100±427.00	117.04±959.00	69.91±2493.00	131.69±168.00	102.17±746.00	102.17±959 ^b
	P2	100±846.00	99.67±417.00	142.54±2964	76.73±649.00	108.73±3684	108.73±1720 ^a
	Mean	100±637.00	108.17±688.00	104.08±2729	96.29±409.00	103.48±2215	
Proline	P1	100±0.00 ^b	88.89±2.00 ^b	125.00±7.00 ^b	60.00±3.00 ^b	91.44±1.00 ^b	91.44±3.00
	P2	100±8.00 ^a	9.52±1.00 ^b	83.33±2.00 ^b	100.00±1.00 ^b	82.57±2.00 ^b	82.57±3.00
	Mean	100±4.00	19.44±2.00	107.14±5.00	73.33±2.00	79.98±2.00	
Polar							
Serine	P1	100±38.00	77.55±8.00	144.74±38.00	67.27±4.00	70.27±3.00	91.97±18.00
	P2	100±8.00	112.00±4.00	89.29±1.00	136.00±2.00	85.29±18.00	104.52±7.00
	Mean	100±23.00	89.19±6.00	121.21±19.00	88.75±3.00	77.46±11.00	
Threonine	P1	100±2.00	106.25±8.00	152.94±42.00	65.38±8.00	108.82±15.00	106.68±15.00
	P2	100±7.00	109.09±4.00	133.33±21.00	112.50±12.00	55.56±1.00	102.10±9.00
	Mean	100±5.00	107.41±6.00	144.83±32.00	83.33±10.00	81.43±8.00	
Cysteine	P1	100±1.00	92.50±8.00	132.83±16.00	136.73±7.00	82.09±0.00	108.75±6.00 ^b
	P2	100±480.00	118.65±21.00	99.43±11.00	109.10±48.00	91.95±5.00	103.83±113 ^a
	Mean	100±241.00	118.37±15.00	99.71±14.00	109.40±28.00	91.82±3.00	
Tyrosine	P1	100±2.00	119.05±6.00	120.00±13.00	80.00±5.00	150.00±15.00	113.81±8.00
	P2	100±7.00	119.35±25.00	67.57±5.00	244.00±29.00	59.02±4.00	117.99±14.00
	Mean	100±5.00	119.23±16.00	88.71±9.00	154.55±17.00	84.71±9.00	
Electrically charged (acidic)							
Aspartic acid	P1	100±7.00	197.78±30.00	106.74±48.00	113.33±30.00	88.54±77.00	121.28±38.00
	P2	100±56.00	161.29±45.00	104.00±116.00	121.54±69.00	120.89±224.00	121.54±102
	Mean	100±32.00	178.28±38.00	105.42±82.00	117.25±50	104.54±151.00	
Glutamic acid	P1	100±156.00	61.35±83.00	164.59±974.00	60.07±134.00	100.80±31.00	97.36±276
	P2	100±901.00	84.60±82.00	85.01±2222	116.88±1.08	100.37±120.00	97.37±687
	Mean	100±529.00	71.18±83.00	124.61±1598	79.54±1.21	100.59±76.00	
Electrically charged (acidic)							
Lysine	P1	100±30.00	63.20±100.00	75.79±283.00	124.87±148	98.22±193.00	92.42±151
	P2	100±15.00	127.10±56.00	77.22±166.00	127.11±4.00	82.34±272.00	102.75±103
	Mean	100±23.00	84.69±78.00	76.51±225.00	126.01±76.00	90.06±233.00	
Arginine	P1	100±0.00	138.10±15.00	89.66±16.00	73.08±0.00	102.63±8.00	100.69±8.00 ^a
	P2	100±1.00	227.27±2.00	84.00±3.00	223.81±34.00	74.47±10.00	141.91±10.00 ^b
	Mean	100±1.00	156.60±9.00	87.95±10.00	116.44±17.00	87.06±9.00	
Histidine	P1	100±0.00	96.88±9.00	187.10±28.00	94.83±2.00	116.36±6.00	119.03±9.00
	P2	100±4.00	83.33±10.00	100.00±0.00	136.67±5.00	92.68±6.00	102.54±5.00
	Mean	100±2.00	89.71±10.00	144.26±14.00	109.09±4.00	106.25±6.00	

^{a b c}: Value with different superscript letter in the same line are significantly different

D-7 on *L. acidophilus* IIA-2B4's and *L. plantarum* IIA-2C12's gel, respectively. At certain time, some specific substrates probably have been completely digested by proteases secreted from both strains, therefore proteolytic activity of both strains is apparently reduced and start to be in an equilibrium state. It is also possible that this phenomena is affected by the stability of proteases from both strains during storage. Further experiment evidences are needed to confirm.

Free amino acids composition: The concentration of free amino acids in the sausages were displayed in Table 2. Glutamin is the predominant amino acid in the sausage inoculated by *L. plantarum* IIA-2C12 and *L. acidophilus* IIA-2B4. Meanwhile, proline and arginine are the least amino acids in *L. plantarum* IIA-2C12's and *L. acidophilus* IIA-2B4's sausages, respectively. The release of free amino acids is attributed to the activities of indigenous proteases (in meat) or proteases secreted by microorganism inoculated in the susages (Candogan *et al.*, 2009; Fadda *et al.*, 2002). Proteolytic activity exhibited by microorganisms in the sausage is considered as part of microorganisms activity to obtain essential amino acids for their growths (Kranenburg *et al.*, 2002). In addition, the free amino acids might affect characteristic of the products, especially in flavor development (Nie *et al.*, 2014).

It is interesting that the sausage inoculated by *L. acidophilus* IIA-2B4 released more aromatic groups containing amino acids. Meanwhile, the sausages inoculated by *L. plantarum* IIA-2C12 has more aliphatic amino acids. Aromatic amino acids have been considered as major precursor for volatile aroma compounds (Kranenburg *et al.*, 2002). This leads both sausages probably have different flavor (aroma) due to the differences of aromatic amino acids. Indeed, organoleptic test showed that *L. acidophilus* IIA-2B4's sausage has different flavor and aroma compared to that of *L. acidophilus* IIA-2B4's sausage (data will be published somewhere else).

Amino acids such as aspartic acid, isoleucine and lysine significantly changes during room storage. Other amino acids like glutamic acid, serine, histidine, threonine, arginine, tyrosine, cystine, valine, methionine, phenylalanine, leucine, alanine and proline does not change significantly during storage on days 0, 7, 14, 21 and 28, respectively. Concentration of aspartic acid, isoleucine and lysine increased during storage. This is consistent with research Aro Aro *et al.* (2010) who also showed changes in aspartic acid and lysine in ripening up to 21 days. Nie *et al.* (2014) stated that the addition of *L. plantarum* ZY40 in fermented beef sausages effectively increase free amino acids in fermented fish sausage. The existence of *L. plantarum* ZY40 showed high level isoleucine and lysine. Nie *et al.* (2014) stated that the peptidase of *L. plantarum*

ZY40 along with muscle aminopeptidase responsible for the increase of free amino acids from the N amino terminus acids of the protein muscle.

Table 2 shows that there is a change of amino acid composition in fermented beef sausages with *Lactobacillus acidophilus* IIA-2B4 in amino acid leucine, alanine and proline. Other amino acids did not change significantly during storage. Increasing the total of this amino acid showed a proteolytic activity of *Lactobacillus acidophilus* IIA-2B4. Proteolytic activity is breakdown of proteins into compounds such simpler amino acids by means of hydrolysis is carried out by microbial proteases (Poedjiadi, 1994). Hristova *et al.* (2006) found that *Lactobacillus acidophilus* has a good proteolytic activity with value 380 AU/mL.

Proteolysis during storage shows an increase of total amino acids composition. Proteolysis or degradation of protein into small peptides is combined action of endogenous cathepsins as well as microbial proteases and aminopeptidases (Latorre-Moratalla *et al.*, 2014). On this case, the common raw meat origin has an identical endogenous enzyme activity. Therefore, the differences in the evolution of some amino acid among product would be partially attributed to the distinct microbial enzymatic activity, such as *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4.

CONCLUSION

In the present study, two fermented beef sausages were prepared with *Lactobacillus plantarum* IIA-2C12 and *Lactobacillus acidophilus* IIA-2B4. Both strains displayed remarkable proteolytic activity toward casein and sarcoplasmic proteins as subsrrates. Proteolytic activity of *L. plantarum* IIA-2C12 is apparently higher than that of *L. acidophilus* IIA-2B4 which might be due to the differences number of proteases encoded by their genomes. In addition, the sausage inoculated by *L. acidophilus* IIA-2B4 produces more aromatic amino acid which might yield difference flavor formation compared to *L. plantarum*'s sausage.

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