THE EFFECT OF ADDITION OF *Lactobacillus plantarum* S4512 ON THE MICROBIOLOGICAL AND CHEMICAL CHARACTERISTICS DURING SORGHUM (*Sorghum bicolor* L. Moench) FERMENTATION

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ABSTRACT

The aim of this study was to investigate the population of selected bacteria and some chemical characteristics during sorghum fermentation with the addition of *Lactobacillus plantarum* S4512. Proteolytic *L. plantarum* S4512 isolated from natural sorghum fermentation was added into sorghum fermentation. Sorghum flour was mixed with sterile water (1:2 w/v) and then was inoculated with 1% v/v (about 10^9 CFU/ml) culture of *L. plantarum* S4512. Fermentation was carried out at 37°C for 24 hours. As a control, natural sorghum fermentation without addition of a starter culture was carried out at 30°C for 24 hours. During fermentation time, the amount of bacteria, acid producing bacteria, coliform and proteolytic bacteria were monitored. The titratable acidity, pH, soluble protein, and proteolytic activity were also measured. Addition of *L. plantarum* S4512 increased significantly the initial population of total bacteria, lactic acid bacteria and proteolytic bacteria to 10^7 CFU/ml and suppressed the growth of coliforms indicated by siginificantly decline of coliforms population after 6 h fermentation. The production of acid was doubled of that in the natural fermentation resulted in the lower pH to 3.14. Both natural sorghum fermentation and that with addition of proteolytic *L. plantarum* S4512 showed some proteolytic activities during fermentation.

Keywords: Lactic acid bacteria, sorghum fermentation, proteolytic activity

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is one of the major food crops of the semiarid regions of Africa and Asia. Compared to other major cereal crops, sorghum has a distinct advatage of being drough-resistant. In Indonesia, especially in the province of East Nusa Tenggara, farmers cultivate sorghum as a staple food crop for consumption, thus sorghum is an important source of energy, protein, vitamins and minerals for people in this region.

A nutritional constraint to the use of sorghum as food is the lower protein and starch digestibility than normal maize flour, especially after cooking (Zhang and Hamaker, 1998; Doudu *et al*., 2003; Elkhalifa *et al*., 2006; De Mesa-Stonestreet *et al*., 2010). Some studies, have shown that sorghum fermentation with naturally-occuring microflora improves the nutritional quality of sorghum. Fermentation increased the protein solubility of sorghum flour in the acidic range, increased oil-binding capacity, emulsifying capacity and emulsifying stability, while it decreased the water-binding capacity (Elkhalifa *et al*., 2005). Starch granules in non-fermented sorghum flours are completely enclosed in a very compact protein matrix. Elkhalifa *et al*. (2006) found that based on the scanning electron microscopy study, the structure of the protein coating was disappeared in the fermented sorghum, leading to the release of the small starch granules. It indicates the involvement of proteolytic enzymes in sorghum fermentation. Improvement of the *in vitro* protein digestibility of *Togwa* (a sorghum based fermented food in Tanzania), suggests that proteolysis takes place during the preparation of the product (Mugula *et al*., 2003b).

Kunene *et al*. (1999) found that sorghum powder was a source of *E. coli*, spore forming bacteria such as *B. cereus* and *C. perfringens*, and lactic acid bacteria. Traditionally, the naturally occuring microorganisms in sorghum flour are utilized in these fermentation. The microflora of fermented sorghum consisted of lactic acid bacteria, coliforms, other acid-producing bacteria, yeasts and molds (Mohammed *et al*., 1991). Lactic acid bacteria were the dominant microflora and their number increased during sorghum fermentation (Mugula *et al*., 2003; Abdel-Rahman *et al*., 2010). The process of fermentation was found to be capable of significantly

reducing the incidence of Gram negative bacteria and non-sporing bacterial pathogen (Kunene *et al*., 1999).

In this study, *Lactobacillus plantarum* S4512 which has a proteolytic activity isolated from natural fermentation of sorghum was addded to sorghum flour fermentation. The purpose of this work was to evaluate the use *L. plantarum* S4512 on the population of selected bacteria and chemical characteristics during during fermentation.

MATERIALS AND METHODS

Preparation of Sorghum Flour

Local variety of sorghum was obtained from a farmer in Belu, East Nusa Tenggara, Indonesia. Sorghum grains were milled to pass a 40-mesh screen.

Sorghum Flour Fermentation

Sorghum flour fermentation was carried out in the traditional way used by Sudanese housewives with some modifications (Mohammed *et al*., 1991). Sorghum flour was mixed with sterile distiled water in a 1:2 (w/v) ratio. The mixture was incubated at 30°C for 24 h in a sterile covered jar. This natural sorghum fermentation was performed by the original microorganisms present in the flour. Sorghum fermentation with addition of proteolytic lactic acid bacteria isolated from natural sorghum fermentation was initiated by inoculation of 1% v/v (about 10^9 CFU/ml) *Lactobacillus plantarum* S4512 into the mixture of sorghum flour with sterile distilled water (1:2 w/v ratio), and then incubated at 37°C for 24 h. There was no sterilization of the sorghum flour. Thus sorghum fermentation was performed by the original microorganism present in the flour and the inoculated *L. plantarum* S4512.

The samples were withdrawn at 0, 2, 4, 8, 12, 16, 20 and 24 h of fermentation for microbial counts, and pH measurement. Titratable acidity, proteolytic acitivity, soluble protein and reducing sugar concentrations were also monitored during fermentation. For soluble protein and reducing sugar determinations, the fermented sorghum flour was dried using oven at 55-60°C until the moisture of the sorghum flour reached 11-13%.

Enumeration of Bacteria Population

Population of bacteria during sorghum fermentation were determined using dilution and plating method. After further serially dilutions in 0.85% NaCl solution, samples were plated in duplicate onto different agar media. Aerobic mesophilic bacteria were enumerated by spread plating onto Plate Count Agar (Oxoid). Numbers of lactic acid bacteria and coliforms were determined by pour plating method using MRS (Criterion) with 1% CaCO3 and Violet Red Bile Agar (VRBA) (Oxoid) respectively. Proteolytic bacteria were enumerated on skim milk agar. After 48 h of incubation at 37°C, the colonies that appeared on the plates were counted and calculated as CFU/ml.

Chemical Analysis

The pH of the supernatant of the fermented material was measured using pH meter (Eutech Instruments pH510). The pH meter was calibrated using standard buffer solution at pH 4.0 and 7.0. The titratable acidity was measured by titrating the sample with 0.1 N NaOH using 1% phenolphthalein as an indicator. Titratable acidity was calculated and expressed as percent lactic acid.

Reducing sugars were determined according to the Nelson-Somogyi method (Anonim, 1990). Sample preparation for soluble protein determination was based on Elkhalifa *et al*. (2005) with some modification. A one gram of sample was dispensed in 60 ml ditilled water. The dispersion was continuously shaken in an orbithal shaker (Lab-line incubator-shaker) at 150 rpm for 2 h at room temperature, and then filtered using whatman paper No 1. The supernatant was collected and the soluble protein was determined by the method of Lowry-Follin.

Proteolytic Activity Measurement

Proteolytic acitivity of the supernatant of the fermented material was measured by the modifications of Nabrdalik *et al*., (2010) and Bruno *et al*., (2010). Casein Hammerstein 2% in phosphate buffer pH 7 was used as a substrate for proteolytic activity analysis. Amount of 0.5 ml Crude enzyme in 2.0 ml phosphate buffer pH 6.0 was pre-incubated for 1 minute at 40°C, then it was added with 0.5 ml substrate solution. Incubation was carried out at 40°C for 30 minutes. Enzymatic reaction was terminated by adding 5 ml of 5% trichloro acetic acid (TCA), and kept at room temperature for 30 minutes. The mixture was centrifuged at 2000 rpm for 15 minutes. One mililiter supernatant was added with 2.5 ml Na_2CO_3 0.4 M and Follin reagent (1:1), incubated at 40°C for 20 minutes, and then determined the absorbance at λ 753 nm. One unit enzyme activity was expressed as the quantity of enzyme that release the equivalent of μmole of tyrosine per minute under assay condition.

RESULTS AND DISCUSSION

Bacterial Population During Sorghum Fermentation

Figure 1A shows the population of selected bacteria during natural sorghum fermentation at 30°C for 24 h under aseptic conditions. The total plate count increased

with increasing fermentation time from 3.21 log CFU/ml to 8.88 log CFU/ml at 24 h. The initial lactic acid bacteria, coliforms and proteolytic bacteria were quite low i.e., 1.43; 2.85; and 2.88 log CFU/ml respectively. As the fermentation progressed the number of lactic acid bacteria and proteolytic bacteria increased to 8.88 log CFU/ml and 7.59 log CFU/ml respectively at the end of fermentation time. In natural sorghum fermentation, coliforms also increased significantly and reached the population of 7.98 log CFU/ml at the end of fermentation time.

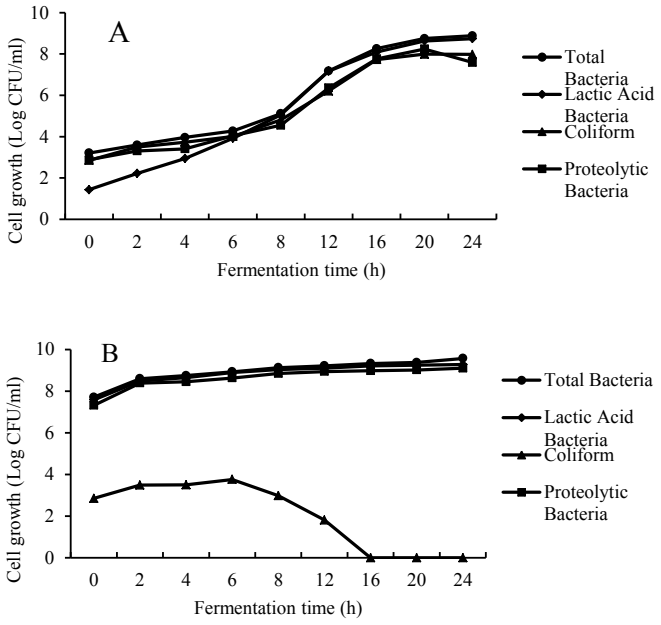

Figure 1. Bacterial growth during natural sorghum fermentation (A),and sorghum fermentation with addition of *Lactobacillus plantarum* S4512 (B)

Similar results were reported in the production of Kisra (Mohammed *et al.*, 1991). The total plate count increased from about 5 log CFU/ml to 9 log CFU/ml, lactic acid bacteria and coliforms constituted very low proportion (about 1 log CFU/ml) at the beginning of the fermentation time to 9 log CFU/ml at 24 h fermentation. Coliforms increased about 2 log cycles after 24 h fermentation of *bushera* (non-alcoholic beverage from sorghum). However, after 3 days, the coliform counts had decrease to less than 4 log CFU/ml and were not detectable after the fourth day (Muyanja *et al*., 2003). This demonstrated the effect of acid production in the suppression of coliform during spontaneous fermentation of *bushera*. Abdel-Rahman *et al*., (2010) reported that *Pediococcus pentosaceous* was the dominant lactic acid bacterium throughout the fermentation process for the three sorghum varieties. Mugula *et al*. (2003) found that lactic acid bacteria was the dominant microbial population in sorghum fermentation with the population increased from 6 log CFU/ml to 9 log CFU/ml, and *L. plantarum* dominated the final stages of fermentation. In spontaneous sorghum fermentation, occuring microorganisms including lactic acid bacteria in sorghum flour is responsible to the fermentation process.

The growth of selected bacteria during sorghum fermentation with the addition of *L. plantarum* S4512 is presented in Figure 1B. addition 1% v/v *L. plantarum* S4512 in the sorghum flour markedly increased the initial number of lactic acid bacteria, proteolytic bacteria and total bacteria to 7.58 log CFU/ml, 7.32 CFU/ml and 7.71 CFU/ml respectively. It means that in the initial fermentation time, bacterial population was dominated by lactic acid bacteria which have proteolytic activity. As the fermentation progressed the number of lactic acid bacteria, proteolytic bacteria and total bacteria increased about two log cycles, reached 9.28 log CFU/ml, 9.10 log CFU/ml and 9.57 log CFU/ml respectively. These growth profiles were similar to the traditional sorghum fermentation with inoculum in Sudan (Mohammed *et al*., 1991). Their growth rates were not as high as the ones in natural sorghum fermentation. High initial population made nutrition competition among microorganism and higher acid production also contributed to the lower growth rate. The proteolytic lactic acid bacteria dominated the initial sorghum flour fermentation due to the inoculation of proteolytic *L. plantarum* S4512. Here sorghum flour was not autoclaved prior to inoculation of *L. plantarum* S4512, thus the microorganims responsible to the sorghum fermentation were naturally occuring microorganism in sorghum flour and inoculated *L. plantarum* S4512.

The growth of coliforms suppressed during sorgum fermentation and no detectable coliform after 16 h fermentation. This could be due to the production of lactic acid by lactic acid bacteria and the low pH value. Figure 2B shows that at 8 h fermentation with the addition of *L. plantarum* S4512 the acid content was almost doubled of the one in natural sorghum fermentation resulted in the drop of pH to 3.89. In the same time the number of coliforms started to decreased. Coliforms can not grow at the pH below 4.0 (Ray, 1996). In the natural sorghum fermentation (Figure 1A) the pH was still above 4.0 at the end of fermentation, thus, coliform could grow well. The similar result was reported by Hidayah (2010) which found that no coliforms were detected in the 16 h sorghum fermentation with addition of *Lactobacillus acidophilus* FNCC 050, in which the fermentation medium pH decreased to 3.49. The reduced levels of Gram negative bacteria and bacterial spores were also found in fermented sorghum samples indicated the effectiveness of fermentation in reducing these populations (Kunune *et al*., 1999).

pH and Titratable Acidity

As the population of lactic acid bacteria increased from the initial low numbers and dominated the fermentation, the amount of acid produced increased with the concomitant drop in the pH. The acid content increased from 0.16% to 0.47% at the end of the natural sorghum fermentation time, which resulted in the drop of pH from 6.13 to 4.75 (Figure 2A).

Significant increase in acid production occured in the sorghum fermentation with the addition of *L. plantarum* S4512 , and at the end of fermentation, the acid content was doubled of the one in natural sorghum fermentation (Figure 2B). Consequently, the pH dropped faster during fermentation, from 5.99 to 4.28 in 6 h fermentation, and reached the pH of 3.41 at the end of fermentaion. The decrease in pH was a result of the production of acid by acid producing bacteria, especially lactic acid bacteria.

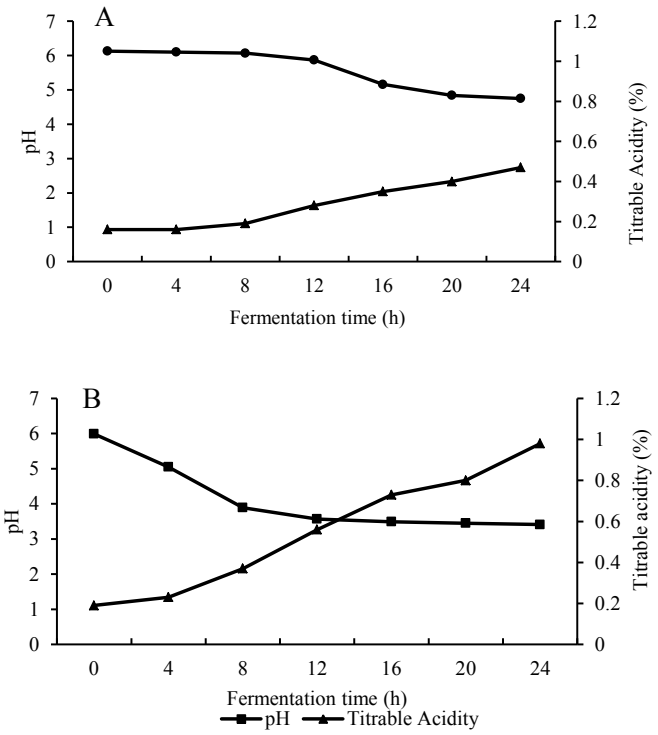

Figure 2. The changes of pH and titratable acidity during natural sorghum fermentation (A),and sorghum fermentation with addition of *Lactobacillus plantarum* S4512 (B)

The initial reducing sugar in the sorghum was 1.29% (Figure 3). The primary sugars present in sorghum grain are fructose, glucose, raffinose, sucrose and maltose (Anglani, 1998). These simple sugars utilized by microorganisms presented in sorghum fermentation for the growth and production of acid. The differences of acid production and pH during sorghum fermentation was affected by the metabolism activities of microorganism present in the fermented sorghum. Sorghum flour inoculated with *L. plantarum* S4512 contained higher lactic acid bacteria in the beginning of fermentation time and the number increased throughout the fermentation time.

Corriea *et al*. (2005) reported that ^{1}H NMR spectra show decreases of glucose, fructose and maltose signals during fermentation of sorghum, and the increased of lactic acid, acetic acid and succinic acids. During 24 h fermentation of *Togwa* the pH decreased from 5,24-5.52 to 3.10-3.34. The organic acids detected during fermentation included lactate, succinate, pyruvate and DL-pyroglutamate, formate, citrate and uric acid (Mugula *et al*., 2003a). Some of *L. plantarum* isolated from *Togwa* were able to hydrolyse starch. *Lactobacillus plantarum* S4512 does not have amylolytic activities. It could be other microorganism naturally occured in sorghum flour responsible to the hydrolysis of sorghum starch.

Proteolytic Activity

In the natural sorghum fermentation, the initial proteolytic activity is 4.1 x 10^{-3} U/ml and then increased to 8.1 x 10^{-3} U/ml at 4 h fermentation and relatively constant until the end of fermentation time. It indicates that naturally microorganism in sorghum flour had proteolytic acitivity. Addition of proteolytic *L. plantarum* S4512 in sorghum fermentation shows similar initial proteolytic activity of the medium fermentation. It could be that in the initial fermentation time *L. plantarum* S4512 have not yet produced protease or its proteolytic activity was low. The proteolytic activity in sorghum fermentation with addition starter culture increased throughout the fermentation up to 16 h fermentation time and then decreased at the end of fermentation time (Figure 3).

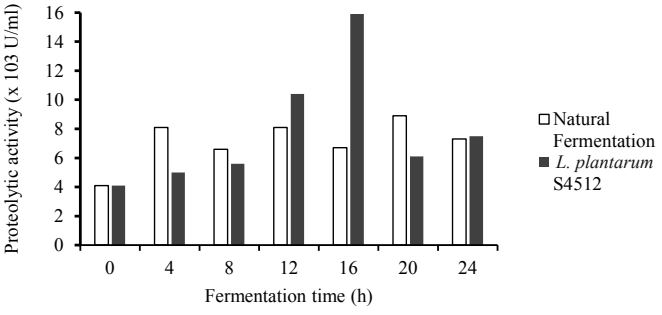

Figure 3. Proteolytic activity during natural sorghum fermentation (■), and sorghum fermentation with addition of *Lactobacillus plantarum* S4512 (□)

In-vitro improvement of protein digestibility in fermented sorghum reported by many researchers indicates that proteolysis take place during sorghum fermentation.

Fermentation of sorghum flour increased the protein solubility of sorghum flour in pH 2-4, meanwhile the unfermented sorghum flour had minimum protein solubility at pH 4 (Elkhalifa *et al*., 2005). The high protein solubility at pH 4 could be due to proteolytic activity. Elkhalifa *et al*., 2006) also reported that protein solubility and SDS-PAGE studies indicated that water-soluble proteins are the main target of hydrolysis during fermentation. Mugula *et al*., (2003b) reported more proteinase and aminopeptidase activities were observed in sorghum-based togwa prepared by natural fermentation than those fermented using starter cultures isolated from native product (*L. brevis*, *L. cellobiosus*, *L. fermentum* and *L. plantarum*). In their research, each starter culture was inoculated into sterile sorghum-based media, thus the enzyme activities were produced only by each lactic acid bacteria used as starter. Although all *togwa* prepared using starter cultures had proteolytic activity, their proteolytic activities were low and varied with different starter cultures. In natural fermentation, proteases were possibly produced by various naturally microorganisms in the sorghum resulted in higher proteolytic activity. In our study, sorghum was prepared without sterilization, thus in sorghum fermentation using addition of *L. plantarum* S4512, the proteolytic activity in the supernatant could be produced by natural microorganisms in sorghum flour and by *L. plantarum* S4512. Our results showed that proteolytic activities during natural sorghum fermentations and with starter culture were not markedly different. It could be that proteolytic activity produced by *L. plantarum* S4512 was not high enough to give significant increase in proteolytic activity in sorghum fermentation.

Soluble Protein and Reducing Sugar Contents in Fermented Sorghum Flour

The change of soluble protein and reducing sugar contents in fermented sorghum flour can be seen in Figure 4 and 5. Soluble protein content in fermented sorghum flour varied within the fermentation time, and relatively higher value was observed in fermentation with addition of *L. plantarum* S4512. Reducing sugar content in fermented sorghum flour from natural fermentation and the one with addition of *L. plantarum* S4512 increased with the increase in fermentation time up to 8 h, and then decreased until the end of fermentation time.

Proteolytic producing microoorganisms in sorghum and *L. plantarum* S4512 produced proteases which degrade sorghum protein into smaller protein/peptides and amino acid thus increased the soluble protein. The free amino acids released will be utilized by microorganism for their growth. Correira *et al*. (2010) carried out sorghum fermentation with strains of lactic acid bacteria, and found various soluble protein, and free amino acid in fermented sorghum. Free

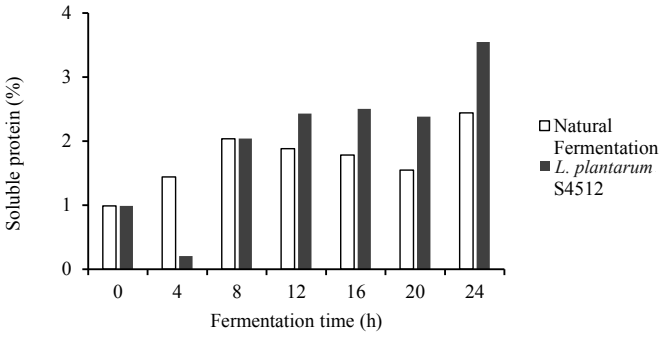

Figure 4. Protein solubility of sorghum flour during natural sorghum fermentation, and sorghum fermentation with addition of *Lactrobacillus plantarum* S4512

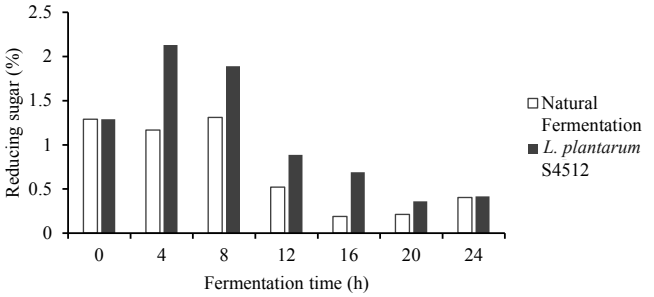

Figure 5. Reducing sugar of sorghum flour during natural sorghum fermentation and sorghum fermentation with addition of *Lactrobacillus plantarum* S4512

amino acids and soluble protein in fermented sorghum with *Lactobacillus brevis* increased compared to the ones in unfermented sorghum. This increase could be due to hydrolysis of insoluble proteins by bacterial proteolysis into peptide and amino acids. However, there was a decrease in soluble protein and amino acids contents in fermented sorghum with *L. plantarum*, *Lactobacillus paracasei* and *Lactobacillus fermentum*. This results indicates that soluble proteins were hydrolyzed to amino acids by bacterial proteases and peptidases, and these amino acids can be readily utilized by microorganism for their growth and metabolism activities.

The amount of soluble protein in the fermented sorghum is affected by the proteolytic activities produced by microorganisms in during fermentation and the rate of consumption of free amino acids for microbial growth and metabolism activities. It could be that higher microbial growth rate in natural sorghum fermentation compared to the one with addition of *L. plantarum* S4512 resulted in higher consumption of free amino acids and thus lower soluble protein content.

Mugula *et al*. (2003b) reported that the SDS-PAGE banding pattern indicated breakdown of high molecular mass protein in *togwa* fermentation. They also found that

natural fermentation increased the content of glutamic acid, proline and ornithine, but the concentration of most of the free amino acids, including the essential amino acids, was reduced during both natural and controlled fermentation. This indicates the utilization of amino acids for growth and production of metabolites. Carreia *et al*. (2005) also noted the reduction of soluble protein and the increase in free amino acid in traditional sorghum fermentation. This supports the involvement of proteolytic enzymes in sorghum fermentation.

The increase in reducing sugar content in fermented sorghum is a consequence of starch hydrolysis. The simpler sugars then can be utilized by microorganism to growth and metabolism activities, thus reduce the reducing sugar content. The decreased in reducing sugar content of fermented sorghum is in agreement with the ^{1}H NMR spectra which show decreases of glucose., fructose and maltose signals (Correia *et al*., 2005). Thus the concentration of reducing sugar in fermented sorghum flour is affected by the activity of microbial amylases during fermentation and the rate of fermentable sugars consumption for growth and production of metabolites.

Correira *et al*. (2010) reported that sorghum fermented with *Lactobacillus brevis* had higher reducing sugar content and total soluble sugars, and lower total starch content compared to the ones in unfermented sorghum. It seems that *L. brevis* has amylolytic and proteolytic activities simultaneously. Proteolytic attack to the proteins could make starch more accessible to bacterial amylases resulted in higher reducing sugar content. Elkhalifa *et al*. (2006) suggested that starch granules in unfermented sorghum flours are completely enclosed in a very compact protein matrix. The protein barrier surrounding the strach granule may reduce the hydrolysis of native starch by amylolytic enzyme. The present of proteases degrades the protein matrix and enhanced starch hydrolysis by increasing surface area and enabling starch to interact with amylases, thus increased the reducing sugar content.

CONCLUSIONS

Addition of *L. plantarum* S4512 in the sorghum flour fermentation increased the initial population of lactic acid bacteria and proteolytic bacteria, and significantly suppressed the population coliform. The production of acid increased significantly, and the acid content of fermented sorghum with addition of *L. plantarum* S4512 was doubled of that in the natural fermentation. Both natural sorghum fermentation and that with addition of proteolytic *L. plantarum* S4512 showed some proteolytic activities during fermentation.

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