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IDENTIFICATION AND CHARACTERISATION OF LACTIC ACID BACTERIA IN MAIZE SOURDOUGHS AND THEIR ROLE IN IMPROVING MAIZE BREAD QUALITY

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ABSTRACT

Lactobacillus plantarum (strain B411) and multiple strains starter culture fermented maize sourdoughs have successfully been used to produce maize bread with improved quality. In this study, the dominant lactic acid bacteria in these sourdoughs were characterised and identified using MALDI-TOF and found to be *L. plantarum*. These dominant lactic acid bacteria were tested for amylolytic and proteolytic properties. It was only the *L. plantarum* from the multiple strains sourdough that exhibited amylolytic property. However, the dominant lactic acid bacteria in both sourdoughs exhibited proteolytic properties. Greater proteolytic activity was observed in the *L. plantarum* sourdough. Relating these findings to the improvement in maize bread quality, it is suggested that the amylolytic and proteolytic activities of the lactic acid bacteria brought about starch modification either directly by hydrolysing the starch granules, thereby creating a larger surface area and hence increased water absorption, and/or increasing the accessibility of water to the starch as a result of hydrolysis of the endosperm protein matrix and proteins soluble in the dough liquid, binding to the starch granules.

Keywords: maize sourdough, proteolytic activity, amylolytic activity

INTRODUCTION

Lactic acid bacteria (LAB) are a group of related bacteria that produce lactic acid from carbohydrates through fermentation as a major metabolic product. LAB has been used to ferment or culture foods for at least 4000 years (Reddy et al., 2008). *Lactobacilli* vary in morphology from long, slender rods to short coccobacilli, which frequently form chains. However, under certain conditions some LAB do not display all these characteristics. Thus, the most profound features of LAB are Gram positiveness and inability to synthesize porphyrin groups (Axelsson, 2004). The inability to synthesize porphyrin (for example haem) results in the LAB being devoid of catalase. The taxanomy of LAB has been based on the Gram reaction and the production of lactic acid from various fermentable carbohydrates. Typical LAB are Gram-positive, non-sporing, catalase-negative, devoid of cytochromes, anaerobic but aerotolerant cocci or rods that are acid tolerant and produce lactic acid as the major end product during sugar fermentation (Axelsson, 2004). LAB grow under anaerobic conditions but they can grow in the presence of oxygen. Because of low energy yields, LAB often grow more slowly than microbes capable of aerobic respiration, and produce smaller colonies of 2-3 mm. LAB can grow at a temperature range of 5–45^{o}C (Reddy et al., 2008). LAB have complex nutritional requirements for amino acids, peptides, nucleotidebases, vitamins, minerals, fatty acids and carbohydrates (Reddy et al., 2008). The

LAB are divided into three groups based on fermentation patterns: homofermentative LAB which produce more than 85% lactic acid from glucose and produce lactic acid as the major product of fermentation; heterofermentative LAB which produce only 50% lactic acid together with ethanol and carbon dioxide; and lastly the less well known heterofermentative LAB species which produce DL-lactic acid, acetic acid and carbon dioxide (Reddy et al., 2008).

Sourdough is a mixture of flour and water fermented by naturally occurring LAB and yeast. Although these sourdough microorganisms originate mainly from the flours and process equipment, the resulting composition of the sourdough microbiota is determined by endogenous (for example, chemical and enzyme composition of the flour) and exogenous (for example, temperature, redox potential, dough yield and time of the fermentation process) factors (Hammes and Gänzle, 1998). LAB are the dominant microorganisms in sourdoughs, and the rheology, flavour and nutritional properties of sourdough-based baked products greatly rely on the activity of LAB (Gobbetti et al., 2005). LAB in mature sourdoughs occur in high numbers $>10^8$ cfu/g (Ehrmann and Vogel, 2005).

Amylolysis and proteolysis are among the enzymic activities of LAB in sourdough (Corsetti et al., 1998). However, these properties vary among LAB organisms. Amylolytic LAB (ALAB) are a group of LAB that have the ability to partially hydrolyze raw starch through the activities of their α-amylases (Rodriguez-Sanoja et al., 2000). ALAB have been reported in different tropical amylaceous fermented foods, prepared from cassava and cereals such as maize and sorghum (Reddy et al., 2008). ALAB are also involved in cereal-based fermented foods such as European sour rye bread, Asian salt bread, dumplings and non-alcoholic beverages production. ALAB are important because they can metabolise starch into lactic acid in a single step fermentation (Reddy et al., 2008). Lactic acid bacteria also possess a variety of proteolytic enzymes that facilitate their growth (Matar et al., 2001). The proteolytic systems of these LAB hydrolyze proteins to peptides and then to amino acids, which is essential for bacterial growth (Liu et al., 2010).

This study focused on identification and characterisation of the dominant LAB in the *Lactobacillus plantarum* (B411) and multiple strains starter culture fermented maize sourdoughs. The possible role of the dominant LAB in improving the quality of the maize bread was discussed.

MATERIALS

Refined maize meal (Impala Special Maize Meal, Premier Foods, Isando, South Africa) with a protein content 8.6 g/100 g (db) and a fat content 2.7 g/100 g (db) was milled into a flour using a laboratory hammer grinder (Mikro-Feinmuhle-Culatti MFC grinder, Janke and Kunkel, Staufen, Germany) fitted with a 0.5 mm opening screen. The *Lactobacillus plantarum* culture (B411) was obtained from the Council for Scientific and Industrial Research, Pretoria, South Africa.

METHODS

Preparation of the sourdoughs

L. plantarum fermented maize sourdough was prepared by mixing maize flour with sterile distilled water containing *L. plantarum* (B411) cells (9.3×10^{10} cfu/ml) in a ratio of 1:1 (w/v). The slurry was fermented at 30^{o}C to a pH range of 3.3-3.6 (approx. 24 h). Multiple strains starter culture fermented maize sourdough was prepared by mixing maize flour with sterile distilled water. The maize dough was left to ferment for 72-96 h at ambient temperature. A portion of the fermented maize dough was used as a starter (backslopping) for a fresh mixture of maize flour and water. The mixture was fermented at 30^{o}C to a pH of 3.4-3.7 (approx. 48 h).

Selection and purification of isolates in the maize sourdoughs

L. plantarum fermented maize sourdough and multiple strains starter culture fermented maize sourdough were plated on mMRS agar (de Man, Rogosa and Sharpe agar modified with 1% (w/v) maltose and 5% (w/v) yeast extract (Coda et al., 2011), pH adjusted to 5.6 with 0.1M HCl). Six isolates each were randomly selected from the plate with the highest dilution (10^{-9}) for each of the sourdoughs. These isolates were streaked on mMRS agar until a pure culture was obtained.

Morphological properties of the colonies

This was done by physically observing the size, shape and appearance of the colonies with the eyes.

Biochemical analyses

Catalase test

This was performed to determine if the isolates were catalase positive or negative. It involved preparing a smear of the isolate using 3% (v/v) hydrogen peroxide (Olutiola et al., 2000). Oxygen bubble formation is an indication of the presence of catalase.

Gram's test

Eighteen to 24 hour old isolates were heat fixed on a slide. Staining was done with crystal violet solution for 2 min and rinsed off with Gram's iodine solution. The slides were washed with 95% alcohol and rinsed under gentle running water. Counter staining was done with safranin. The slides were then washed, blotted dry and viewed under the microscope (Olutiola et al., 2000). A purplish colour is an indication that the isolate is Gram positive, while a pinkish colour is an indication that the isolate is Gram negative.

Amylolytic activity of isolates

Isolates were tested for their ability to hydrolyze starch. Starch agar was prepared (beef extract (3 g), soluble starch (10 g), agar (12 g) in 1 L of distilled water). The isolates were grown on the starch agar for 48 h at 30^{o}C, after which the plates were flooded with Lugol's iodine solution. Clear zones around the colonies is an indication of starch hydrolysis (Hashim et al., 2004).

Proteolytic activity of isolates

Isolates were tested for proteolytic activity. MRS-caseinate agar (MRS agar (62 g), sodium caseinate (10 g), tri-sodium citrate (3.8 g), calcium chloride (2.2 g) in 1 L of distilled water) was prepared. The isolates were grown on the starch agar for 48 hr at 30^{o}C (Williams and Banks, 1997). White zone formation around the colonies is an indication of proteolytic activity (Vermelho et al., 1996).

Identification of the isolates

The isolates were identified using a MALDI Biotyper 3.0, Bruker Daltonik, Bremen, Germany (Standing et al., 2013). The MALDI Biotyper identifies microorganisms using MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry measuring the unique protein fingerprint of an organism. Specifically, the MALDI Biotyper measures highly abundant proteins that are found in all microorganisms (such as ribosomal or nucleic acid-binding proteins) and uses it as a biomarker (Pineda et al., 2003). The characteristic patterns of these proteins are used to reliably identify a particular microorganism by matching the respective patterns with an extensive open database (MALDI Biotyper Real Time Classification 3.0) to determine the identity of the microorganism.

RESULTS

All the isolates from the *L. plantarum* or multiple strains starter culture fermented maize sourdoughs were puntiform, dome shaped with entire edge (Tables 1 and 2). They also had opaque and smooth surface. All the isolates were catalase negative. The isolates from both the *L. plantarum* and multiple strains starter culture fermented maize sourdough were purplish when viewed by light microscopy. Also, isolates from

the *L. plantarum* sourdough appeared rod-like in shape. In contrast, the isolates from the multiple strains sourdough appeared more like cocci, joint together like short chains. All the isolates from the multiple strains sourdough showed clear zones when Lugol's iodine was poured over the growth area. However, isolates from the *L. plantarum* sourdough did not show clear zones.

TABLE 1: CHARACTERISATION AND IDENTIFICATION OF ISOLATES FROM *L. PLANTARUM* FERMENTED MAIZE SOURDOUGH

Isolate Codes	Morphology of the isolates	Gram Staining Test	Catalase Test	Amylolytic Property	Proteolytic Property	Organisms as Identified by the MALDI-TOF
ISMA	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	+++	*Lactobacillus plantarum*
ISMB	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	+++	*Lactobacillus plantarum*
ISMC	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	++	*Lactobacillus plantarum*
ISMD	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	++	*Lactobacillus plantarum*
ISME	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	++	*Lactobacillus plantarum*
ISMF	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	+	*Lactobacillus plantarum*

TABLE 2: CHARACTERISATION AND IDENTIFICATION OF ISOLATES FROM MULTIPLE STRAINS STARTER CULTURE FERMENTED MAIZE SOURDOUGH

Isolate Codes	Morphology of the isolates	Gram Staining Test	Catalase Test	Amylolytic Property	Proteolytic Property	Organism as Identified by the MALDI-TOF
ISMG	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	-	*Lactobacillus plantarum*
ISMH	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	-	-	*Lactobacillus plantarum*
ISMI	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	+	-	*Lactobacillus plantarum*
ISMJ	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	+	+	*Lactobacillus plantarum*
ISMK	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	+	-	*Lactobacillus plantarum*
ISML	Puntiform, dome shaped with entire edge. Opaque and smooth surface	+	-	+	-	*Lactobacillus plantarum*

All the isolates from the *L. plantarum* sourdough showed white zones around the colony growth (Fig 1). However, only one of the isolates from the multiple strains sourdough showed white zone around the colony. All the isolates were identified as *L. Plantarum*

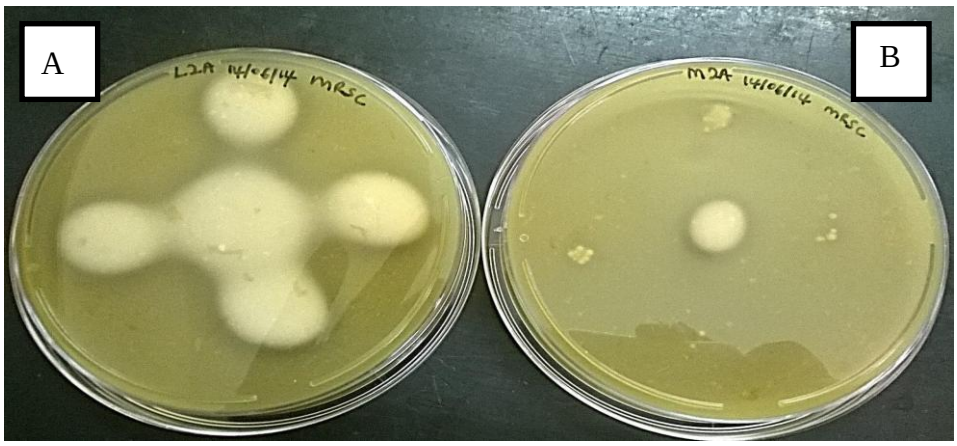

Figure 1: Proteolytic activity of the Isolates from, (A): *L. plantarum* fermented maize sourdough, and (B): Multiple Strains Starter Culture fermented Maize Sourdough Proteolytic activity: +: zone formed < 5 mm)

DISCUSSION

Catalase test

The isolates being catalase negative means that they did not produce phorphyrin and suggests that they were all LAB (Axelsson, 2004). According to this author, the inability of LAB to synthesize porphyrin groups is one of their profound characteristics. This inability results in the LAB being devoid of catalase.

Gram's test

Purplish colour of the isolates when viewed under the microscope indicates that they were Gram positive. This is a key feature of LAB (Axelsson, 2004).

Amylolytic activity of isolates

Apppearance of clear zones when Lugol's iodine was poured over the growth area is an indication of amylolytic activity (Hashim et al., 2004). According to Wehrle and Arendt (1998), the rapid drop in pH level in sourdough can cause reduced amylolytic activity, whereas the slower drop in the pH level in spontaneously fermented dough permits further starch degradation. Since the *L. plantarum* sourdough was incubated for a shorter time period (24 h) than for the multiple strains sourdough (48 h), it can be speculated that this may be the reason for the absence of amylolytic property in the isolates from the *L. plantarum* sourdough. However, this speculation was disproved when the actual *L. plantarum* culture used as a starter culture for the *L. plantarum* sourdough did not exhibit amylolytic property.

Proteolytic activity of the isolates

White zones around the colony growth are an indication of proteolytic activity (Vermelho et al., 1996). However, only one of the isolates from the multiple strains sourdough showing white zone around the colony suggests lower proteolytic activity in the multiple strains sourdough isolates than in the isolates from the *L. plantarum* sourdough.

Identification of isolates from the sourdoughs

L. plantarum has been shown to be the dominant organism at the end of the fermentation of maize-derived products like ogi (fermented maize gruel popularly consumed in West Africa) (Steinkraus, 1995). There are generally four factors that account for the dominance of lactobacilli in a sourdough, namely their highly adapted carbohydrate metabolism, their growth requirements for temperature and pH that match the conditions encountered during sourdough fermentation, their possible stress responses, and their excretion of antimicrobial compounds which may inhibit the growth of other microorganisms (reviewed by De Vuyst and Neysens, 2005). Some LAB occurring in sourdoughs are sensitive to low pH and therefore will not survive for long. More acid-resistant species will be able to survive for longer and eventually, may become dominant (Clarke and Arendt, 2005). Since the pH of the sourdoughs were low (< 4), a more suitable environment was probably created for the *L. plantarum* to thrive better than the other microorganisms in the dough. The dominance of *L. plantarum* at the late stages of cereal fermentation has been attributed to its high acid tolerance (Oyewole and Odunfa, 1990; Hounhouigan et al., 1993), or perhaps better substrate utilization (Oyewole and Odunfa, 1990). Weckx et al. (2010) attributed the dominance of *L. plantarum* in sourdoughs not only to its high acid-tolerance but also to its ability to transport and metabolize different plant carbohydrates.

PROBABLE RELATIONSHIP OF THE PROTEOLYTIC AND AMYLOLYTIC ACTIVITIES OF LAB TO IMPROVEMENT IN MAIZE BREAD QUALITY

From our previous study, improvement in maize bread quality by sourdough fermentation is primarily due to starch granule modification (Falade et al. 2014). It is therefore suggested that the proteolytic and/or amylolytic activities of the dominant LAB in the maize sourdough may be related to starch granule modification. However, the way in which each activity brings about this modification differs.

As stated, amylolytic LAB are a group of LAB that have the ability to partially hydrolyze raw starch through the activities of their α-amylases (Rodriguez-Sanoja et al., 2000). It is speculated that hydrolysis of starch probably brought about starch modification by increasing the rate of water absorption by the starch granules. This effect probably influenced the rheological properties of the maize dough and eventually improved maize bread quality. Edema et al. (2013) attributed the improvement in fonio bread quality brought about by sourdough fermentation specifically to starch modification (slight granule swelling and probably some leaching of starch molecules) by endogenous amylases from the sourdough microorganism whose activities were favoured at low pH.

As also stated, the proteolytic systems of LAB hydrolyze proteins to peptides and then to amino acids, which are required for growth of the LAB (Liu et al., 2010). Though starch cannot be hydrolyzed by the proteolytic activities of the LAB, the proteolytic activities of LAB can be linked to starch modification. It can be proposed that proteolytic activities of LAB hydrolysed the endosperm protein matrix and proteins soluble in the dough liquid, binding to the starch granules allowing increase accessibility of water to the starch granules, hence a form of starch modification. This effect probably influenced the rheological properties of the maize dough and eventually improved maize bread quality. Schober et al. (2007) attributed the improvement of sorghum bread to the effect of sourdough fermentation on the endosperm protein matrix and proteins soluble in the dough liquid. Sourdough fermentation brought about degradation of these proteins, preventing them from interfering with the starch gel.

CONCLUSIONS

The role of the dominant LAB in improving the quality of maize bread may be attributed to their amylolytic and proteolytic activities in the maize

sourdough. These activities probably brought about starch modification. Starch modification thereby, positively influenced the rheological properties of the maize dough and bread properties.

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