Natural and Lactic Acid Bacteria Fermentations of Pastes of Soybeans and Soybean-Maize Blends: Effect on Nutritional Quality, Microbial Diversity, Food Safety and Consumer Acceptance

Naturlig fermentering og fermentering med melkesyrebakterier av soyabønne- og soyabønne-mais blandinger: Effekt på ernæringsmessig kvalitet, mikrobiell diversitet, mattrygghet og forbrukeraksept

Philosophiae Doctor (PhD) Thesis

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Tinna Austen Ng'ong'ola-Manani.

Ås, Norway, September, 2014.

DEDICATION

To

My Dad, *Austin Henderson Supuni Ng'ong'ola* and my mum, *Grace Chapasi Ng'ong'ola*.

For your love and for instilling love for education in your five daughters and your son.

&

To

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For your love, patience and endurance during my studies.

ABBREVIATIONS

100S	Paste composed of 100% soybeans and subjected to natural fermentation
100SBS	Paste composed of 100% soybeans and subjected to lactic acid bacteria fermentation through back-slopping
100SC	Paste composed of 100% soybeans and fermented with *Lactobacillus fermentum*
75S	Paste composed of 75% soybeans and 25% maize and subjected to natural fermentation
75SBS	Paste composed of 75% soybeans and 25% maize and subjected to lactic acid bacteria fermentation through back-slopping
90S	Paste composed of 90% soybeans and 10% maize and subjected to natural fermentation
90SBS	Paste composed of 90% soybeans and 10% maize and subjected to lactic acid bacteria fermentation through back-slopping
90SC	Paste composed of 90% soybeans and 10% maize and fermented with *Lactobacillus fermentum*
BSP	Pastes fermented through back-slopping
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
LAB	Lactic acid bacteria
LFP	Lactic acid bacteria fermented pastes
NFP	Naturally fermented pastes
PCA	Principal component analysis
PCR	Polymerase chain reaction
SCP	Pastes fermented with *Lactobacillus fermentum* as starter culture
TI	Trypsin inhibitors
TIA	Trypsin inhibitor activities

SUMMARY

Traditional Malawian diets are predominantly maize-based and have been associated with widespread inadequate intakes of several nutrients. In addition to maize, legumes are an important source of protein and other nutrients in diets of many people in developing countries. Soybeans have the highest protein content among legumes and when consumed together with cereals, a high quality protein is provided because cereals and legumes are complementary in terms of limiting amino acids. However, soybean utilization in Malawi is minimal due to limited knowledge in processing.

In an effort to increase utilization and consumption of soybeans by all age groups in Malawi, solid-state fermented pastes of soybeans and soybean-maize blends were developed. The fermented pastes were to be used as relish and to serve as major sources of protein in maize-based diets. Spontaneous solid state fermentation of soybeans favors growth of *Bacillus subtilis*, a highly proteolytic organism that produces high amount of ammonia. High ammonia levels result in strong odor which some people find objectionable. On the other hand, lactic acid bacteria (LAB) are weakly proteolytic and do not lead to accumulation of high levels of organoleptically unpleasant metabolic products.

In this study, *thobwa*, a Malawian fermented cereal gruel prepared from maize flour and co-fermented with malt flour from finger millet was used as a back – slopping material to facilitate lactic acid bacteria fermentations in LAB fermented pastes (LFP). Whereas pastes fermented without inoculum were referred to as naturally fermented pastes (NFP). Pastes composed of 100% soybeans, 90% soybeans and 10% maize, and 75% soybeans and 25% maize. Naturally fermented pastes were designated 100S, 90S and 75S, while LFP were designated 100SBS, 90SBS and 75SBS. Metabolite changes, microbial diversity, growth and survival of enteropathogens, sensory properties and consumer acceptance of pastes of soybeans and soybean-maize blends fermented naturally and by LAB were compared.

Both types of fermentation resulted in increases in soluble protein which were pronounced at 48 hrs in most samples and were highest in 100S (49%). High decreases in total amino acids were also observed at 48 hrs, with 6.8% and 7.4% reductions in 100S and in 100SBS, respectively. On a positive note, the limiting amino acids, cysteine (in 100S and 90S) and methionine (in 90S) increased throughout fermentation. Whereas in LFP, cysteine increased during 48 hrs of fermentation and this trend was also observed with methionine in 75SBS.

Both types of fermentation degraded anti-nutritional factors, phytic acid and trypsin inhibitors. However, natural fermentation was more effective in degrading phytic acid than LAB fermentation. In NFP, 33 to 54% reduction in phytic acid was achieved during 24 hrs fermentation and by 72 hrs, 85% reduction was noted and the phytate was not detected in some samples. Whereas, 18 to 32% reduction was achieved in LFP after 24 hrs, and by 72 hrs, 37 to 49% reduction had been achieved.

Lactic acid was the major end product of fermentation in both LFP and NFP. High lactic acid production in LFP was consistent with pH reduction. The pH was reduced from 6.44 - 6.48 to 4.20 – 4.64 representing a 28 – 35% reduction after 24 hrs fermentation. After 72 hrs, the pH was reduced to 3.91 – 4.26, representing 34 – 39% reduction. In NFP, pH was reduced from 6.88 – 6.95 to 6.15 – 6.74 during 24 hrs and to 5.36 – 5.81 during 72 hrs representing 3 – 12% and 17 – 23% reductions, respectively. Higher pH reduction in LFP could have been due to a higher LAB population which was 3 $\log_{10}$ cfu/g higher than in NFP at the beginning of the fermentations.

The fermenting LAB microflora in both NFP and LFP were heterofermentative rods and homofermentative cocci. The microbiota were phenotypically characterized as *Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus buchneri, Lactobacillus collonoides, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus pentosus*, *Leuconostoc mesenteroides*, *Weissella confusa, Lactococcus lactis* subsp. *lactis*, *Pediococcus pentosaceus* and *Pediococcus damnosus*. The dominant microflora were *Lb. fermentum, Lb. brevis*, *W. confusa* and *P. pentosaseus*. These four species were confirmed as the dominant fermenting microflora by 16*S* rDNA genotyping. In addition, *Bacillus* spp. and *Enterococcus faecium*/ *Enterococcus durans* were identified as part of the microflora.

Denaturing gradient gel electrophoresis confirmed *Lb. fermentum, W. confusa*/*Weissella cibaria*, *P. pentosaseus* as dominant microflora. DGGE revealed microbial succession in NFP in which *Bacillus* spp. and *Lactobacillus linderi* were succeeded during later fermentation. Microbial diversity was similar throughout fermentation in LFP. The following microorganisms were present in both NFP and LFP at the end of the fermentations: *P. pentosaceus*, *Lb. fermentum*, *W. confusa/W. cibaria*, and *Weissella koreensis.*

In paper IV, natural fermentation, LAB fermentation through back-slopping and starter culture fermentation using *Lb. fermentum* were inoculated with *Escherichia coli.* All

fermentations could not reduce the pH to ≤ 4.4, the critical value for *Escherichia coli* growth. Nevertheless, back-slopping inhibited *E. coli* growth more than the other fermentations. In back-slopped pastes, *E. coli* counts increased from 2.4 to 3.5 $\log_{10}$ cfu/g during 24 hrs and remained constant during further fermentation. While *E. coli* population increased from 2.0 – 2.3 $\log_{10}$ cfu/g to 6.8 – 7.6 $\log_{10}$ cfu/g in *Lb. fermentum* fermentation and from 2.3 $\log_{10}$ cfu/g to 8.8 – 9.2 $\log_{10}$ cfu/g in NFP during 24 hrs fermentation. The cell counts were above the infectious dose of 100 cells implying food safety concerns for some Shiga-toxin producing *E. coli* in the event of contamination during fermentation.

In fermentations inoculated with *Bacillus cereus*, only back-slopping reduced the pH to below 5.0, the critical value for *B. cereus* growth. After 72 hrs, *B. cereus* cell counts ranged between 0 to 3 $\log_{10}$ cfu/g in back-slopped pastes. In *Lb. fermentum* fermentation, pH values ranged between 5.30 and 5.35 while cell counts were 3.7 to 5.3 $\log_{10}$ cfu/g after 72 hrs of fermentation. In natural fermentation, pH increased from 5.87 at 24 hrs to 7.2 during 72 hrs of fermentation in 90S. Consequently, *B. cereus* population increased from 2.2 $\log_{10}$ cfu/g to above 8.0 $\log_{10}$ cfu/g during 24 hrs of fermentation. Since the infectious dose for *B. cereus* is ≥ 3.0 $\log_{10}$ cfu/g, it was concluded that back-slopping has a potential of producing pastes that are safe with regards to *B. cereus* poisoning. Nevertheless, a thermal treatment of the pastes prior to consumption was recommended to ensure safety.

Consumers unconsciously used type of fermentation to determine their preference patterns and preference was biased towards natural fermentation. Naturally fermented pastes were characterized by yellow color, higher pH, fried egg-like appearance and aroma, sweetness, softness, rancid odor, and raw soybean odor. These attributes were also considered as drivers of liking. Lactic acid bacteria fermented pastes were characterized by brown color, sourness, bitterness, saltiness, umami, burnt roasted soybeans and maize aromas. Optimization by enhancing the drivers of liking while suppressing drivers of dislike would increase utilization of soybean fermented pastes.

SAMMENDRAG

Tradisjonell malawisk kost inneholder mye mais og blir ofte satt i sammenheng med mangel på flere viktige næringskomponenter. I tillegg til mais er belgvekster en viktig kilde til protein og andre næringskomponenter for mange mennesker i utviklingsland. Soyabønner har det høyeste innholdet av proteiner spesielt sammenliknet med andre bønner og belgvekster. Sammen med kornråstoff vil disse to utfylle hverandre med tanke på essensielle aminosyrer. Konsumet av soyabønner er imidlertid lavt i Malawi da kunnskap om riktig prosessering er mangelfull.

Som et tiltak for å øke konsumet av soyabønner for alle aldersgrupper i Malawi, er det gjort forsøk med å fermentere en grøtblanding av soyabønner og soyabønne-maisblandinger. Den fermenterte grøten kan brukes som mellommåltid eller som en viktig proteinkilde i en diett basert på mais. Ved spontanfermentering av moste soyabønner vil *Bacillus subtilis,* en bakteriekultur som produserer mye ammoniakk, være dominerende. Dersom det er mye ammoniakk tilstede i produktet vil dette føre til en særegen lukt som mange finner ubehagelig. Melkesyrebakterier (MSB) er svakt proteolytiske organismer og fører ikke til et tilsvarende høyt innhold av ubehagelige lukter (odører). I dette forsøket ble det brukt *thobwa*, et malawisk fermentert produkt, laget av maismel og maltet mel av hirse som starterkultur for å sikre et riktig fermenteringsforløp for et MSB fermentert produkt, kalt melkesyrefermentert puré/grøt (LFP). Grøt fermentert uten tilsetning av starterkultur ble kalt naturlig fermentert grøt (NFP). Deretter ble forskjeller i metabolske produkter, mikrobiell diversitet, vekst og overlevelse av enteropatogener, sensoriske egenskaper og konsumentpreferanser sammenliknet mellom soyabønne og soyabønne/maisblandinger med henholdsvis spontanfermentering og med melkesyrebakteriefermentering.

Begge typer fermentering resulterte i økning i innholdet av løselig protein, mest markert etter 48 timer i de fleste prøvene og høyest i 100S (49%). Høyest nedgang i totalt aminosyreinnhold fant en etter 48 timer, med 6,8 % og 7,4 % reduksjon i henholdsvis 100S og 100SBS. De begrensende aminosyrene cystein (i 100S og 90S) og meteonin (i 90S) økte derimot i løpet av fermenteringen, mens en fant økning i cystein i alle LFP etter 48 timers fermentering. Dette ble også observert for metionin ved 75SBS.

Ved begge fermenteringsformene fant en nedgang i antinæringsstoffene, fytinsyre- og trypsinhemmere. Spontanfermentering reduserte innholdet av fytinsyre mer effektivt enn LAB fermentering. Ved NFP fant en nedgang på mellom 33 og 54% i fytinsyre etter 24 timers

fermentering og 85% nedgang etter 72 timers fermentering. I noen prøver fant en ikke fytinsyre. Ved LFP fant en 18-32 % nedgang i innholdet av fytinsyre etter 24 timers fermentering og 37-49% nedgang etter 72 timers fermentering.

Melkesyre var hovedproduktet dannet både fra fermentering av LFP og NFP. Høyt innhold av melkesyre samsvarte med reduksjon av pH. pH gikk ned fra 6,44-6,48 til 4,20-4,64, noe som gir 28-35 % reduksjon etter 24 timer. Etter 72 timer var pH gått ned til 3,91-4,26, noe som gir en reduksjon på 34-39%. I NFP gikk pH ned fra 6,88-6.95 til 6,15-6,74 i løpet av 24 timer og til 5,36-5,81 etter 72 timer, noe som gir en nedgang på henholdsvis 3-12 % og 17-23 %. Høyere nedgang i LFP kan komme av et høyere antall MSB, som var 3 $\log_{10}$ cfu/g høyere enn i NFP ved starten av fermenteringen.

MSB mikrofloraen i både NEP og LEP bestod av heterofermentative staver og homofermentative kokker. Mikrofloraen ble fenotypisk karakterisert som *Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus buchneri, Lactobacillus collonoides, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus pentosus, Leuconostoc mesenteroides, Weissella confusa, Lactococcus lactis* subsp. *lactis, Pediococcus pentosaceus og Pediococcus damnosus.* Den dominerende mikrofloraen var *Lb. fermentum, Lb. brevis, W. confusa* og *P. pentosaseus.* Ved 16S rDNA genotyping ble disse fire artene bekreftet som de dominerende fermenteringsmikroorganismene. I tillegg ble *Bacillus* spp. og *Enterococcus faecium/Enterococcus durans* identifisert som en del av mikrofloraen.

Denaturerende gradient gel elektroforese (DGGE) bekreftet *Lb. fermentum, W. confusa*/*Weissella cibaria*, *P. pentosaseus* som de dominerende mikroorganismene. DGGE bekreftet mikrobiologisk endring i NFP hvor *Bacillus* spp. og *Lactobacillus linderi* ble etablert etter hvert i fermenteringsforløpet. Det mikrobiologiske mangfoldet var tilsvarende i LFP. En fant følgende mikroorganismer ved slutten av fermenteringsperioden i både NFP og LFP: *P. pentosaceus*, *Lb. fermentum*, *W. confusa/W. cibaria*, og *Weissella koreensis.*

I manus IV, hvor forsøk med naturlig fermentering, fermentering med MSB ved poding med tilsetning fra en tidligere produksjon ("back-slopping") og bruk av starterkultur med *Lb. fermentum* ble alle tilsatt *Escherichia coli.* Ingen av disse fermenteringene fikk den nødvendige nedgangen i pH til $\leq 4{,}4$ som er det kritiske nivået for vekst av *E. coli*. En fant imidlertid bedre hemming av *E. coli* ved "back-slopping" enn ved de andre fermenteringsformene. I grøt med "back-slopping" økte *E. coli* celletall fra 2,4 til 3,5 $\log_{10}$

cfu/g i løpet av 24 timer og dette holdt seg på samme nivå gjennom fermenteringsperioden. Mengden *E. coli* økte fra 2,0-2,3 $\log_{10}$cfu/g til 6,8-7,6 $\log_{10}$ cfu/g ved fermentering med *Lb. fermentum* og for NFP økte antallet fra 2,3 $\log_{10}$ cfu/g til 8,8-9,2 $\log_{10}$ cfu/g i løpet av 24 timers fermentering. Disse celletallene var over det anbefalte nivået på 100 bakterier totalt og kan utgjøre et mulig problem med hensyn til mattrygghet, dersom shiga-toksin produserende *E. coli* er tilstede under fermenteringen.

Ved fermentering med tilsatt *Bacillus cereus* var det bare ved "back-slopping" at en fikk nedgang i pH til <5,0 som er det kritiske nivået for vekst av *B. cereus*. Etter 72 timer var antallet *B. cereus* celler mellom 0 og 3 $\log_{10}$ cfu/g i grøtblandingene hvor "back-slopping" var brukt. Ved *Lb. fermentum* fermentering ble det målt en pH på mellom 5,30 og 5,35, mens celletallet varierte fra 3,7 til 5,3 $\log_{10}$ cfu/g etter 72 timers fermentering. Ved naturlig fermentering økte pH fra 5,87 etter 24 timer til 7,2 i 90 S i løpet av 72 timers fermentering. Antallet *B. cereus* celler økte derfor fra 2,2 $\log_{10}$ cfu/g til over 8,0 $\log_{10}$ cfu/g i løpet av 24 timers fermentering. Siden en regner med en infektiv dose for *B. cereus* > 3,0 $\log_{10}$ cfu/g kan en anta at en ved "back-slopping" kan produsere en grøt som vil være trygg med hensyn på *B. cereus* matforgiftning. Varmebehandling av grøten vil imidlertid være å anbefale for å være på den sikre siden.

Forbrukerne brukte ofte ubevist fermenteringsmetode ved bestemmelse av sin preferanse for produkt, og preferansene var oftest mot naturlig fermentering. Disse grøtene ble karakterisert med gulere farge, høyere pH, utseende og aroma som stekt egg, søt, myk, harsk lukt og lukt av rå soyabønner. Disse egenskapene var også viktige for hvordan konsumentene likte produktet. Grøt fra melkesyrefermentering ble karakterisert med brun farge, sur, bitter, salt, umami, stekte/brente soyabønner og maislukt. Dersom en i videre arbeid kan få fram de positive og redusere de negative sensoriske egenskapene vil dette kunne føre til økt bruk av fermentert grøt laget av soyabønner.

LIST OF PAPERS

PAPER I

Tinna Austen Ng'ong'ola-Manani, Hilde Marit Østlie, Agnes Mbachi Mwangwela, Trude Wicklund. (2014). Metabolite changes during natural and lactic acid bacteria fermentations in pastes of soybeans and soybean-maize blends. *Food Science & Nutrition (In press). Doi: 10.1002/fsn3.171*

PAPER II

Tinna Austen Ng'ong'ola-Manani, Trude Wicklund, Agnes Mbachi Mwangwela, Hilde Marit Østlie. (2014). Identification and characterization of lactic acid bacteria involved in natural and lactic acid bacteria fermentations of pastes of soybeans and soybean-maize blends using culture-dependent techniques and denaturing gradient gel electrophoresis. Accepted for publication in *Food Biotechnology*.

PAPER III

Tinna A. Ng'ong'ola-Manani, Agnes M. Mwangwela, Reidar B. Schüller, Hilde M. Østlie & Trude Wicklund. (2014). Sensory evaluation and consumer acceptance of naturally and lactic acid bacteria-fermented pastes of soybeans and soybean–maize blends. *Food Science & Nutrition*, 2: 114-131.

PAPER IV

Tinna Austen Ng'ong'ola-Manani, Trude Wicklund, Agnes Mbachi Mwangwela, Hilde Marit Østlie. (2014). Effect of natural and lactic acid bacteria fermentations on growth and survival of *Bacillus cereus* and *Escherichia coli* in pastes of soybeans and soybean-maize blends. *Manuscript.*

1 INTRODUCTION

1.1 Background to the study

About 85% of the Malawian population lives in rural areas {156} and works in agriculture, with a primary aim of producing maize for home consumption {75}. Traditional Malawian diets are predominantly maize-based {64, 129}). The major staple food is a stiff, unfermented maize-based porridge (*nsima*) consumed with relishes prepared from green vegetables, legumes, fish {63, 64} and occasionally meat, while complementary foods given to young children are thin gruels made from unfermented and unrefined maize {218}. Such predominantly maize-based diets have been associated with widespread inadequate intakes of several nutrients {61, 64}. The maize-based diet has consequences on the content and bioavailability of iron, zinc, preformed vitamin A, vitamin B-12 and calcium due to the presence of anti-nutrients like phytic acid, polyphenols and oxalate {64, 80}. Increased risks of various types of malnutrition including micronutrient deficiencies in children and pregnant women have been attributed to such diets {81, 128, 129}.

According to the National Statistical Office & O.R.C Macro {157}, the extent of chronic malnutrition in Malawi has not changed for decades. Among under-five children, stunting was reported as high as 47% (and 20% were severely stunted), while wasting and underweight were estimated at 4% and 13%, respectively {156}. Nine percent women of the age group 15 – 49 years had chronic energy deficiency nationwide. The levels of malnutrition were higher among rural women with highest levels (12%) in Mangochi district (site of the NUFU project) {157}. Micronutrient deficiencies were also reported amongst under – five children, women and even men {157}. Malnutrition is caused by inadequate dietary intake of energy and nutrients {64, 126, 143} among other causes. Foods of animal origin are good sources of protein, vitamins and other micronutrients but because of economic constraints, their consumption is generally low in the populace {62}. Therefore, there is need for an alternative low cost source of high quality protein and other nutrients that can be incorporated in the diet and can be consumed by both adults and children.

Legumes such as soybeans provide good quantities of protein, carbohydrate, fiber, B vitamins, calcium, iron and fat hence are an appropriate supplement to carbohydrate rich diets {8}. Soybean (*Glycine max)* has the highest protein content (35-40%) among the edible grain

legumes, it is cheaper than animal source proteins {8} and is cultivated in most parts of Malawi {83}. Like all legumes, soybean proteins are relatively low in sulphur-containing amino acids (methionine and cysteine) and tryptophan but are a rich source of lysine which is a first limiting essential amino acid in cereals {8, 46}. With respect to lysine and sulphur-containing amino acids, cereal and legume proteins are nutritionally complementary because limiting amino acids in soybean protein are adequately complemented by those found in cereals such as maize {46}. Therefore, consumption of soybeans together with cereals provides a high quality protein equivalent to that of meat and eggs {125}. Soybean has been used in the prevention and treatment of protein energy malnutrition in young children, as well as in improving the nutritional status of communities {223}. Therefore, soybean is a suitable substitute or alternative for expensive animal products as there is a worldwide shortage of affordable protein {223}.

1.2 Statement of the problem

In spite of the high nutritional value of soybean, its utilization in Malawi is minimal and it is mainly consumed as a porridge locally known as *Likuni Phala* {87, 126}. Utilization of soybeans to their maximum potential is held back due to limited knowledge in processing {35}. Soybeans require processing before utilization because they contain anti-nutritional factors such as lectins, trypsin inhibitors and phytic acid {137, 168, 171, 207}. The major disadvantage of soybeans regarding consumer acceptance is the strong off-flavors associated with its products. Soybeans have a characteristic “beany” or grassy flavor with bitter and astringent tastes {8} which some people find disagreeable. In addition, adoption of soybean utilization at home level in Africa is challenged by the long cooking time that is required before palatability is attained and the subsequent high fuel requirements {122, 223}. In order to maximise nutritional benefits from soybeans, there is a need for low cost household food processing methods that can reduce or eliminate anti-nutrients and off-flavors and at the same time reduce the energy requirements by reducing the cooking time. It was against this background that fermentation of soybeans into soybean pastes or soybean-maize blends to be consumed as side dishes or relishes along with *nsima* was proposed.

1.3 Justification of the study

Although numerous intervention efforts to combat various types of malnutrition mainly focused on under-five children, it is now recognized that malnutrition are prevalent in the

general population {66}. In addition, regardless of several trials involving multiple micronutrient supplements or fortificants conducted in developing countries, results have been disappointing. In some cases, no effect of multi-micronutrients on growth was seen whereas in others, the actual increase in linear growth in children receiving the multi-micronutrients was less than the potential increment expected {236}. The mixed results highlighted the constraints on growth in children in developing regions, imposed by prenatal and/or intergenerational maternal malnutrition {236}. Therefore, there is critical need for sustainable dietary interventions to improve the nutrient adequacy of plant-based diets across generations in developing countries {236}.

Fermentation is one of the oldest food technology applications that has been used for survival since the primitive pottery age. In Malawi, fermentation is an indigenous technique used in the production of cereal fermented gruels (*thobwa*) consumed as refreshing beverages {64}. The traditional fermentation of foods has several benefits including enrichment of the diet through development of a diversity of flavors, aromas, and textures in food substrates, preservation of substantial amounts of food through lactic acid, alcoholic, acetic acid, and alkaline fermentations, enrichment of food substrates biologically with protein, essential amino acids, essential fatty acids, and vitamins, detoxification and decrease in cooking times and fuel requirements {210, 212}.

Moreover, in a study to promote dietary diversification in order to increase consumption and bioavailability of micronutrients (zinc and iron) in maize-based staples in rural Malawi, Gibson & Hotz {62} recommended use of fermented maize and legume flours. They also recommended consumption of additional fermented products. Fermented soybeans or a combination of soybean and grains have been widely consumed in Far East Asia and in some parts of Africa as protein sources and as flavoring ingredients {39, 95, 106, 193, 211}. Some of the fermented soybean products like Nepalese *kinema* are fried and served as side dishes along with rice and they act as meat substitutes or meat alternatives {194}. Therefore, in order to increase utilization of soybeans in Malawian diets, fermented soybean pastes and their maize blends to be used as meat alternatives were developed.

2 LITERATURE REVIEW

2.1 History, production and uses of soybeans

Soybean [*Glycine max* (L.) Merr.] belongs to the *Leguminosae* family and is thought to have originated in Eastern Asia, particularly in north and central China. It is believed that cultivated varieties were introduced into Korea and Japan about 2000 years ago {16}. Today, 220.9 million metric tons of soybeans are cultivated worldwide and the United States of America, Brazil and Argentina are the leading producers {8}. In 2008, U.S.A produced 33% of the total soybean in the world, followed by Brazil (28%), Argentina (21%), and China (6%) {8}. In Malawi, 73,000 tonnes of soybeans were produced in 2010 and most (63,000 tonnes) were used within the country {130}. Demand for soybean production in Malawi is driven by the poultry feed industry {130} and limited demand comes from the corn–soy blend industry that produces composite flours mainly for children consumption {220}.

The existence of numerous utilization alternatives have earned soybean the title of " **the wonder bean"** {16}. Utilization options for soybeans can be divided into two groups: those based on the whole seed and those which start with the fractionation of the soybean into oil and meal (Fig 1). Crude soybean oil undergoes several operations during refining to produce soybean oil for human consumption while the by-products are used as animal feedstuff {16}. Roasted whole soybean flours are used as ingredients of traditional confectionery products and snacks in China, Japan, Korea and Indonesia. Immature whole green soybeans are consumed as a vegetable while mature dry soybeans are rarely used as cooked legume (as is done in navy beans, black beans, chick peas or lentils) even in the traditional areas of soybean consumption {16}. This is probably due to the persistent bitterness and "green beany taste" of soybeans, the low starch content, the relatively low water adsorption (swelling) capacity, long cooking time and poor digestibility {16}. Therefore, all the traditional routes of utilization of soybeans as food involve some sort of processing or fractionation to overcome these disadvantages.

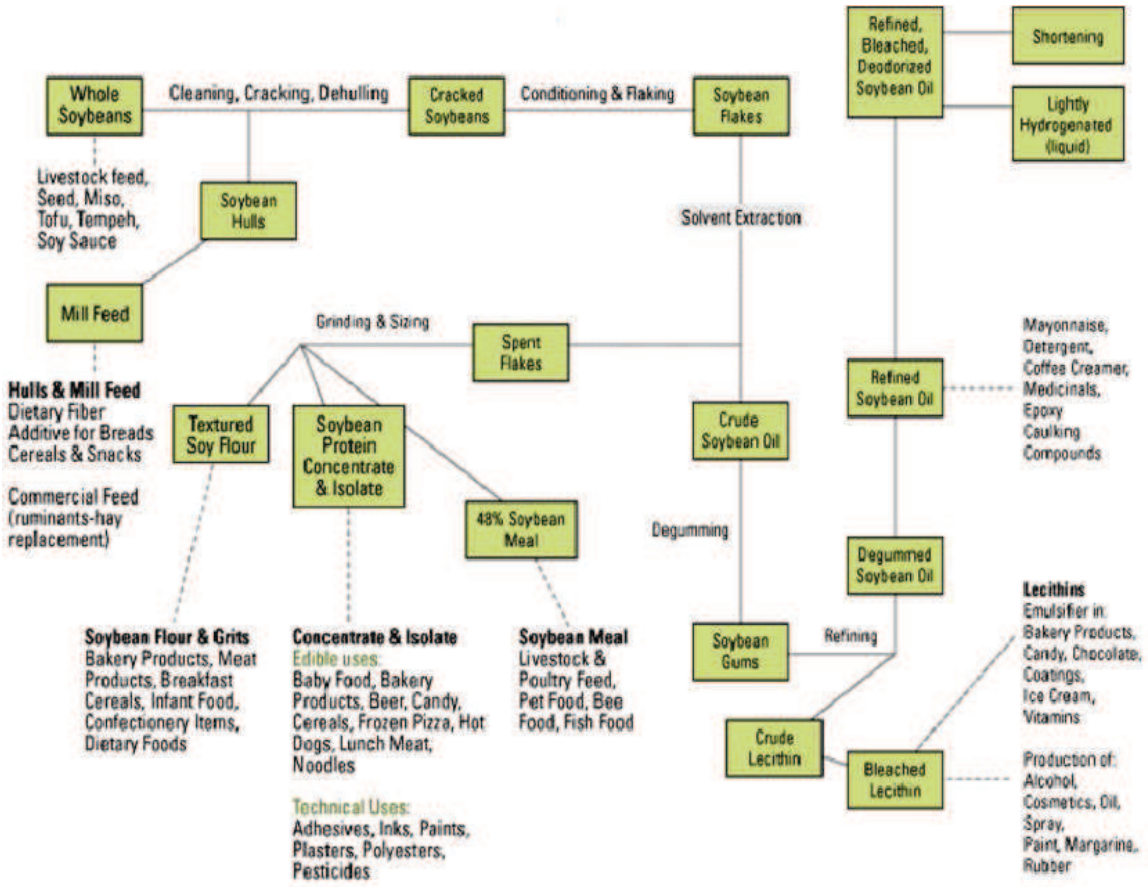

Fig 1: Flow chart of soybean processing, products and how they are used. Source: National Soybean Research Laboratory {155}.

2.2 Chemical and nutritional composition of soybeans

2.2.1 Proteins and amino acids

Soybeans constitute an important component of the traditional diets of many people particularly in Asia and Africa {16, 134, 216}. Soybeans are valued because of their high protein and fat contents {8, 132}. The chemical composition of soybeans varies according to genotype and growing conditions {70}. Soybean seeds contain on average 40 – 41% protein on a dry matter basis {132} although a range of 33.2 to 51.3% protein in some genotypes has been reported {16, 60, 84, 137, 168, 181, 184}. The main proteins are two globulins, glycinin and β-conglycinin and they account for 65 – 80% of the total seed protein {132}.

Soybean proteins contain all amino acids essential to human nutrition; hence soybean's protein quality is regarded as almost equivalent to animal sources {44}. Protein quality is evaluated using chemical score, biological value, protein efficiency ratio, net protein utilization, and true protein digestibility-corrected amino acid score {8, 16, 46}. The chemical

score of soybeans is estimated to be about 70% {222} because the percentage of limiting sulfur containing amino acids, methionine and cysteine (Table 1) in soybean protein is about 70% of that of whole egg protein {16}. Protein digestibility-corrected amino acid score (PDCAAS) is now widely used as a routine assay for protein quality evaluation {8}. This method compares the amino acid pattern of a protein and human amino acid requirements and digestibility, to arrive at a value for protein quality {8}. According to PDCAAS method, soybean protein products received scores between 0.95 and 1.00 {8}. The PDCAAS values for beef protein, milk protein, and egg protein are 0.92, 1.00, and 1.00, respectively {8, 27}. Thus according to the PDCAAS method, the quality of soybean protein is comparable to animal protein {8}.

Overall nutritional quality of soybeans is lower than that of animal proteins because of low concentration of sulfur containing amino acids in soybeans. {46, 132}. Nevertheless, for a plant protein, soybean protein is rich in lysine and tryptophan and hence serves as a valuable supplement to cereal foods where the two amino acids are limiting factors {16, 168}. The most abundant amino acid in soybeans is glutamic acid followed by aspartic acid (Table 1). These two amino acids are responsible for the monosodium glutamate (umami) flavor in soybean products {92}. The other amino acids contribute to sweetness and bitterness in soybean products {92, 119}.

2.2.2 Lipids

The amount of oil in soybeans varies from 15 – 23% depending on genotype {16, 84, 138, 184}. The contents of unsaturated fatty acids such as oleic, linoleic and linolenic acids are relatively higher compared to the saturated fatty acids, palmitic and stearic acids. Total unsaturated fatty acids in different soybean varieties range from 78.8 to 88.3% {138}. Soybean oil is a rich source of linoleic and α-linolenic acids which are essential fatty acids belonging to the ω-6 and ω-3 families, respectively {44, 132}. However, the high content of unsaturated fatty acids makes soybean oil relatively unstable and susceptible to oxidation in the presence of lipoxygenases leading to rancidification and consequently to off-flavor development {44, 132, 138}.

169 Table 1: Amino acid composition of soybeans (dry matter basis)

Component	Literature values	Literature reviewed
Essential amino acids	g/100g (g/16g N)	{16, 23, 56, 69, 70, 141, 168, 171}
Arginine	2.58 – 3.45 (5.68 – 7.23)	
Histidine	0.91 – 1.23 (2.28 – 2.53)	
Isoleucine	1.33 – 2.08 (3.40 – 4.54)	
Leucine	2.77 – 3 .65 (5.88 – 7.84)	
Lysine	2.07 – 2.96 (5.23 – 6.68)	
Methionine	0.22 – 0.65 (1.19 – 1.39)	
Phenylalanine	1.84 – 2.36 (3.88 – 4.94)	
Threonine	1.42 – 2.13 (3.34 – 3.99)	
Valine	1.54 – 2.22 (3.40 – 4.80)	
Nonessential amino acids	g/100g (g/16g N)	{16, 23, 56, 69, 70, 141, 168, 171}
Alanine	1.53 – 2.09 (3.38 – 4.26)	
Aspartic acid	4.12 – 5.59 (9.26 – 11.70)	
Cystine	0.45 – 1.04 (1.33 – 1.39)	
Glutamic acid	6.65 – 8.77 (14.3 – 18.87)	
Glycine	1.52 – 2.06 (3.29 – 4.18)	
Proline	1.85 – 2.61 (4.35 – 5.61)	
Serine	1.97 – 2.58 (4.61 – 5.28)	
Tyrosine	1.12 – 1.75 (2.49 – 3.76)	
Tryptophan	0.30 – 0.80 (0.71 – 1.28)	
Hydroxyproline	0.01 – 0.1	

170 **2.2.3 Carbohydrates**

171 Carbohydrates are among the most abundant components in soybeans accounting for ca. 35%
172 of the dry seed weight {132}. Other seed lines with a total carbohydrate content ranging from
173 19.8 – 38.1% are known {16, 60, 168, 171}. High proportion of the carbohydrates is in seed
174 coat (hulls) and some carbohydrates are located in the embryo {132}. Almost half of the total
175 carbohydrates are structural, composed of cell-wall polysaccharides such as cellulose,
176 hemicellulose, and pectins, whereas the nonstructural carbohydrates include starch and
177 different mono-, di-, and oligosaccharides {132}. The cell-wall polysaccharides together with
178 lignin, enzyme-resistant starch and oligosaccharides are constituents of dietary fiber, a non-
179 digestible portion of food and feed {132}. Soybeans are rich in dietary fiber (Table 2) which
180 is mostly removed during a dehulling process.

181 The proportion of soluble carbohydrates varies between 11 and 25% and includes 15 – 20
182 different sugar species {132}. The most abundant soluble sugars are sucrose, stachyose and
183 raffinose (Table 2). The galactooligosaccharides, raffinose, stachyose and verbascose are
184 considered as anti-nutritional factors because their consumption is associated with flatulence
185 and digestive disturbance in humans and nonruminant animals {108, 132, 229}. Raffinose,
186 stachyose and verbascose contain one, two, and three galactose molecules, respectively,

attached to sucrose via α-1→6 glycosidic bond {132}. Mammals do not synthesize α-galactosidase enzyme required to hydrolyze galactooligosaccharides to D-galactose and sucrose in the small intestine {27, 132}. Consequently, the galactooligosaccharides pass to the lower intestine where they become substrates for bacterial fermentation that generates carbon dioxide, methane and other flatulence–producing gases {27, 132}.

Table 2: Carbohydrate composition and dietary fiber content of soybeans (dry matter based)

Carbohydrate	Literature values (g/100g)	Literature reviewed
Glucose	0.03 – 2.7	{16, 132, 168, 171, 184}
Fructose	0.02 – 2.5	
Maltose	0.30 – 0.50	
Sucrose	1.1 – 9.47	
Raffinose	0.1 – 1.4	
Stachyose	0.07 – 6.9	
Verbascose	0 – 0.19	
Starch	0.2 – 1	
Dietary fiber	19.7 – 24.4	
Crude fiber	4 – 8	

2.2.4 Minerals and vitamins

Soybeans contain about 3.9 – 5.36% minerals determined as ash {16, 60, 171}. The major mineral constituents are potassium, calcium, magnesium and phosphorus. However, a substantial amount of the phosphorous in soybeans is found in bound form hence is not available for human use {132}. Mohamed *et al.* {137} estimated total phosphorus in soybeans as 20.82 mg/g while available phosphorus was 11.97 mg/g. In addition, the biological utilization of most minerals such as zinc (Zn), iron (Fe), magnesium (Mg) and calcium (Ca) is impaired by phytic acid {16, 132, 137}.

Soybean is a good source of niacin, B-vitamins, folic acid and vitamin E (α-tocopherol) but is deficient in vitamin B_{12} and vitamin C {44}. In human diet, soybean oil is considered as good source of vitamins K and E even though some proportion of the vitamins may be lost during processing of the oil {168}.

2.2.5 Anti-nutritional factors

In addition to oligosaccharides, soybeans contain other anti-nutritional factors namely trypsin inhibitors, lectins, and phytic acid {30, 168, 205, 224}. Levels of the anti-nutritional factors are presented in Table 3.

There are two types of trypsin inhibitors known as Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) and both are active against trypsin, while the latter is also

active against chymotrypsin {16, 27, 56, 168}. These protease inhibitors interfere with digestion of proteins resulting in decreased animal growth {27, 168}. Ingestion of trypsin inhibitors can result in increased pancreatic secretion and hypertrophy of the pancreas {118}. Since pancreatic enzymes such as trypsin and chymotrypsin are particularly rich in sulfur-containing amino acids, then pancreatic hypertrophy causes a drain on the body tissue of these particular amino acids {118}. This loss intensifies an already critical situation which cannot be compensated with dietary soybean protein, which is inherently deficient in sulfur-containing amino acids {16, 27, 118}. The activity of the inhibitors is destroyed when soybeans are toasted or heated during processing {48, 168}.

Table 3: Anti-nutrient composition of soybeans

Anti-nutrient	Literature values	Literature reviewed
Lectins	0.11 – 9.4 HU[1]/mg DM[2]	{168, 171}
Phytic acid	1.12 – 4.07 g/100 g DM	{137, 207}
Trypsin inhibitor	24.71[3] – 184[4] TIU[5]/mg DM	{137, 168}

[1] HU = Hemaggluttination units
[2]DM = dry matter
[3] in TIU/mg of raw soybean based on dry matter
[4] in TIU/mg in protein content of crude defatted soybean seed extract
[5]TIU = Trypsin inhibitor units

Lectins were formely known as hemagglutinins and are proteins capable of agglutinating red blood cells {16, 180}. Some lectins can be highly toxic but the lectins present in raw soybeans have no observable dietary effects (good or bad) in humans and they are heat-labile {16, 180}. However, resistance to dry heat has been reported in some studies {168}.

Phytic acid (myoinositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) is the main storage form of phosphorus in legumes and cereals {27, 132, 137}. It accounts for 65–80 % of the total soybean seed phosphorous {132} and its contents varies (Table 3). In soybeans, phytic acid is concentrated mostly in the cotyledons {132}. Humans cannot utilize phytic acid phosphorus because of lack of phytase, the digestive enzyme required to release phosphorous from the phytic acid molecule {27, 132}.

Charged phytin salts bind with nutritionally important minerals like Zn, Ca, Mg and Fe and forms phytate-metal complex rendering the minerals biologically unavailable to humans and non-ruminant animals {30, 132, 137, 224, 237}. In addition, phytic acid decreases the solubility, functionality and digestibility of proteins by forming protein-phytate complexes {27, 180}. The protein-phytate complexes are more resistant to digestion by proteolytic

enzymes; hence they reduce utilization of dietary protein {27}. Phytic acid also interacts with enzymes, such as trypsin, pepsin, α-amylase and β-galactosidase decreasing their activities {30, 180}.

There is an increasing number of reports suggesting that phytic acid may have a positive effect on animal and human health by acting as an anti-carcinogen and as an antioxidant through formation of complexes with Fe leading to a decrease in free radical generation and peroxidation of membranes {67, 132, 134}. However, in populations dependent on cereals and legumes, where micronutrient deficiencies such as Zn deficiencies and anemia are widespread, emphasis should be on reducing or eliminating phytic acid in the diet.

Mohamed & Rangappa {138} considered lipoxygenase as an anti-nutritional factor prevalent in soybean. Lipoxygenase activities are responsible for the development of off-flavors described as grassy or beany {8}. Lipoxygenase activities in soybean ranges from 829.8 to 4750.4 units/min/mg and the variations are influenced by genotype {138}. Lipoxygenase-free cultivars were developed to eliminate the off-flavors and increase acceptability of soybean foods in Western cultures {132}. However, sensory characteristics only improved in tofu and soymilk but not in bread, meat patties, and beverage products because of auto-oxidation of oil {132}. As a result, lipoxygenase-free cultivars have not been used extensively {132}.

2.2.6 Other compounds

Soybean is the most abundant source of isoflavones, containing about 0.1 to 5 mg/g (dry weight){44, 107}. Isoflavones are a sub-group of plant phenolic compounds called flavonoids {44, 134}. The isoflavones in soybeans are of three basic types, diadzein, genistein and glycitein and they exist as aglycones but they can also exist in three other conjugate forms as glucosides, acetylglucosides or malonylglucosides {44, 85, 107, 168}. Lately, the effects of isoflavones in humans have become an active area of research. Isoflavones are phytoestrogens with structural similarity to mammalian estradiol; hence they have the ability to bind estrogen receptors and exhibit weak estrogenic activities {44, 132, 134}. Other biological and beneficial health effects include hypocholesterolemic effects, anticancer effects, improved digestive tract function, improved lipid metabolism, bone health, prevention of menopausal symptoms, antidiabetic effects and antioxidative effects {12, 44, 85, 106, 132, 134}. However, isoflavones are associated with bitter and astringent tastes which are increased by the hydrolysis of isoflavones aglycones through the action of β-glucosidases {8}.

Soybean is one of the eight foods that account for most of the immunoglobulin E (IgE) - mediated food allergies {27, 168, 209}. The prevalence of soybean allergy in the general population is between 0.3 – 0.7% and the prevalence is high in children with atopic eczema {168}. Many cases of soy allergy are outgrown during childhood {55, 168}. Allergic reactions are similar to those elicited by other food allergens and most severe reactions like anaphylaxis and death are rare {168}. Proteins in soybeans that are considered as potential allergens include β-conglycinin, glycinin and P34; and the P34 protein is responsible for most of the soybean allergic reactions {168}.

2.3 Effect of domestic processing on composition of soybeans

Different processing techniques such as soaking, cooking, roasting, germination and fermentation are employed before consumption of soybeans. The processing causes changes in a number of physicochemical, biochemical, nutritional and sensory properties. The processing methods enhance the nutritional value of soybean by increasing the availability of amino acids, increasing vitamin content, improving protein digestibility and reducing the contents of the anti-nutritional factors {199}. In many cases, the use of only one method does not completely remove a given anti-nutritional compound and a combination of two or more methods is required {46}. Processing of raw soybeans is also required in order to eliminate the disagreeable beany flavors so as to increase market potential and acceptability {179, 233}. Processing also improves appearance, texture, cooking quality and palatability {48, 207}.

2.3.1 Dehulling

Soybeans possess a fibrous seed coat, or testa or husk which is indigestible because it contains most of the dietary fiber. Therefore, in most cases soybeans are dehusked or dehulled before use or before further processing. Dehulling improves palatability and digestibility and it also reduces cooking time {207, 233}. Digestibility is improved through removal of the indigestible cell-wall polysaccharides, tannins and trypsin inhibitors {132, 233}. Dehulling can lead to 48% reduction {90} or complete elimination of tannins {48}.

Combining dehulling and cooking can completely eliminate tannins and at the same time substantially (82%) reduce trypsin inhibitor {48}. Lower reduction (17%) in trypsin inhibitor activity (TIA) was reported due to dehulling and cooking {90}. However, the combined treatment led to an increase in phytic acid content by 21% {48}. Removal of the germ along with the husk during dehulling results in loss of thiamine {207}.

2.3.2 Soaking

Soaking reduces the amount of soluble compounds like oligosaccharides, reducing sugars and total soluble sugars. Soaking reduced total soluble sugars by 17%, reducing sugars by 23%, starch content by 14% {89}, sucrose, raffinose and stachyose, by 26.68%, 25% and 20%, respectively {48}. The combined effect of soaking, dehulling, washing and cooking resulted in 61%, 47.5% and 63% reductions in sucrose, raffinose and stachyose, respectively {48}. While soaking and cooking reduced raffinose by 88% and stachyose by 75%, the two oligosaccharides along with sucrose were completely hydrolysed after fermentation {189}. On the contrary, Kaushik *et al.* {89} reported buildup of reducing sugars from 34 to 44% and of total soluble sugars from 2 to 10% after cooking of soaked seeds. In the same study, cooking greatly reduced the starch content with 35 to 39% and the reductions were highest in pressure cooking (57%).

Soaking reduced anti-nutritional factors such as tannins by 15 – 54.6%, sapponins by 29% and trypsin inhibitor activities (TIA) by 8% {30, 199} but phytic acid content was increased by 34% {30}. Soaking is also employed prior to a number of other processing treatments such as germination, cooking and fermentation because it reduces cooking time {199, 233}. In a combined treatment of soaking and cooking, tannins, phytic acid, sapponins and TIA were reduced to a greater extent, 43%, 45%, 56% and 26%, respectively, than soaking only {199}.

Mineral losses occur when legumes are soaked in water which is generally discarded before further processing {30}. Kaushik *et al.* {89} reported decreases in potassium (K), Ca, Mg, phosphorus (P) and Fe and Kayembe {90} reported a 21% decrease in Fe but slight increases in Ca and P. Significant reductions were also reported in riboflavin, niacin, pyridoxine and ascorbic acid {89}. Vitamin losses were explained in terms of their relative solubility in water which defined the corresponding vitamin diffusion to the soaking medium and its subsequent loss {89}. Sometimes salts such as sodium bicarbonate are added to soaking or cooking water to reduce the cooking time {233}. However, such alkali treatments result in considerable losses (up to 80%) of thiamine {233}.

2.3.3 Heating

Proper heat treatment is an absolute requirement if the essential nutrients in soybean products were to be used maximally {179}. According to Chitra *et al.* {30}, both wet-heating and dry-heating were effective in reducing phytic acid content, although wet-heating was more effective than dry-heating. Reddy & Pierson {180} reported 14% and 10.3% reductions in

phytic acid content due to boiling and steaming, respectively. Boiling was also more effective than autoclaving in reducing phytic acid and trypsin inhibitor contents. Boiling reduced phytic acid by 65 – 67% and and trypsin inhibitor contents by 77 – 89%, while autoclaving resulted in 4 – 13% and 54 – 57% reductions in phytic acid and tryspin inhibitor contents, respectively {60}. On the other hand, boiling soybeans that were previously germinated had no effect on the phytic acid content {222}. This could be due to reduced extractability of phytic acid due to heat processing {137} because insoluble complexes between phytate phosphorus and other components are formed during cooking {60}.

Friedman *et al.* {56} demonstrated the effect of duration of heating in reducing trypsin, chymotrypsin and lectin activities. Autoclaving soybeans at 121 ^{o}C for 10 min resulted in 31%, 81% and 75% reductions in trypsin, chymotrypsin and lectin activities, respectively. When the heating time was increased to 30 min, there was complete destruction in chymotrypsin activity while 81% and 97% reductions were achieved in trypsin and lectin activities, respectively. Vasconcelos *et al.* {228} did not detect TIA after 5 min of incubation at boiling temperature (92 ^{o}C). Trugo *et al.* {222} also reported complete inactivation of trypsin inhibitor in soybeans that were germinated prior to boiling for 20 min. Although heating is effective in destroying protease inhibitor activities, it does not completely eliminate the protease inhibitors {55}. Friedman *et al.* {56} and Friedman & Brandon {55} reported 24% and about 1% retention of KTI and BBI, respectively, in soybeans heated for 30 min. A retention of 5 – 20% of the original trypsin and chymotrypsin inhibitory activity has also been reported {228}. Residual inhibitory activity is attributed to minor protease inhibitors and nonspecific inhibitors {55}. The extent to which anti-nutritional factors are destroyed by heating is a function of temperature, duration of heating, particle size and moisture conditions {228}.

Heat processes such as roasting, autoclaving and boiling result in non-significant increases in crude protein content {30, 222} although up to 10% increases due to boiling were reported by Kaushik *et al.* {89}. The increases were attributed to loss of soluble solids, which increased the concentration of protein in the cooked seeds. On the other hand, slight decreases in protein content due to autoclaving (0.9 – 1.1%) and boiling (1.7 – 5.6%) were reported {60}. Protein digestibility was increased due to roasting and autoclaving, although to a lesser extent than fermentation and germination {30}. Increase in protein digestibility due to heating is

attributed to the destruction of heat-labile protease inhibitors and to the denaturation of globulin proteins that are highly resistant to proteases in their native state {30, 122, 233}.

Heating can also lead to non-significant increases in amino acids {56, 222} although it is generally agreed that heat treatments often damage lysine, arginine, and the sulfur amino acids {76, 228}. Up to 27% reduction in lysine content of previously soaked chickpeas that underwent microwave cooking for 23 min was reported {76}. However, Van Buren *et al.* {226} associated lysine loss with excessive heating. Heating at 121 ^{o}C, 165 ^{o}C, and 182 ^{o}C did not cause lysine loss, but when soymilk powders were heated from 226 ^{o}C to 315 ^{o}C, there was an increasing loss in lysine availability. Therefore, although heating improves protein quality by increasing its digestibility {46, 233}, loss of protein quality on continued heating may occur due to increasing Maillard browning rendering lysine unavailable {226, 233}. As such, heat treatments have to be kept to a minimum to avoid destroying sensitive amino acids like cysteine, methionine and lysine {55, 228}.

The loss of lysine due to thermal processing may be followed by the formation of new amino acids such as lysinoalanine, lanthionine and ornithinoalanine. The loss of cysteine probably occurs through desulphurization reactions which produce unstable residues of dehydroalanine which may then condense with cysteine or lysine to form lanthionine, or lysinoalanine {173}. Although not an essential amino acid, cysteine has a sparing effect on the dietary requirement for methionine {55}. Therefore, cysteine destruction is of importance in many vegetable proteins which are limiting in the sulphur amino acids {173}. Bioavailability studies have shown that lysinoalanine is not available as a source of lysine {55, 173}. Similarly, lanthionine, as a source of cysteine is partially available {173}. Methionine on the other hand may undergo oxidation to methionine sulfoxide and methionine sulfone, or may undergo racemization to D-methionine which may be degraded to compounds with undesirable flavors {55}. Protein-bound methionine is poorly utilized because of poor digestibility {55}.

Sensory characteristics such as flavor and texture may also be improved by heating, particularly roasting {204}. Soy-*dawadawa* made from roasted soybeans was most preferred by consumers than *dawadawa* made from boiled beans {39}. Roasting can also serve as a preliminary step in facilitating husk removal {204}. However, roasting substantially (22%) decreased in vitro protein digestibility due to an increased (21%) tannin content {90}.

Heating increased total carbohydrate content by 15 – 20% {60} and reduced dietary fiber content to almost the same level as in germinated soybeans, with an advantage of less mineral loss than germination {30}. Mineral loss is high in wet-heating because of leaching {30} and the extent depends on cooking method and the mineral in question {89}. Similarly, vitamin losses are vitamin dependent and cooking method dependent with microwave cooking resulting in more vitamin retention than boiling and pressure cooking because of the shorter cooking time in the microwave cooking {89}.

Extrusion cooking, although not a domestic technique is becoming more popular because of its numerous applications including precooked food mixes for infants and texturized vegetable proteins (TVP) that are used as meat analogues {8, 206}. Texturized vegetable protein is cheaper than animal proteins and it can be flavored {8}. In a consumer acceptance study of fermented soybeans in Malawi, 17.8% (n = 129) of the respondents indicated using TVP popularly known as *soy pieces* as relish {158}. The use of TVP as protein source will become increasingly important as the cost of animal source proteins is still escalating. Therefore, it is important to review some of the nutritional changes that may occur during extrusion.

Extrusion cooking is a high-temperature, short-time process in which moistened, expansive, starchy and/or proteinacious food materials are plasticized and cooked in a tube by a combination of moisture, pressure, temperature and mechanical shear, resulting in molecular transformation and chemical reactions {206}. The extrusion process denatures undesirable enzymes (lipoxygenase, peroxidase); inactivates some anti-nutritional factors (trypsin inhibitors, haemagglutinins, tannins and phytates); sterilises the finished product; and retains natural colors and flavors of foods {26, 206}. There is nutritional concern regarding extrusion when the process is used specifically to produce foods that are nutritionally balanced or enriched like weaning foods, meat replacers, animal feeds, and dietetic foods {26, 206} .

Protein digestibility value of extruded products is higher than non-extruded products, possibly because of the denaturation of proteins and inactivation of anti-nutritional factors that impair digestion {17, 26, 206}. Texturization under moderate conditions (160 – 170 ^{o}C) does not lead to formation of lysinoalanine and lanthionine in appreciable amounts {26}.

However, extensive lysine loss can take place when legumes or cereals are extruded under severe conditions {26, 206}. Lysine loss increases with increasing temperature (Tm > 180°C) and decreasing moisture content (%H_2O < 15), especially in the presence of reducing sugars

{17, 26, 206}. Decreases in total lysine content, available lysine and lysine bioavailability of up to 37%, 32 – 80% and 50%, respectively were reported {17, 26, 206}. Since lysine is limiting in cereals, its loss would immediately result in a decrease in protein nutritional value {206}. Hence, lysine may serve as an indicator of protein damage in extruded products. In addition, availability of arginine, tryptophan, cysteine, aspartic acid, histidine and tyrosine may be decreased in low moisture extrusion {17, 206}. Cheftel {26} suggested supplementing extruded flours with free lysine or methionine (together with vitamins and minerals).

2.3.4 Germination

Germination is one of the effective means of reducing anti-nutritional factors like phytic acids. During germination, the activities of endogenous phytases are increased leading to hydrolysis of phytic acid {80}. Chitra *et al.* {30} reported a 38.9% reduction in phytic acid content of soybeans as a result of germination. Germination for 3 and 6 days reduced TIA by 22.6% and 32.4%, respectively {141}. Six days of germination decreased tannins (54%) but increased polyphenols (18%) {90}.

Germination led to variable effects in reducing sugars. For instance, 4.5% reduction in reducing sugars was observed after one day of germination and this was explained in terms of leaching out of the soluble fractions during the soaking period {141}. As the germination period increased, reducing sugars decreased by 27% {141}. The reduction in reducing sugars was attributed to utilization of simple sugars as a source of energy during the germination process {141}. On the contrary, Kaushik *et al.* {89} reported a progressive increase in the concentration of both total soluble and reducing sugars with germination time. Increases of 10% and 32% were observed in total soluble and reducing sugars, respectively, while starch content was reduced by 26% {89}. Increases in sugars were probably due to mobilization and hydrolysis of seed polysaccharides including starch to oligosaccharides and ultimately to monosaccharides, resulting in more available sugars and decreased starch content {89}.

Chitra *et al.* {30} and Kaushik *et al.* {89} reported higher increases in protein content between 13% and 15%. According to Mostafa *et al.* {141}, a major change was observed in the non-protein nitrogen fraction which increased by 54% after 6 days of germination. This was attributed to the activity of proteolytic enzymes and hydrolysis of protein molecules. Although germination does not increase protein content significantly, hydrolysis of proteins

leads to improved digestibility {141}. Increased in vitro protein digestibility between 12% - 26% were reported {90, 141}.

Mostafa *et al.* {141} also showed that germination increased amino acid contents of soybeans. Total essential amino acids increased from 8.9 to 22.4% during 3 days and 6 days of germination while the corresponding increases in total non-essential amino acids were 17.6 and 17.5% after 3 and 6 days' germination, respectively. The greatest increases were, in descending order: leucine > tyrosine > phenylalanine and glutamic acid whereas methionine and histidine contents slightly decreased {141}.

Germination also increased fat content (30%) and decreased dietary fiber content (12%), starch content (30%), calcium and Mg {90, 141}. A major advantage of germination is the pronounced increases in vitamin contents. The levels of vitamins A, E, B_1, B_2, B_6 and C increased significantly with germination {89} and increases exceeded 500% in vitamins E and B_2 {176}.

2.3.5 Fermentation

During fermentation of soybeans, proteases, lipases, a variety of carbohydrases, and phytases are produced {164}. These enzymes cause degradation of macromolecules into substances of lower molecular weight such as peptides, amino acids, fatty acids, and sugars {107}. Cell walls and intracellular material are partly solubilized {164} contributing to texture, flavor, aroma and functionality of the product {107, 164}. In addition, fermentation can yield products with decreased cooking times and improved digestibility {210}. Generally, fermentation improves the nutritional and functional properties of legumes {3}.

Variable effects of fermentation on anti-nutritional factors have been reported. In cowpea and groundbean, TIA could not be detected after 24 hrs of fermentation while the TIA increased in soybeans during 48 hrs of fermentation {48}. Reddy & Pierson {180}, reported about 90% reduction in TIA in *tempe* fermented with *Rhizopus* spp. for 24 hrs and 48 hrs. On the other hand, phytic acid content increased during 24 hrs of fermentation before a 31% reduction was observed after 36 hrs {48}. Phytic acid reductions ranging from 19 - 67% have been reported in naturally-, *Bacillus* and *Lactobacillus* fermented soybeans {7, 30}. Variations in the extent to which phytic acid is reduced are attributed to the differences in culture inoculum which produce varying levels of phytase activities {30}. Phytic acid was also degraded to various extents when other processes were combined with fermentation {180}. There was a 54.5% reduction in fermented *tempe*, 77% reduction in fried *tempe*, 89% reduction in *tempe* stored

for two weeks at 5 ^{o}C and a 94.5% reduction in fried *tempe* that had been stored for two weeks at 5 ^{o}C. Loss of phytic acid during fermentation is due to the activities of endogenous phytases from both raw ingredients and due to production of phytases by fermentative microorganisms {30, 80, 180}.

Other anti-nutritional factors like saponins, lectins and oligosaccharides; and sucrose are also hydrolyzed during fermentation. Up to 55.8% hydrolysis of saponins and complete hydrolysis of lectins in *tempe* made from germinated soybeans and fermented for 36 hrs were reported {180}. *Rhizopus oligosporus*, *Lactobacillus curvatus* R08, *Leuconostoc mesenteriodes*, *Lactobacillus fermentum*, *Bifidobacterium* spp. were reported to produce α-galactosidase, an enzyme that hydrolyses oligosaccharides {27}. Stachyose and raffinose decreased by 56.8% and 10%, respectively, in soybeans fermented by *R. oligosporus* {27}. Raffinose, stachyose and sucrose were completely hydrolyzed in cooked soybeans fermented with either *Bacillus subtilis, Enterococcus faecium, Geotrichum candidum, Candida parapsilosis* or their mixture {189}. Similarly, in *Leu. mesenteriodes* JK55 and *Lb. curvatus* R08 fermented soymilk, the oligosaccharides were completely hydrolyzed during 18 – 24 hrs of fermentation {27}.

Proteins and lipids are partially hydrolyzed during fermentation resulting in more digestible products {91, 210}. Minor protein increases (1 – 2%) were reported during fermentation of soybeans that were either roasted or boiled prior to fermentation {39}. Chitra *et al.* {30} reported 11% increase in protein content and 13% increase in vitro protein digestibility due to fermentation. Solid state fermentation of soybeans by *Lactobacillus plantarum* Lp6 caused polypeptide degradation due to proteolytic enzymes of the bacteria {3}. Free amino acid contents increased significantly from 0.33 to 8.86 g/100g, although the contents of essential amino acids did not increase {3}. The increases were in non-essential amino acids like leucine, isoleucine, valine, aspartic acid and proline {3}. During natural fermentation to produce *kinema*, total nitrogen, soluble nitrogen and free fatty acids increased significantly {193}. While *B. subtilis* fermentation to produce *kinema* resulted in 18 – 21% reduction in protein nitrogen {163}, *B. subtilis* also increased solubility of soybean proteins during 48 hrs fermentation from 22 to 54 – 64% depending on strain used {91}.

Different types of fermentation result in varying effects in the chemical composition of fermented soybeans. Cooked soybeans were subjected to natural fermentation, or were prepared into *dawadawa* and naturally fermented with potash or were fermented by *Lactobacillus* {7}. The amino acid profiles of soybeans fermented with *Lactobacillus* were

preferable while most of the amino acids (except glutamic acid which was highest) were lowest in *dawadawa* compared to the naturally and *Lactobacillus* fermented soybeans. *Lactobacillus* fermented soybeans had highest proline (3.59%), methionine (1.34%), leucine (7.44%), tyrosine (3.46%) and phenylalanine (4.50%) contents while these amino acids were lowest in *dawadawa* fermentation {7}. The variations were attributed to differences in fermentation microorganisms associated with the different methods {7}. The different microorganisms might have used different nutrients to varying extents as sources of energy and protein for their growth and survival {7}.

Song *et al.* {209} also showed that different microorganisms used in fermentation of soybeans resulted in varied protein and amino acid contents of the fermented products. Soybean meal that underwent natural and *Saccharomyces cerevisiae* fermentations increased in protein content from 47 to 50% and 58%, respectively {209}. There were no significant changes in methionine in natural, *Lb. plantarum* and *S. cerevisiae* fermentations but there was a 15% decrease in methionine in *Bifidobacterium lactis* fermentation. Cysteine decreased in natural, *Lb. plantarum* and *B. lactis* fermentations and increased by 56% in *S. cerevisiae* fermentation {209}. Sarkar *et al.* {190} also observed variations in amino acid profiles of *kinema* due to *B. subtilis* and yeast fermentations. In *Bacillus* fermentation, free amino acids increased from 0.2 to 26% of the total dry matter representing a 60-fold increase. This was attributed to the high proteolytic nature of the organism which was evidenced by a 40-fold increase in ammonia. The proteolytic activities were accompanied by depletion of methionine, cysteine, tryptophan, lysine, histidine, arginine, alanine, isoleucine and hydroxyproline. The depletions were more pronounced in arginine and cysteine. On the other hand, yeast fermentation resulted in smaller increases in glycine, leucine, glutamine, lysine, histidine, phenylalanine and proline. The variations in the profiles were attributed to the nutritional requirements of the fermenting organisms {190}.

The extent to which amino acids are increased or decreased depends on fermentation time and temperature. Proteolytic activities were higher in *dawadawa* fermented at 30 – 40 ^{o}C, with highest activities observed at 35 ^{o}C throughout fermentation while there were no proteolytic activities until 12 hrs in soybeans fermented at 25 ^{o}C {166}. The proteolytic activities coincided with increases in free amino acids which were highest at 35 ^{o}C and lowest at 25 ^{o}C {166}. Free amino acids also increased with fermentation time with most being produced during secondary fermentation (from 48 hrs to 2 months) of *douchi* fermentation {239}. Total amino acids in *douchi* were lower than total amino acids of boiled soybeans used as a raw

material {239}, suggesting reduction of total amino acids with fermentation. Methionine was not detected after 15 days of fermentation, while arginine decreased more than the other amino acids because it was a preferred source of nitrogen for microorganisms during secondary fermentation {239}.

Crude lipid content increased from 28.8 to 32.7 – 37.2% during 48 hrs of *B. subtilis* fermentation of soybeans {91}. Crude lipid increases between 20 – 30% in *kinema* fermentation were reported {191}. Contrasting results by Dakwa *et al.* {39} indicated decreases in lipid content by 10 to 15% in *dawadawa*. Slight decreases in crude lipid content were also reported in soybean fermentation of *douchi* {239}.

Fermentation can alter the distribution pattern of fatty acids, although linoleic acid remains the major fatty acid in both unfermented and fermented soybeans {191}. In *tempe* fermentation, there was preferential hydrolysis of α-linolenic acid resulting in slight increases in oleic and linoleic acids at the expense of linolenic acid and the levels of total free fatty acids were higher in the final product {164}. *Kinema* fermentation led to increases in free fatty acids {194} which were significant in palmitic and stearic acids and non-significant in other fatty acids, resulting in 9% and 6% increases in saturated and unsaturated fatty acids, respectively {191}. The changes were due to lipases produced during fermentation which hydrolyzed glycerides into fatty acids that can be easily assimilated {191}.

Vitamin or provitamin formations during *tempe* fermentation by fourteen strains of *Rhizopus* were studied and all the strains could form riboflavin, pyridoxine, nicotinic acid and ergosterol {43}. Some of the strains were able to form β-carotene in significant amounts and a fourfold increase in β-carotene was detected between 34 and 48 hr fermentation {43}. According to Steinkraus {210}, riboflavin nearly doubles, niacin increases sevenfold and vitamin B_{12} is synthesized during *tempe* fermentation. Vitamin B_{12} levels in *tempe* are estimated in the range of 2 – 40 ng/g and *Klebsiella pneumoniae* and *Citrobacter freundii* are responsible for its production {163}. Soybean is considered as a good source of vitamin K. A Japanese fermented soybean product, *natto*, is a rich source containing about 6-9 ug/g vitamin K_2 {27}. *Natto* that was produced by a mutated *B. subtilis* strain showed an even higher content of vitamin K_2, up to 12.98 ug/g {27}.

Fermentation can be used to eliminate or reduce allergic reactions in soybean sensitive individuals. During fermentation, soybean allergens are degraded by microbial proteolytic enzymes {27, 209}. In fermented soy products such as soy sauce, *miso* and *tempe*, soy protein is hydrolyzed into smaller peptides and amino acids and the structure of antigen epitopes may be altered, and may become less reactive {27, 104}. In soybean meal naturally fermented and fermented with *Lb. plantarum*, *B. lactis*, and *S. cerevisiae,* immunoreactivity was reduced by 79.8%, 78%, 77% and 77%, respectively, when plasma of soy sensitive individuals was used. The reductions were higher, 87.5%, 86.9%, 86.3%, and 88.7%, respectively, when pooled plasma of soy sensitive individuals was used {209}.

Lately, the study of fermented soybeans has received a lot of attention from researchers because of formation of functional biopeptides. During fermentation large protein molecules are degraded into small peptides and amino acids. Some of the peptides are biologically active and play important roles as angiotensin-I converting enzyme (ACE) inhibitor and as antioxidants {27, 82, 164, 202}.

2.4 Fermentation as a small-scale and household technology

Fermentation is one of the oldest food processing techniques that has stood the test of time because of its numerous benefits including improvement of nutritional quality through degradation of anti-nutritional factors and biological enrichment with nutrients, improvement of organoleptic characteristics, prolonging shelf-life of food, ensuring food safety and reduction in cooking times and fuel requirements {18, 22, 77, 142, 170, 212}. Fermentation has allowed dietary diversification through utilization and acceptance of certain foods that may have been considered unpalatable in unfermented state {77, 201}. Fermentation is a cheap and reliable technology that is accessible to all populations even in less developed countries {116, 142}, where it may still serve as an economic means of preserving food where refrigeration or other means may not be available {78, 142, 170}. In addition, fermentation has the potential of enhancing food safety by controlling growth and multiplication of some enteropathogens {99, 100, 142, 214}. Fermentation still has wide applications to date in spite of industrialization as evidenced by a variety of fermented products {78} including dairy, bakery, alcoholic and non-alcoholic beverages, vegetables and meat products. Lately, the contribution of functional attributes to a food as a result of fermentation has become a major research theme {22, 27, 82, 202}.

Fermented foods are described as food substrates that are invaded or overgrown by edible microorganisms whose enzymes, particularly amylases, proteases and lipases hydrolyze the polysaccharides, proteins and lipids to non-toxic products with flavors, aromas and textures pleasant and attractive to the human consumer {211, 212}. Microorganisms contribute to the development of taste, aroma, visual appearance, texture, shelf-life and safety due to their metabolic activities {77, 78}. Enzymes indigenous to the raw materials may also play a role in enhancing these characteristics {77, 78}. Traditional skills for controlling technical parameters during fermentation were developed through trial and error {78}. Traditional approaches include spontaneous or natural fermentations and back-slopping {78, 116} which serves as a traditional mixed starter system of inoculation.

2.4.1 Natural fermentations

Natural fermentations are processes initiated without the use of a starter inoculum {78}. A starter inoculum or starter culture is a preparation or material containing large numbers of viable cells of at least one microorganism, which is added to a raw material to accelerate its fermentation process {77, 78, 116}. Natural fermentations result from competitive activities of a variety of contaminating microorganisms on raw materials, utensils and from the environment {78, 116}. The microorganisms best adapted to the food substrate and to the fermentation conditions and with highest growth rate dominate different stages of the fermentation process {77, 78}. The production of metabolites (e.g. organic acids) inhibitory to other contaminating microorganisms may provide an additional advantage during fermentation {78}. Natural fermentations have been applied for thousands of years and are still practiced in a majority of small-scale and household fermentations all around the world, but today more extensively in Africa and Asia {18, 38, 58, 78, 95, 144, 148, 170, 174, 186, 187, 216}.

The quality of naturally fermented foods depends on the microbiological load of the raw material {116}. Usually, the initiation of the fermentation process takes a long time and there is a risk of fermentation failure which can lead to spoilage or survival of pathogens, thereby creating unexpected health risks in food products generally regarded as safe {58, 78}. Because processing conditions are not sterile, natural fermentations are difficult to control {162}, and results in products of inconsistent and unstable qualities {58, 186}. To accelerate natural fermentations, malted grains have been added to the fermentation media. Available endogenous amylolytic enzymes in the malted grains increase the rate of fermentation {78,

100, 101, 165}. Acceleration of natural fermentations have also been achieved through back-slopping {58, 116, 162, 165}.

2.4.2 Traditional mixed starter systems of inoculation

These systems involve the inoculation of raw materials with a small quantity of a previous successfully fermented batch and are commonly referred to as back-slopping {78, 116}. The microorganisms are in an active state during the time of inoculation {78}. This reduces the initiation time, shortens the fermentation process and reduces the risk of fermentation failure {116}. Use of back-slopping is common in developed countries although it is still practiced in industrialized countries in fermentation of some products such as sauerkraut, sourdoughs, fermented meats and vegetables {22, 78, 116}. Up to 10% of previously fermented material can be used as back-slopping material {161} and its continuous recycling results in selection of the best adapted strains {116}. In this study, 10% of traditional fermented cereal gruel, *thobwa*, was used as back-slopping material to initiate and accelerate lactic acid bacteria fermentations.

2.4.3 Preservation of inoculum in traditional fermentations

Use of back-slopping has allowed preservation of "starter cultures" using simple techniques such as dehydration or use of carriers. A fermenting substrate containing mixed cultures can be preserved by dehydration (air- or sun-drying) in form of flat cakes, hard balls or powders {78, 162}. Dehydration enhances the viability of microorganisms over relatively long periods, provided the product is maintained in the dehydrated state {78}. The extended viability of fermentation strains allows extended storage and improved distribution of a 'starter' for traditional food fermentations {77}.

Inoculum carriers include use of piece of cloth in a fermentation so that the cloth becomes microbiologically impregnated and provides inoculum in subsequent fermentations {219}. This is practiced in fermentation of cassava dough into *agbelima* {219}. Another carrier of similar nature is an "inoculation belt" for *pito* beer fermentations in Ghana in which a fiber belt is immersed into fermenting brew to immobilize microorganisms {219}. The microbiologically impregnated cloth is dried and used to initiate next batch of fermentation {78, 219}. A porous material of a gourd and fermentation utensils are also used to preserve inoculum {78}. Such simple preservation techniques usually result in mixed strain cultures which offer several advantages to small-scale processing that are usually limited in infrastructure. The mixed strain cultures are less susceptible to deterioration, are relatively

unaffected by fluctuating conditions of handling, storage and applications and they contribute to a more complex sensory quality {78}. The mixed strain cultures produce favorable synergistic effects such as degradation of undesirable factors, flavor production and accelerated ripening and maturation {78}. The disadvantage of the cultures is the variation in product quality which can be minimized through proper process control and the risk of contamination of the back-slopping cultures {78, 162, 186}.

2.4.4 Starter cultures in small-scale fermentations

Defined (multiple–strain starter) and undefined (mixed-strain starter) cultures that are used in large-scale fermentations {131} offer advantages of improving both process control and the predictability of product quality {186}. However, they possess a number of challenges for small-scale operations because the pure cultures relatively undergo deterioration in performance easily due to bacteriophage infection and loss of key physiological properties that may be plasmid encoded {78, 116}. Besides, the equipment for the preparation, handling and application of pure strain cultures and the strict process control required at all stages of the fermentation may not be feasible in most small-scale operations {78}. Another barrier to the application of starter cultures in small-scale fermentation is the loss of uniqueness of the characteristics that made the fermented products popular {116, 225}.

Nevertheless use of defined starter cultures becomes inevitable due to upscaling of manufacturing processes as products become popular and as demand grows {22}. This makes it imperative to determine roles and technological properties of microorganisms involved in natural fermentations. Therefore, studies on isolation and characterization of wild strains that dominate natural and back-slopping fermentations remain important as these strains serve as sources of desirable properties for starter culture development.

2.5 Microbiology of lactic acid bacteria fermented cereal gruels of Africa

Africa boasts abundant types of fermented foods that can be found in all categories of Steinkraus {211} classification of fermented foods. Among the fermented foods in Africa, lactic acid bacteria fermented products form the majority, and of which cereal based fermented foods dominate {170}. Lactic acid bacteria fermented cereal based foods include gruels and beverages, alcoholic beverages, acid leavened breads and pancakes, while non-cereal based foods include root crops, milk products and vegetable products {170}. The main LAB implicated in fermentations of both cereal and non-cereal based foods belong to four genera, namely *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* {170}. Some of

728 the cereals gruels are used as complementary foods therefore are of nutritional significance
729 {18, 170, 211}.
730
731 The names, raw materials, preparation techniques and recipes for fermentation of cereal
732 gruels vary from country to country (Table 4). The Zimbabwean *tobwa* is made from left
733 overs of stiff maize porridge (*sadza*) which are broken into small pieces, suspended in water
734 and left to ferment naturally {58}. On the other hand, the Malawian *thobwa* is prepared like
735 some of the Tanzanian *togwa* in which maize flour slurry is boiled for 20 – 30 min, cooled
736 down to 45 – 60 ^{o}C, and then malt flour of either millet, sorghum, or maize is added before
737 the mixture is left to ferment naturally {102, 144, 158}. Lactic acid bacteria are the dominant
738 flora of the fermented cereal gruels (Table 4). The general principle of lactic acid bacteria
739 fermentation is that lactic acid bacteria convert fermentable sugars in the cereals to lactic acid
740 and other metabolites {99, 133, 211}. The pH is subsequently reduced to 4.0 or less and the
741 final product is generally regarded as safe {100, 133, 214}. Inhibition of proliferation of
742 enteropathogens including toxigenic *Escherichia coli, Campylobacter jejuni, Shigella*
743 *flexineri, Salmonella typhimurim*, *Bacillus cereus* and *Aeromonas*, during fermentation of
744 maize and sorghum based gruels (*togwa* and *mahewu*) have been reported {58, 99, 100, 214}.
745 Therefore, promotion of wide use of fermented cereals as complementary foods was
746 suggested as a solution to many cases of infant diarrhea that arise due to contamination of
747 infant foods in Africa {170}.
748
749

750 Table 4: Microorganisms isolated in some fermented cereal-based African non-alcoholic gruels

Product name	Substrate	Place/Country	Microorganisms	References
Ogi	Maize, sorghum, or millet	Nigeria, West Africa	*Lactobacillus plantarum, Lactobacillus fermentum, Saccharomyces cerevisiae, Candida mycoderma, Candida krusei, Debaromyces hasenii, Klebsiella* spp., *Staphylococcus* spp., *Corynebacterium, Aerobacter cloacae, Rhodotorula, Cephalosporium, Fusarium, Aspergillus, Penicillium*	{18, 170, 186}
Mahewu	Maize, sorghum or millet, wheat	South Africa, Zimbabwe	*Lactobacillus delbrueckii* subsp. *delbrueckii, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus* spp., *Streptococcus lactis, Lactococcus lactis.*	{18, 170, 186}
Bogobe	Sorghum	Botswana	unknown	{18}
Ilambazi lokubilisa	Maize	Zimbabwe	LAB[1], yeasts and moulds	{18}
Koko or kenkey	Maize, sorghum or millet	Ghana	*Enterobacter cloacae, Acinetobacter, Lb. plantarum, Lactobacillus brevis, S. cerevisiae, C. mycodema,*	{18}
Mutwiwa	Maize	Zimbabwe	LAB, bacteria, moulds, *Pediococcus pentosaceus*	{18, 58}
Tobwa	Maize	Zimbabwe	LAB	{18, 58}
Thobwa	Maize, millet	Malawi	LAB	{158}
Uji	Maize, sorghum, millet	Kenya, Uganda, Tanzania,	*Leuconostoc mesenteroides, Lb. plantarum, Lactobacillus* spp.	{18, 186}
Togwa	Maize, sorghum, millet,	Tanzania	*Lb. plantarum, Lb. brevis, Lb. fermentum, Lactobacillus cellobiosus, P. pentosaceus, Weissella confusa, Issatchenkia orientalis, S. cerevisiae, Candida pelliculosa and Candida tropicalis*	{144}
bushera	Sorghum, millet	Uganda	*Lb. plantarum, Lactobacillus. paracasei* subsp. *paracasei, Lb. fermentum, Lb. brevis, Lb. delbrueckii* subsp. *delbrueckii, Streptococcus thermophilus, Lactococcus* sp., *Leuconostoc* spp., *Lactobacillus* sp., *Weissella* spp., *Enterococcus* spp.	{148}

751 [1]LAB = Lactic acid bacteria

2.6 Microbiology of alkaline fermented seeds and soybean products

Africa and Asia have a number of important fermented foods that are produced from seeds to provide flavor and serve as cheap sources of protein in the diet {167, 187, 211, 216}. In Africa, these include *dawadawa (soumbara* or *iru), ogiri*, *ugba, aisa*, *okpehe, bikalga* and *awoh* {4, 174}. *Ogiri*, *ugba, aisa*, *okpehe, bikalga* and *awoh* are produced from mellon seeds (*Citrullus vulgaris*), African oil been seeds (*Pentaclethra macrophylla*), rain or saman tree (*Albizia saman*), *Prosopis Africana*, roselle (*Hibiscus sabdariffa*) and cotton seeds (*Gossypium hirsutum*), respectively {174}. *Dawadawa,* as is known in Nigeria and Ghana, is also called *soumbara* in Burkina Faso and *iru* in Nigeria, *nenetu* in Senegal, *afitin, iru* and *sonru* in Benin and *kinda* in Sierra Leon {1, 4, 39, 174}. *Dawadawa* is produced by natural fermentation of soaked, cooked and dehulled locust bean seeds (*Parkia biglobosa*) and *B. subtilis* is the main fermenting microorganism {4, 39, 174, 211}. The seeds are spread in calabash trays and wrapped in jute sacks or packed in earthenware pots and their fermentation results in a stringy mucilaginous coating accompanied by strong ammoniacal odor {174}. Soybeans can substitute locust beans or can be used in combination with locust beans in the production of soy-*dawadawa* {4, 39, 174}.

Popular alkaline fermented foods of Asia include Japanese *natto*, Thai *thua-nao*, Korean *doenjang*, and Indian *kinema*, all made from soybeans and where *B. subtilis* is the major microorganism during their fermentation {38, 96, 193, 211}. The principle behind alkaline fermentation is that the fermenting bacteria are highly proteolytic, hydrolyzing proteins into peptides and amino acids with subsequent production of ammonia {2, 113, 114, 133, 174, 193, 211}. The ammonia is released and the pH rapidly increases to 8.0 or higher {113, 211}. The combination of high pH, free ammonia and rapid growth of the fermenting microorganisms at relatively high temperature (above 40 ^{o}C) make it difficult for competing microorganisms that may be present in the product to grow {192, 211}. Therefore, alkaline fermented foods are quite stable, safe and well preserved especially when dried {211}.

Natto is consumed fresh without further processing in boiled rice while *kinema* is fried and eaten as a side dish with boiled rice {113, 191, 194}. Traditional *kinema* is a non-salted fermented food prepared by washing, soaking, cooking and crushing soybeans to grits {187, 215}. The soybeans are then wrapped in fern or banana leaves and sackcloth and allowed to ferment for 1-3 days at ambient temperature {187, 194}. After about 12 h, the surfaces of the

beans are covered with a rough, white, viscous mass {187}. Fresh *kinema* is fried in oil and then added to vegetables and spices to prepare a curry {187, 192}. Shelf-life of fresh *kinema* without refrigeration is 2 – 3 days in summer and 5 – 7 days in winter and the shelf-life is extended by sun drying for 2 – 3 days {216}. A bonus characteristic of *kinema* is the 50% reduction in cooking time compared to raw soybeans {192}. Japanese *hikiwari-natto* differs from *kinema* because dehulled soybeans cracked into two or four pieces are used, while the more common Japanese *itohiki-natto*, Thai *thua-nao*, and Chinese *schui-douchi* are prepared like in *kinema* except that whole soybeans rather than crushed ones are used {37, 187, 192}.

Korean *cheonggukjang* differs from *kinema* because steamed soybeans are naturally fermented or are fermented using dried rice straws (*jip*) for over three days {152}. On the other hand, Korean *doenjang* is prepared from another fermented product called *meju*. *Meju* serves as basis for preparation of soy sauce (*ganjang*), soybean paste (*doenjang*) and hot pepper paste (*gochujang)* {98, 111, 153}. Traditional *meju* is prepared by soaking, steaming, and molding soybeans, followed by aging for one or two months under natural environmental conditions {98}. During *doenjang* preparation, *meju* is mixed with salt and water or red pepper or rice and the mixture is further fermented for 2 or 3 months {95, 152}. The quality and functionality of *doenjang* are affected by microorganisms, fermentation process and by basic ingredients such as soybeans or grains used {151}.

High ammonia production in alkaline fermented foods results in strong pungent odor which readily reaches objectionable levels {2, 174}. As such, consumption of alkaline fermented foods is limited because some people find the atypical odor unpleasant, offensive, undesirable or objectionable {4, 174}. The strong ammonia-like smell can be reduced by drying the fermented product as in the case of *thua nao* or frying as is done in *kinema* {113, 192}. Alternatively, ammonia production can be restricted during fermentation by limiting growth and metabolism of the bacteria without inhibiting the action of flavor-generating proteolytic enzymes {2}. Addition of humectants such as NaCl or glycerol or limitation of the amount of initial O_2 by doing the fermentation in sealed containers achieves this {2, 174}. Use of NaCl inhibits both growth of bacteria and enzymatic activities while glycerol inhibits growth but allows enzymatic activities to continue {2}. Amoa-Awua *et al.* {4} reported 1.5 and 1.7 mol per kg as optimal concentrations of salt and glycerol, respectively, for controlling *Bacillus* growth for a reduced *dawadawa* odor. For a successful fermentation, addition of the humectants after the beans have started fermenting actively is recommended {4}. Limiting the

initial amount of O_2 to 10 – 20% reduced growth of *Bacillus* and allowed some increase in pH without ammonia production {2}. In *natto*, ammonia formation is reduced by storage at low temperature (5 – 10 ^oC) which restricts bacterial growth but allows the proteolytic enzymes to continue to work {113, 174}.

Dominant microorganisms in many alkaline fermented foods are *B. subtilis* {113, 174}. In numerous *dawadawa* samples, *B. subtilis* accounted for 31% of all isolates while other *Bacillus* spp. accounted for 13%, and in some samples *B. subtilis* represented as high as 61 to 69% of all isolates {174}. Other microorganisms in *dawadawa* included LAB, *Micrococcus* spp. and *Staphylococcus* spp. (Table 5). The main microflora in *thua nao* include *Bacillus* spp. whose count can reach as high as 10^{10} cfu/g, followed by LAB with a count of 10^6 cfu/g and moulds with a count of 10^3 cfu/g {113}. Chukeatirote *et al.* {33} reported 10^{12} cfu/g moulds and bacteria counts were 10^{13} cfu/g and LAB were detected after 12 and 24 hrs of *thua nao* fermentation. In *kinema*, *B. subtilis* is the functional bacterium during fermentation {216}. *Bacillus* spp. population ranged from 3 – 5 x 10^8 to 10^{10} cfu/g and the growth of *Bacillus* did not affect growth of *E. faecium* whose population ranged from 5 x 10^7 to 10^9 cfu/g during fermentation {187, 194}. Other commonly isolated microorganisms in *kinema* are yeasts {194}(Table 5). Presence of *E. coli* and other *Enterobacteriaceae* and *B. cereus* in market *kinema* have also been reported {163}.

Complicated and diverse microbial structures have been reported in *meju, doenjang* and *dajiang* (Table 5). Contrary to other reports that suggested *B. subtilis* and its relatives as the dominant and only important species in *meju* fermentation, LAB were also found to be dominant {98}. *Bacillus* spp. represented 52% of total isolates in *meju* while LAB represented 44% of the isolates and pyrosequencing confirmed *Firmicutes* (composed of *Bacillus* and LAB) as the dominant phyla {98}. LAB genera in *meju* include *Enterococcus, Pediococcus, Lactococcus,* and *Leuconostoc* (Table 5). According to Kim *et al.* {95}, *B. subtilis* and *B. licheniformis* are the dominant organisms that play an important role during fermentation in *doenjang*. However, *Bacillus* species are not always the dominant species because LAB were reported dominant in other *doenjang* samples {151}. Although *Bacillus* species were dominant in some samples of *Cheonggukjang*, levels of *B. subtilis, B. licheniformis* and *B. amyloliquefaciens* varied between samples and the three species were not the dominant microorganisms in all samples {152}. Unclassified *Bacillus* species and LAB dominated various samples of *Cheonggukjang* {152}.

853 Table 5: Microorganisms isolated from locust bean and soybean fermentations

Product name	Substrate	Country/Place	Microorganisms	References
Dawadawa, Soumbara, iru, nenetu, afitini, sonru, kinda	Locust bean (*Parkia biglobosa*)	Nigeria, Ghana, Burkina Faso, Senegal, Benin, Sierra Leon	*Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, Bacillus brevis, Bacillus megaterium, Bacillus polymyxa, Bacillus cereus, Bacillus firmus, Bacillus sphaericus, Bacillus badius, Bacillus thuringiensis, Bacillus mycoides, Pediococcus acidilactici, Enterococcus faecium, Paenibacillus alvei, Paenibacillus larvae, Brevibacillus laterosporus, Brevibacillus borstelensis, Brevibacillus parabrevis, Leuconostoc* spp., *Pseudomonas aeruginosa, Micrococcus* spp.,*Staphylococcus vitulinus, Staphylococcus aureus, Staphylococcus saprophyticus, Staphylococcus* spp.,*Tetragenococcus halophilus, Morganella morganii, Ureibacillus thermosphaericus, Salinicoccus jeotgali, Brevibacterium* spp.	{1, 11, 52, 86, 169, 174, 188}
Soy(a)-dawadawa/ soy-daddawa	Soybeans, locust beans	Nigeria, Ghana	*B. subtilis***,** *Bacillus licheniformis, Bacillus circulans, B. cereus, B. firmus, B. pumilus, B. megaterium, S. saprophyticus, Staphylococcus epidermidis, Micrococcus luteus, P. aeruginosa,* LAB	{39, 167, 174}
Thua nao	Soybeans	Thailand	*B. subtilis*, *B. megaterium, B. cereus, B. pumilus,* LAB, *Lactobacillus* spp., moulds,	{113} {33}
Kinema	Soybeans	Nepal, India	*B. subtilis, B. cereus, B. licheniformis, B. circulans, B. thuringiensis, B. sphaericus, Enterobacteriaceae, E. faecium, Candida parapsilosis, Geotrichum, Escherichia coli*	{163, 188, 194}
Cheonggukjang	Soybeans	Korea	*Bacillus amyloliquefaciens, Bacillus coagulans, B. licheniformis, Bacillus sonorensis, B. subtilis, Bacillus thermoamylovorans, Bacillus vallismortis, Enterococcus faecalis, E. faecium, Lactobacillus pentosus, Lactobacillus plantarum, Streptomyces rangoonensis,*	{152}
Doenjang	Soybeans, grains	Korea	*B. subtilis, B. licheniformis, B. sonorensis, B. cereus, B. amyloliquefaciens, Brochothrix* spp., *E. faecium, E. faecalis, Enterococcus casseliflavus, Lactobacillus halophilus,*	{94, 95, 151}

854 Table 5 continued: Microorganisms isolated from locust bean and soybean fermentations

Product Name	Substrate	Country/Place	Microorganisms	References
Doenjang	Soybeans, grains	Korea	*Pediococcus pentosaceus, P. acidilactici, Leuconostoc mesenteroides, Lactobacillus sakei, Pseudomonas* spp., *T. halophilus, Lb. plantarum, Weissella viridescens, Weissella confusa, Weissella hellenica, Weissella salipiscis, Carnobacterium pleistocenium, Staphylococcus gallinarum, Staphylococcus lentus, S. saprophyticus, Staphylococcus sciuri, Staphylococcus nepalensis, Mucor, Debaryomyces, Galactomyces, Zygosaccharomyces, Pilaira, Zygomycete,Candida, Absidia, Pichia, Aspergillus, Sterigmatomyces, Clavispora,* Coliforms	
Meju	Soybeans	Korea	*P. acidilactici, Leu. mesenteroides* ssp. *mesenteroides, Enterococcus durans, Leuconostoc citreum, Lactobacillus coryniformis* ssp. *torquens, P. pentosaceus, Lactococcus lactis* ssp. *lactis* bv. *diacetylactis, B. subtilis, Lactobacillus sakei, B. licheniformis, Thermoactinomyces sanguinis, E. faecium, Enterococcus thailandicus, Pichia, Absidia, Aspergillus, B. sonorensis, Mucor, Penicillium, Candida, Acinetobacter, Staphylococcus, Hafnia, Enterobacter, Brevibacterium, Leuconostoc, Pediococcus, Leucobacter, Lactobacillus,*	{98, 111}
Dajiang	Soybeans	China	*Bacillus* spp., *S. gallinarum, S. epidermidis*, *Aspergillus oryzae, Zygosaccharomyces rouxii, Candida etchellsii, Absidia corymbifera, B. cereus, B. megaterium, Lb. plantarum, Lb. fermentum, E. faecium, B. firmus, B. amyloliquefaciens, Lactobacillus delbrueckii, Lactobacillus kefiri, Leu. mesenteroides, Leuconostoc gasicomitatum, Streptococcus mutants, Streptococcus thermophilus, T. halophilus, Lb. sakei, Bifidobacterium, Candida humilis, Kluyveromyces, Zygosaccharomyces, Williopsis, Paenibacillus, Oceanobacillus*	{96, 235, 241}
Miso	Soybeans	Japan	*S. gallinarum, Leuconostoc pseudomesenteroides, Staphylococcus kloosi, T. halophilus, Weissella cibaria, Leu. citreum, P. pentosaceus, Pichia, Aspergillus, Zygosaccharomyces, Clavispora*	{96}

2.7 Safety of fermented soybean products

Alkaline fermentations are generally regarded as safe because the high pH value and the release of ammonia make the substrate unsatisfactory for invasion by other microorganisms {2, 211}. However, Sarkar & Tamang {193} showed that *B. subtilis* and *E. faecium* co-existed during natural fermentation of *kinema* and the two organisms did not affect each other's growth. *Bacillus subtilis* population increased from 8.0 x 10^5 cfu/g to 4.55 x 10^8 cfu/g while *E. faecium* increased from 2.0 x 10^5 cfu/g to 8.23 x 10^7 cfu/g during 48 hrs fermentation {193}. The two organisms were also dominant in market *kinema* at levels of 3-5 x 10^8 cfu/g and 5-9 x 10^7 cfu/g for *B. subtilis* and *E. faecium*, respectively {194}. In addition, *B. cereus, E. faecium, E. faecalis*, and *E. coli* have been isolated from some alkaline fermented products {33, 39, 95, 111, 151, 152, 163, 188, 235, 241}. Furthermore, some *B. cereus* strains isolated from *kinema* were able to produce diarrheal type enterotoxin, BCET {188}.

The presence of these undesirable microorganisms suggests that alkaline pH and ammonia production do not inhibit growth of all potential spoilage and pathogenic bacteria. Besides, *E. coli* can grow at a wide pH range of 4.5 to 9.0, with minor differences in generation time between pH 7.3 (0.4 hrs) and pH 9.0 (0.5 hrs){65}. *Escherichia coli* can also survive lactic acid fermentation because of its inducible acid-tolerance response system {21, 99, 100, 117, 142}. Thus, growth of *E. faecium* and presence of *B. cereus* and *E. coli* suggest food safety concerns in alkaline fermented foods.

The fact that a number of foodborne hazards are not completely inhibited by fermentation means that fermentation alone cannot be relied upon for complete elimination of these hazards. However, controlled fermentations of *kinema* and *doenjang* with starter cultures reduced the microbial diversity of these products {151, 188}. In addition, a *kinema* strain, *B. subtilis* DK- Wl, was able to suppress growth and BCET formation by a selected toxin producing strain (BC7-5) of *B. cereus* {188}. Thus, controlled fermentation could be used to reduce the presence of pathogenic bacteria that may be found as opportunistic contaminants in natural fermentations. Further, safety of soybean fermented pastes could be ensured if the fermentation is combined with other processing operations, such as cooking {142}. *Escherichia coli* can be controlled readily through sufficient heat treatment {21} and frying *kinema* was able to reduce BCET from levels exceeding 256 ng/g soybeans to 8 ng/g {188}.

Another concern in fermented soybeans is the formation of various biogenic amines during fermentation. Biogenic amine levels change depending on the ratio of soybean in the raw

material, microbiological composition, duration of fermentation, and many other factors {93, 203}. High levels of histamine, cadaverine, putrescine and tyramine were reported in fermented soy products {198, 203}. Consumption of food containing biogenic amines can lead to several types of foodborne diseases, including histamine poisoning or scombroid poisoning and tyramine toxicity or cheese reaction {198}.

2.8 Methods for detection of microorganisms in fermented soybean products

Traditionally, detection of microorganisms in different environmental systems including fermented foods have relied on culture-dependent methods which are based on cultivation and isolation of microorganisms on suitable substrates {51}. The isolates may be phenotypically characterized through their morphology, physiology, biochemical or protein profiles {217}. Alternatively, genotypic methods based on polymerase chain reaction (PCR) that enables selective amplification of specifically targeted DNA fragments through the use of oligonucleotide primers are used {217}. Particular gene fragments such as the 16*S* rDNA can be amplified {1}. Genotypic techniques are robust in discriminatory power and in differentiation of microorganisms to strain level {217}. The DNA resulting from PCR can be used in fingerprinting methods such as restriction fragment length polymorphism, amplified ribosomal DNA restriction analysis, randomly amplified polymorphism of DNA, amplified fragment length polymorphism and pulsed field gel electrophoresis {1, 217}. The results provide information on isolate identities and clonal relationships through dendrograms of gel fingerprints {1}.

One major limitation of culture-dependent techniques is bias in cultivation of isolates because it is difficult to develop media that accurately resembles the growing conditions of most bacteria in natural habitat {50, 51}. The identification of isolates using phenotypic methods such as sugar fermentation patterns may sometimes be uncertain, complicated and time-consuming {51}. Uncertainty arise because some strains that display similar phenotypes do not always correspond to similar or even closely related genotypes {217}. Additional weaknesses include poor reproducibility and poor discriminatory power {217}. Culture-dependent methods do not always give a true reflection of microbial diversity in complex ecosystems such as fermented foods and there is failure to account for minor microbial populations, stressed and injured cells that are present in low numbers {1, 51, 217}. Nevertheless, cultivation and isolation of microorganisms cannot be ignored since most

industrial applications and health effects of microorganisms depend on specific characteristics of particular strains {217}. Learning the properties of the strains also depends on cultivation.

Culture-independent methods were developed to overcome the limitations of selective cultivation {50}. Microorganisms in a food matrix can be detected at genus, species or strain level using specific primers for target organisms in microbial DNA extracted from the sample {217}. The limitation of this approach is that only targeted microorganisms are detected, hence the PCR assays are of limited value in the analysis of complex ecosystems or samples with unknown species composition {217}. Probing techniques based on hybridization of synthetically designed oligonucleotides to specific target sequences in bacterial DNA, such as colony, dot blot and in situ hybridizations have similar limitations {217}.

Lately, denaturing gradient gel electrophoresis (DGGE) has proven to be one of the most suitable and widely applied methods to study complex microbial communities originating from various ecosystems. Denaturing gradient gel electrophoresis is a PCR-based genetic fingerprinting technique that provides a profile of genetic diversity in a microbial community based on physical separation of unique nucleic acid species {149}. During DGGE, there is sequence-dependent separation of a mixture of amplified DNA fragments of the same length on polyacrylamide gels containing a linear gradient of DNA denaturants {50, 149, 150, 217}. The separation is based on decreased electrophoretic mobility of a partially melted double-stranded DNA molecule {149, 150}. The DNA fragments melt in discrete melting domains, with each domain having a melting temperature (*Tm*) at a particular position in the denaturing gradient gel. Partial denaturation of the domains occurs at *Tm* causing the migration of the domains to practically halt, forming discrete bands in the gel {53, 150}. Sequence variation and % G + C content within such domains causes the *Tm* to differ, and molecules with different sequences will stop migrating at different positions in the gel {150, 217}. Addition of a 30- to 40-bp GC clamp to one of the PCR primers insures that the fragment of DNA remains partially double-stranded and that the region screened is in the lowest melting domain {50}. Fig 2 summarizes the principle of DGGE.

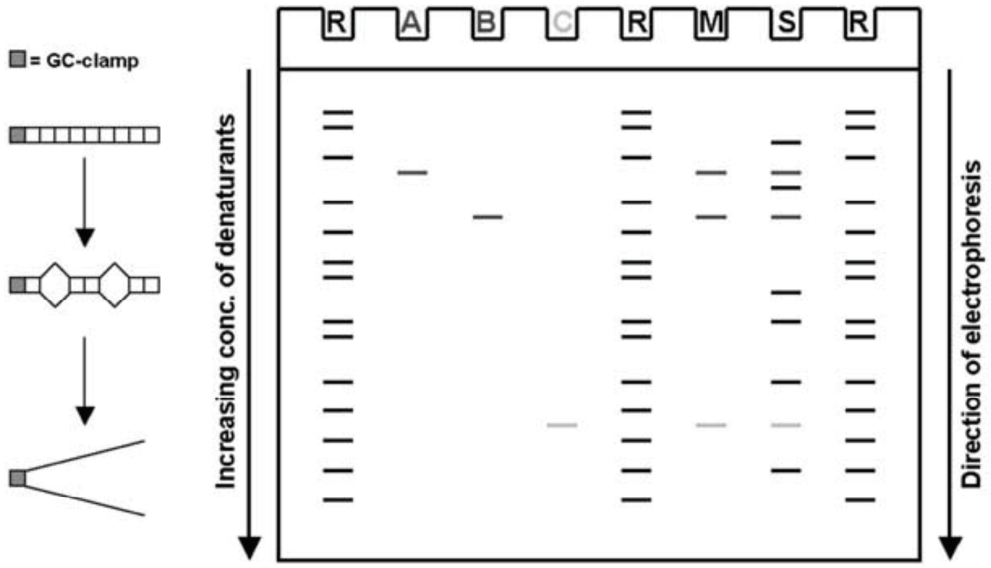

Fig. 2: Principle of DGGE. PCR amplicons of equal length are electrophoretically separated in a sequence-dependent manner. The increasing gradient of denaturing components along the gel separates the double stranded amplicons into single stranded DNA through melting domains. A GC-clamp attached to the 5' end of one of the PCR primers prevents the amplicons from completely denaturing. Different sequences will result in different origins of melting domains and as a consequence also in different positions in the gel where the DNA fragment halts. Source: Temmerman *et al.* {217}

Prior to DGGE, PCR amplifications are done commonly targeting the ribosomal DNA because it is a very conserved region of the genome that also includes variable regions {50}. Different bacteria species have differences in base pair composition within the variable regions of 16*S* rDNA, making it possible to distinguish them by PCR-DGGE {50}. Several primer pairs have been designed and employed to amplify the variable regions of the 16*S* rDNA for bacteria and 18*S* rDNA or 26*S* rDNA for eucaryotes {50, 159, 160}. Amplified variable V3 region of the 16*S* rDNA has been used widely in identification of food microbial communities {5, 51, 115, 135, 146}

Denaturing gradient gel electrophoresis provides the possibility of identifying microbial community members by sequencing of excised bands {50, 53} or by hybridization with specific probes, which is not possible with other fingerprinting techniques {149}. Since DGGE allows simultaneous analysis of multiple samples {149}; it can be used to monitor microbial fermentations during ripening and analyze the community dynamics in food in response to environmental changes {50, 95}

However, like other molecular techniques DGGE is also affected by errors and biases due to sample handling, extraction and purification of nucleic acids {50, 149, 150}, differential or preferential amplification of rRNA genes by PCR {50, 51} and the possibility of formation of chimeric molecules due to PCR amplification of mixed target DNAs {50, 149, 150}. Limitations specific to the DGGE itself include separation of relatively small DNA fragments, co-migration of DNA fragments with different sequences, presence of heteroduplex molecules, possibility of species giving identical band positions and limited sensitivity of detection of rare community members {50, 135, 149, 150}. In fact, detection limits ranging from 10^4 to 10^8 cfu/ml have been reported depending on species or strain {50, 51}.

The resolution of DGGE analysis in mixed microbial systems can be improved by using more than one primer pair {28, 29}. In other cases, even nested PCR-mediated DGGE approaches have been used to improve the sensitivity of detection when the targeted microbial population is low {95, 96, 111}.

There is no doubt that DGGE reveals the complexity of microbial communities in food fermentations. However, currently next generation sequencing or pyrosequencing has found its place in the study of food fermentations. Pyrosequencing involves the synthesis of single-stranded DNA and the detection of light generated by pyrophosphate released in a coupled reaction with luciferase {115}. Pyrosequencing allows over 100-fold higher throughput rapid and accurate sequencing of nucleotide sequences {98, 115}. The information generated makes it possible to process large numbers of samples simultaneously {98} and analyze population structure, gene content and metabolic potential of the microbial communities in an ecosystem {115}. Pyrosequencing has highlighted DGGE's failure to detect a large number of predominant or diverse rare species in fermented soybeans {152}.

In soybean fermentations, pyrosequencing has revolutionalized the dominant thought that *Bacillus subtilis* was the only important microorganism. Currently, from the thousands of sequences generated and analyzed from soybean paste fermentations, *Bacillus* species and lactic acid bacteria seem to be the dominant species and there are a diverse range of rare species that accompany the fermentations that had never been reported before {98, 151, 152}.

2.9 Sensory properties and acceptance of fermented soybean products

Sensory and consumer studies play an important role in food science and industry for the understanding of food properties, human acceptance, preference and buying behavior. The use and application of descriptive sensory testing offers the ability to determine relationships

between descriptive sensory and instrumental or consumer preference measurements {147}. Thus descriptive sensory testing provides understanding of consumer responses in relation to products' sensory attributes, and aids in sensory mapping and product matching {147}. It may also be used to investigate the effects of ingredients or processing variables on the final sensory quality of a product, and to investigate consumer perceptions of products {147}. In this study descriptive sensory analysis was used in combination with data from chemical and physical analyses and consumer acceptance to generate preference maps.

Descriptive sensory analyses of fermented soybean products have indicated the complexity of sensory properties of these products. Sensory panels generated 10 – 48 attributes describing appearance, odors, aromas, taste, mouthfeel and texture {34, 92, 110}. Brown and yellow colors are used to describe the appearance of fermented soybean pastes that include *miso, natto, doenjang* and *chungkukjang* {34}. Strong desirable flavors associated with the fermented products include soy sauce, boiled soy sauce, nutty, briny, roasted bean, sweet aroma, *meju*, *gatsuo*, sesame leaf flavor, alcohol flavor, chemical flavors and sweet grain flavor while undesirables odors include earthy, metallic, fermented fish, burnt, sulfury, ammonia, fermented, beany, and acetic acid odors {34, 92, 110}. Fermented soybeans pastes elicit all the taste sensations, namely, sweet, salty, bitter, sour and monosodium glutamate (umami) in addition to astringency {34, 92}. The products' texture vary in particle sizes depending on production methods and they are usually sticky or slippery with a mouth coating mouthfeel {34, 92, 110, 238}.

Kim *et al.* {92} determined the sensory attributes that influenced consumer acceptance in *doenjang*. Drivers of liking were strong intensities of sweetness, umami, sweet grain flavor, soy sauce flavor, sesame leaf flavor while drivers of disliking were strong intensities of bitterness, earthy, metallic, astringency, saltiness and fermented fish. Apart from sensory characteristics, consumer preference was also significantly influenced by consumer segments {92}. Consumers tendency to rate the intensities of negative attributes highly for products that originated from other countries was reported in fermented soybean products {34}. This signifies the challenge that could be encountered when introducing food products that consumers are not culturally accustomed to.

Lee & Ahn {112} noted correlations among some volatile compounds and some sensory data. The sweet aromas were associated with furfuryl alcohol, 1-octen-3-ol, 1, 2-dimethoxy-benzene and acetyl pyrrole; while beany flavors were associated with isoamyl acetate, 2-

phenylethyl acetate and 4-ethyl-2-methoxy-phenol. They suggested heat treatment during the manufacturing process and ingredients of the fermented products as main sources of variation in aroma characteristics. Ingredients and fermentation methods also determined aroma characteristics in different fermented soybean pastes {34}.

2.10 Metabolism of the major microorganisms involved in fermentation of soybean pastes

2.10.1 Carbohydrate fermentation of *Bacillus* species

Bacillus subtilis has usually been found as the dominant microorganism in fermented soybean foods {230}. There are 8 members within the *B. subtilis* group, including *B. subtilis* subsp. *subtilis, B. licheniformis, B. amyloliquefaciens, B. atrophaeus, B. mojavensis, B. vallismortis, B. subtilis* subsp. *spizizenii,* and *B. sonorensis* {178}. *Bacillus* spp. are ubiquitous and grow in diverse environments including soils, on plant roots, and within the gastrointestinal tract of animals {47, 178}. *Bacillus* spp. belong to the phylum *Firmicutes,* class *Bacilli*, order *Bacillales* and family *Bacillaceae* {57}. *Bacillus* spp. are Gram-positive, endospore-forming and catalase positive bacteria that have spreading colonies with fringed margins {194}. *Bacillus subtilis* is capable of utilizing a variety of carbohydrates although glucose is the preferred source of carbon and energy {19, 195, 208}.

Glucose is taken up and is phosphorylated by the sugar:phosphoenolpyrvate phosphotransferase system {195}. Further metabolism of glucose involves the Embden–Meyerhof–Parnas or the glycolytic pathway, pentose phosphate pathway and the Krebs cycle (Fig 3) {19, 40, 195}. Other sugars and polyols are phosphorylated and converted to intermediates of either pathways {19, 195}. Pyruvate formed during glycolysis is further oxidized to acetyl-CoA or is used to generate NAD^+ {19} resulting in fermentation products such as lactate, acetate and acetoin which are excreted into the extracellular environment {208}. Acetyl-CoA can be converted to acetate through substrate-level phosphorylation {19}; which is coupled to ATP synthesis by the activities of phosphotransacetylase and acitate kinase {208}.

Utilization of organic acids produced during glycolysis requires conversion of the acids to intermediates of the Krebs cycle, gluconeogenesis or pentose phosphate pathway {195}. Glycolysis is linked to the Krebs or citric acid cycle by pyruvate dehydrogenase {19}. When glucose is completely consumed to pyruvate, *B. subtilis* cells metabolize lactate, acetoin and acetate using lactate dehydrogenase, acetoin dehydrogenase and acetyl-coA synthatase,

respectively, via the Krebs cycle (Fig 3), generating additional ATP and reducing power {208}. *Bacillus subtilis* is also able to grow anaerobically in the presence of nitrate as an electron acceptor {47}.

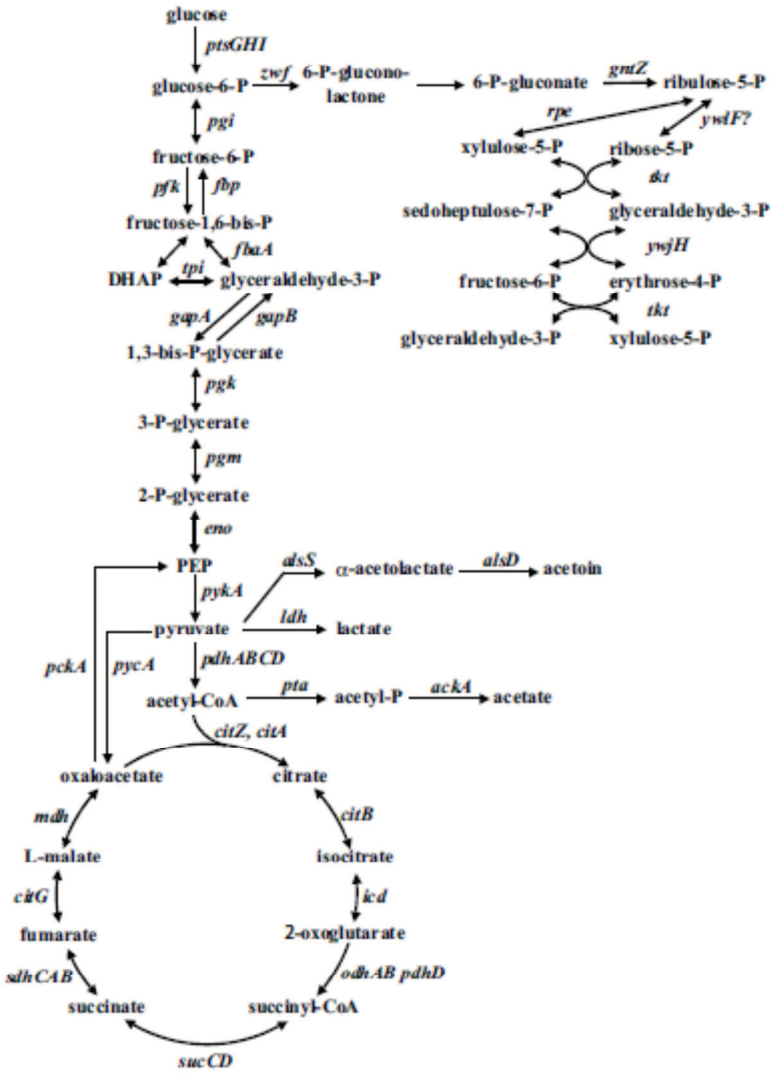

Fig 3: Overview on glycolysis, pentose phosphate shunt, Krebs cycle and their interconnections in *B. subtilis*. Abbreviations: DHAP, dihydroxyacetone phosphate; PEP, phosphoenol pyruvate. Enzymes are indicated by the names of the corresponding genes as follows: *ptsGHI*, glucose-specific enzymes for sugar:phosphoenolpyruvate phosphotransferase system; *pgi*, phosphoglucoisomerase, *pfk*, phosphofructokinase; *fbp*, fructose-1,6-biphosphatase; *fbaA*, fructose-1,6-biphosphate aldolase; *tpi*, triose phosphate isomerase; *gapA*/*gapB*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *pgm*, phosphoglycerate mutase; *eno*, enolase; *zwf*, glucose-6-phosphate 1-dehydrogenase; *gntZ*, 6-phosphogluconate dehydrogenase; *rpe*, ribulose-5-phosphate epimerase; *ywlF*, ribose-5-phosphate isomerase; *tkt,* transketolase; *ywjH,* transaldolase; *pykA*, pyruvate kinase; *pdhABCD*, pyruvate dehydrogenase (pyruvate decarboxylase); *alsS*, α-Acetolactate synthase; *alsD*, α-acetolactate decarboxylase; *ldh*, lactate dehydrogenase; *pta,* phosphotransacetylase; *ackA*, acetate kinase; *citZ*, *citA*, citrate synthase; *citB*, aconitase; *icd*,

isocitrate dehydrogenase; *odhAB pdhD*, 2-oxoglutarate dehydrogenase; *sucCD*, succinyl-CoA synthetase; *sdhCAB*, succinate dehydrogenase; *citG*, fumarate hydratase; *mdh*, malate dehydrogenase; *pckA*, phosphoenolpyruvate carboxykinase; *pycA*, pyruvate carboxylase. Source: Blencke *et al.* {19}.

2.10.2 Proteolysis in *Bacillus subtilis*

An important process during soybean fermentation is enzymatic degradation of proteins by proteolysis. Proteolysis contributes to texture, appearance and characteristic aroma and taste of fermented soybeans {230}. *Bacillus subtilis* exhibits relatively high proteolytic activities, hence is able to digest soybean proteins, releasing amino acids and other biological compounds {178}. Soybean fermentation with *Bacillus* is characterized by increases in pH and release of ammonia due to deamination of amino acids {230}. Proteolysis also increases TCA-soluble peptides and α-amino acids {230}. Extracellular proteases secreted by *B. subtilis* hydrolyse soy proteins to oligopeptides, smaller peptides and amino acids and these are subsequently converted to γ-polyglutamic acid, a major component of the viscous material on the surface of fermented soybeans {124, 230}. The smaller molecules can either be assimilated directly into microbial protein or fermented with the production of ammonia and volatile fatty acids {124}.

Glutamine is the preferred nitrogen source for *B. subtilis*, followed by arginine {54}. In the presence of exogenous amino acids, *B. subtilis* can produce glutamate by the glutamate synthase and α-ketoglutarate reactions and by the degradation of glutamine, proline and arginine {14}. Catabolism of arginine to glutamate proceeds via the first three steps of the Roc pathway (Fig 4) through ornithine and γ-glutamic semialdehyde {14}. Two enzymes of the three steps, arginase and ornithine aminotransferase are induced in the presence of L-arginine, L-ornithine, and L-citrulline {13}. The reaction that converts Δ^1-pyrroline-5-carboxylate to glutamate is shared with the proline utilization pathway {13, 14}. The final step in arginine and proline utilization pathways is catalyzed by glutamate dehydrogenase and leads to formation of α-ketoglutarate and ammonium {14}.

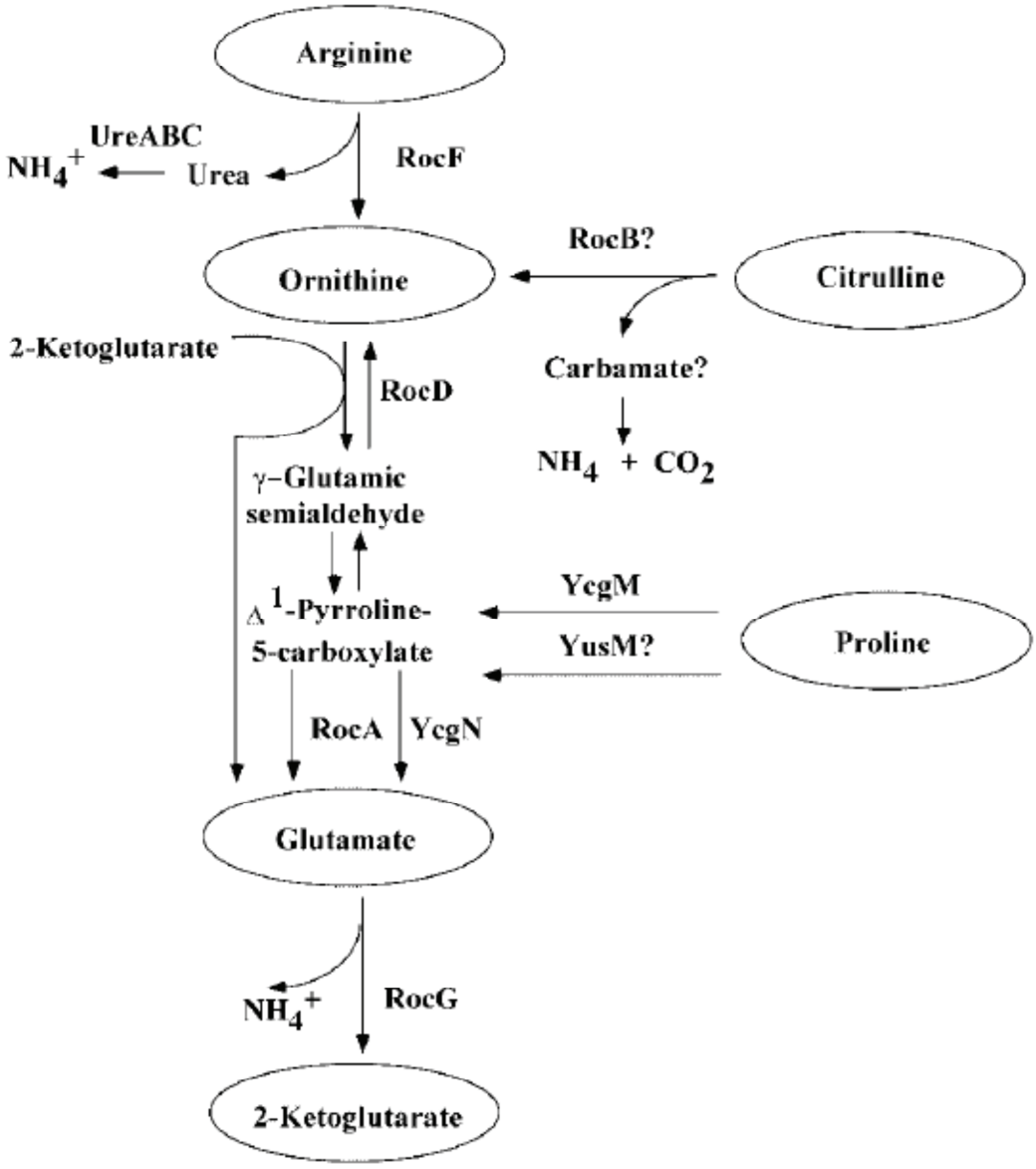

Fig 4: Roc pathway of utilization of arginine-related amino acids and proline in *B. subtilis*. Enzymes are indicated by the names of the corresponding genes as follows: *UreABC*, urease; *RocF*, arginase; *RocD*, ornithine aminotransferase; *RocA* and *YcgN*, Δ^1-pyrroline-5-carboxylate dehydrogenases; *RocG*, glutamate dehydrogenase; *RocB*, cetrullinase; *YcgM* and *YusM*, proline oxidases (proline dehydrogenases). Source: Belitsky & Sonenshein {14}.

2.10.3 Carbohydrate fermentation of lactic acid bacteria

Lactic acid bacteria are found as the second largest group in most fermented soybeans {98} and they were found to be the dominant organisms in some *doenjang* samples {151}. A typical LAB has been described as a Gram-positive, catalase negative, non-spore forming, devoid of cytochromes, of nonaerobic habitat but aerotolerant, fastidious, acid-tolerant, strictly fermentative organism, with lactic acid as the major end product of sugar fermentation {9}. Lactic acid bacteria belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*,

and include the following families: *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostoceae* and *Streptococceae* {232}. *Bifidobacteria* are not included among the LAB because they have more than 55 % G + C content in their DNA {213}. The LAB of importance in foods belong to the genera: *Aerococcus*, *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* {213}.

Lactic acid bacteria can be categorized into two groups based on their hexose fermentation pathways. Those with a homolactic fermentation use glycolysis resulting in almost exclusively lactic acid as the end product of sugar fermentation under standard conditions {9, 88, 232}. Lactic acid bacteria with heterolactic fermentation use pentose phosphate pathway or 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway and produce significant amounts of other fermentation products such as ethanol, acetic acid and CO_2 in addition to lactic acid {9, 88, 232}.

Glycolysis is characterized by the splitting of fructose-1,6-disphosphate (FDP) with an FDP aldolase into two triose phosphate moieties which are further converted to lactic acid, Fig 5A {88}. Glycolysis occurs in aerococci, enterococci, tetragenococci, vagococci, lactococci, streptococci, pediococci and homofermentative lactobacilli {9, 10, 88}. In obligately homofermentative LAB, sugars only can be fermented by glycolysis {9}. Obligately homofermentative LAB possess a constitutive FDP aldolase and lack phosphoketolase hence they are unable to ferment pentoses and gluconate {9, 88}. Obligately homofermentative lactobacilli such as *Lb. acidophilus, Lb. delbrueckii, Lb. helveticus,* and *Lb. salivarius* are referred to as group I lactobacilli {9}.

Although most species of enterococci, lactococci, pediococci, streptococci, tetragenococci and vagococci are grouped as homofermentative LAB, but they are in fact intermediate regarding fermentation {9}. They possess a constitutive FDP aldolase and use glycolysis for hexose fermentation; at the same time they are able to ferment pentoses via the 6-PG/PK {9, 88}. Therefore, they are homofermentative with respect to hexose fermentation and heterofermentative with respect to fermentation of pentoses and other substrates and are as such more “correctly” called facultatively heterofermentative LAB {9, 88}. However in general terms “homofermentative LAB” refers to those in the group that use the glycolytic pathway for glucose fermentation and “heterofermentative LAB” refers to those that using the

6-PG/PK pathway. Group II lactobacilli includes *Lb. casei, Lb. curvatus, Lb. plantarum, Lb.sakei* among others are falcutative heterofermentative organisms {9, 232}.

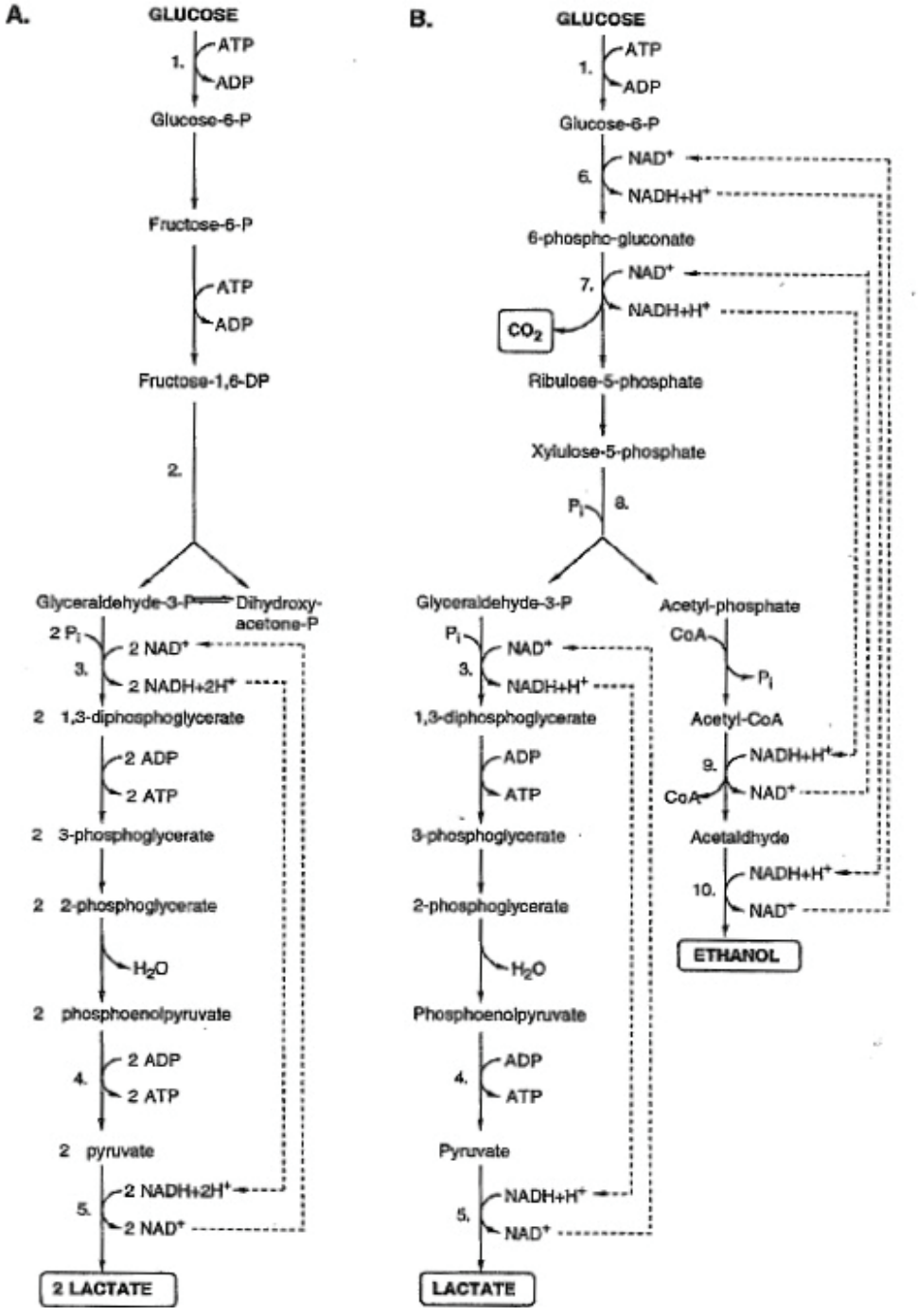

Fig 5. Main pathways of hexose fermentation. (A) Homolactic fermentation (Embden–Meyerhof–Parnas pathway or glycolysis). (B) Heterolactic fermentation (pentose phosphate pathway/6-phosphogluconate/phosphoketolase pathway). Selected enzymes are numbered. 1, Glucokinase; 2, fructose-1,6-diphosphate aldolase; 3, glyceraldehyde-3-phospho dehydrogenase; 4, pyruvate kinase; 5, lactate dehydrogenase; 6, glucose-6-phosphate dehydrogenase; 7, 6-phosphogluconate dehydrogenase; 8, phosphoketolase; 9, acetaldehyde dehydrogenase; 10, alcohol dehydrogenase. Source: Axelsson {9}

Heterofermentation, is initiated by the oxidation of glucose-6-phosphate to gluconate-6-phosphate followed by decarboxylation and splitting of the resulting pentose-5-phosphate into C-2 and C-3 moieties, Fig 5B {88}. The key enzyme for heterofermentation is phosphoketolase, therefore obligately heterofermentative LAB constitutively express this enzyme but lack FDP aldolase {9}. Group III lactobacilli such as *Lb. brevis, Lb. buchneri, Lb. fermentum*, and *Lb. reuteri*, leuconostocs, oenococci, and weissellas are obligately heterofermentative {9, 232}. In heterofermentation, ethanol is produced from acetaldehyde when additional hydrogen acceptors are not available and the ratio acetate/ethanol formed depends on redox potential of the system {88}. In the presence of additional hydrogen acceptors such as O_2 or fructose, O_2 is reduced to H_2O_2 and H_2O while fructose is reduced to mannitol and ethanol is not formed {72, 88, 231}. Although LAB are fermentative, the presence of O_2 as an electron acceptor is often stimulatory to their growth {232}. Oxygen is reduced by the action of NADH oxidase resulting in conversion of acetyl phosphate to acetic acid and formation of an additional ATP {232}. This phenomenon is common among heterofermentative LAB {232}.

Other hexoses such as fructose, mannose and galactose enter the two fermentation pathways at the level of glucose-6-phosphate or fructose-6-phosphate after isomerization and/or phosphorylation {9, 232}. Disaccharides can be split by specific hydrolases into monosaccharides, which then enter the major pathways. Maltose, for instance is phosphorylated by maltose phosphorylase yielding glucose and glucose-1-phosphate {72, 232}. The glucose-1-phosphate is converted to glucose-6-phosphate which can be further metabolized via either glycolysis or 6-PG/PK pathways while the glucose can be phosphorylated by homofermentative LAB or converted to glucose-6-phosphate by heterofermentative LAB and follow their respective pathways {10, 42, 72, 231}. Sucrose is cleaved into fructose and glucose-6-phosphate by sucrose-6-phosphate hydrolase and the two monosaccharide units enter the major pathways {9, 10}. Sucrose also acts as a donor of monosaccharides for dextran or exopolysaccharide formation in certain LAB. Sucrose is cleaved by a cell-wall associated enzyme, dextransucrase, resulting into a glucose moiety that is used for dextran formation while the fructose is fermented in the usual way {9, 232}.

Pentoses in heterofermentative LAB are readily fermented via phosphorylation of the pentose sugar to ribulose-5-phosphate or xylulose-5-phosphate by epimerases or isomerases {9}. The phosphorylated compounds can then be metabolized by the lower part of the 6-PG/PK pathway {9, 88}. Products of pentose fermentation are different from those of glucose

fermentation because there is no CO_2 production and dehydrogenation is not required to produce the intermediate xylulose-5-phosphate {9}. Acetyl phosphate is used by acetate kinase in substrate-level phosphorylation yielding acetate and ATP. The products of this fermentation are equimolar amounts of lactic acid and acetic acid {9}.

2.10.4 Alternative fates of pyruvate

In a lot of fermentations, pyruvate serves as an electron (or hydrogen) acceptor for regeneration of NAD^+ from reduced form NADH necessary for continued fermentation of the cells {9}. As such, pyruvate is not always reduced to lactic acid but may undergo alternative pathways. Alternative pathways of pyruvate may result in a flavor compound such as diacetyl (butter aroma) and flavorless compounds acetoin/2,3-butanediol which are important in fermentation of milk {9, 88}. Other products from pyruvate pathways include acetate, formate, acetyl-coA, ethanol, acetolactate, active acetaldehyde, CO_2 and H_2O_2 {9, 88}. When pyruvate enters alternative pathways, homolactic fermentation may be converted to heterolactic fermentation with acetate, ethanol and formate as end products {88}.

2.10.5 Proteolysis in lactic acid bacteria

Lactic acid bacteria are weakly proteolytic compared to many other bacteria {109}, but they are amino acid auxotrophs requiring preformed amino acids for their growth {105, 109}. The amino acid requirements are strain dependent and can vary from 4 to 14 amino acids {105}. Growth of LAB in milk has been intensively studied and it is concluded that LAB depend on proteolytic systems which allow them to degrade milk proteins {105, 109}. The proteolytic systems are important because they make protein and peptide nitrogen available for microbial growth, and at the same time they play an active role in the maturation processes which give fermented foods their characteristic rheological and organoleptic properties {109}.

LAB proteolytic systems are comprised of three major components: (i) extracellular proteinases that breakdown proteins into oligopeptides, (ii) peptide transport systems that translocate the breakdown products across the cytoplasmic membrane and (iii) various intracellular peptidases that degrade peptides into shorter peptides and amino acids {105, 109, 120}. The amino acids can be further converted into various flavor compounds such as aldehydes, alcohols, acetaldehyde and esters {109, 120}. Proteolysis may also contribute to bitter defects in cheese {109}.

3 OBJECTIVES

3.1 Main objective

To improve soybeans' nutritional quality, utilization and consumer acceptance through natural and lactic acid bacteria fermentations.

3.2 Specific objectives

1. To determine the effects of natural and lactic acid bacteria fermentations of pastes of soybeans and soybean-maize blends on the levels of protein, amino acids, anti-nutritional factors (phytic acid and trypsin inhibitor), organic acids, reducing sugars and enzyme activities. PAPER I
2. To isolate, identify and characterize lactic acid bacteria involved in natural and lactic acid bacteria fermentations of pastes of soybeans and soybean-maize blends using culture-dependent techniques and denaturing gradient gel electrophoresis. PAPER II
3. To determine sensory properties driving consumer acceptance of naturally and lactic acid bacteria fermented pastes of soybeans and soybean-maize blends. PAPER III
4. To assess the effect of natural and lactic acid bacteria fermentations on growth and survival of *Bacillus cereus* and *Escherichia coli* in pastes of soybeans and soybean-maize blends. PAPER IV

3.3 Hypotheses

- There are no significant differences in the effects of natural and lactic acid bacteria fermentations on levels of protein, amino acids, anti – nutritional factors, organic acids, sugars, and enzyme activities in pastes of soybeans and soybean-maize blends.
- There is no difference in microbial composition between naturally fermented and lactic acid bacteria fermented pastes of soybeans and soybean-maize blends.
- There is no significant difference in consumer acceptance between naturally fermented and lactic acid bacteria fermented pastes of soybeans and soybean-maize blends.
- There are no significant differences in the effects of natural and lactic acid bacteria fermentations on growth of *Bacillus cereus* and *Escherichia* coli in pastes of soybeans and soybean-maize blends during fermentation.

4 MAIN RESULTS AND GENERAL DISCUSSION

4.1 Effect of fermentation on nutritional quality of pastes of soybeans and soybean-maize blends

In papers I – III, pastes composed of 100% soybeans, 90% soybeans and 10% maize, and 75% soybeans and 25% maize were naturally fermented (NFP) and lactic acid bacteria fermented (LFP). Lactic acid bacteria fermentations were facilitated through back-slopping using traditional fermented cereal gruel, *thobwa* as an inoculum. Naturally fermented pastes were designated 100S, 90S and 75S, while LFP were designated 100SBS, 90SBS and 75SBS. In paper I, changes in levels of protein, amino acids, enzyme activities and anti-nutritional factors were studied to determine the effects of natural and lactic acid bacteria fermentations on nutritional quality of the fermented pastes. Organic acids and sugars were also studied in paper I but will be discussed in later sections.

Both types of fermentation did not significantly affect total protein content, but significant fluctuations in soluble protein content suggested proteolysis of soybean proteins and subsequent utilization of the hydrolyzed protein by fermenting microorganisms. Highest increase in soluble protein was observed at 48 hrs in all samples except 75SBS; and 100S had the highest (49%) soluble protein increase. After 72 hrs, increase in soluble protein content was observed only in 75S (27%) while there were significant losses in the remaining samples. Proteolytic activities were confirmed by fluctuations in free amino acid contents in most of the 21 free amino acids analyzed during fermentation.

The free amino acids analyzed included cyst(e)in (Cys), methionine (Met), aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), arginine (Arg), glutamine (Gln), asparagine (Asn), citrulline (Cit), γ-aminobutyric acid (GABA), ornithine (Orn) and tryptophan (Trp). Arginine was the most abundant free amino acid at the beginning of the fermentations but it was rapidly depleted during fermentation and its depletion was more pronounced in LFP. Rapid depletion of Arg during fermentation was also reported in *kinema* and was attributed to its preferential uptake by *B. subtilis* as source of nitrogen {190}. In LAB, Arg provides energy via substrate level phosphorylation {32}. Arginine may enter the arginine-deiminase pathway where Arg is converted via Orn to CO_2 and NH_3 which contributes to the acid tolerance of lactobacilli {72}. Glutamate was the only free amino acid that increased throughout fermentation in all

samples and it became the most abundant free amino acid at the end of the fermentations. In *B. subtilis*, Glu is the preferred nitrogen source followed by Arg, and Glu can be synthesized from Arg and Pro {14, 54}. Other amino acids also increased throughout fermentation in some samples and these included Ala (all LFP), GABA and Lys (100SBS and 90SBS) and Asp (90S). Decreases in free amino acids during early fermentation followed by increases during late fermentation were observed in NFP in Ala, Val, Ile, and Leu; in LFP in Asn and Leu; in 100S in Asn and Gly and in 100SBS in Val. Free amino acids that had significantly higher levels at the end of the fermentation than at the beginning were Glu, Ala, Lys in all samples; Leu in NFP; Gln in LFP, 100S and 90S; Thr and GABA in 100SBS and 90SBS; Asn, Cit and Ile in 100S; Gly in 100S, 90S and 100SBS; Phe in 100S and 90S; and Val in 90S and 100SBS. Amino acids contribute to taste and aroma in fermented soybean foods {37}.

Seventeen total amino acids including Cys, Met, Asp, Thr, Ser, Glu, Pro, Gly, Ala, Val, Ile, Leu, Tyr, Phe, His, Lys, and Arg were identified. The sums of the total amino acids were higher in NFP than in LFP except in 75S at 48 hrs and 72 hrs. Slight percent increases in sums of total amino acids were observed in 90SBS at 48 hrs (1.5%) and 72 hrs (1.7%) and in 75SBS at 48 hrs (3.2%). On the other hand, percent reductions were noted at 48 hrs in all NFP (ranging from 0.01% in 90S to 6.8% in 100S) and in 100SBS (7.4%). This could have been due to utilization of the amino acids to sustain higher metabolism rate at this fermentation time since enzyme activities were also highest at this time. Higher total amino acid percent losses were also noted at 72 hrs in 75S (4.7%) and in 100SBS (7.4%).

Nonetheless, significant increases ($p<0.05$) in the sulfur-containing amino acids (Cys and Met) which are limiting in soybeans were observed throughout fermentation in some samples. The increases in NFP with respect to Cys were in100S and 90S and with respect to Met were observed in 90S. In all LFP, Cys increased during 48 hrs of fermentation while this trend was observed with Met in 75SBS. In this study, Glu, Asp, Leu, Arg, Lys, Ser and Phe were considered the main amino acids (> 20 g per kg sample) throughout fermentation. With the exception of Ser and Glu, similar results were reported in other studies {39, 190}.

Alpha-galactosidase activities were highest at 48 hrs in NFP while α-amylase activities were highest at 48 hrs in 75S. Significant increases in α-amylase activities for 90S and α-galactosidase activities for 100S were observed at 24 hrs. High enzyme activities at 48 hrs could suggest that metabolic activities of the fermenting pastes were highest at this fermentation time. On the other hand, LFP had high enzyme activities at the beginning of the

fermentations due to use of malt in back-slopping material, and the enzyme activities declined with fermentation time, although fluctuations were observed in 75SBS and significant increases in α-amylase activities were observed in 90SBS at 72 hrs. The inconsistencies in the trends in enzyme activities in both fermentations underscore one of the limitations of processes based on natural fermentations, which is variability in product quality {78, 186}.

The increases in α-amylase and α-galactosidase activities with fermentation time in NFP could suggest increases in starch hydrolysis and degradation of α-galactooligosaccharides, respectively. Starch hydrolysis would reduce the dietary bulk density and provide the possibility of increasing energy density through use of increased amounts of raw materials {74, 123, 140} but with a product of acceptable consistency. Degradation of α-galactooligosaccharides would reduce flatulence and its related gastrointestinal discomforts {229}and hence could increase consumer acceptance of soybean products.

Both types of fermentation degraded the anti-nutritional factors, phytic acid and trypsin inhibitors (TI). Heat treatment was most effective in deactivating TI {48}, because TI content was 19 mg/g sample in raw soybeans, but after boiling TI could not be detected in 100S while the highest TI at 0 hrs was 0.169 mg/g sample signifying a 99% reduction. Nevertheless, fermentation also hydrolyzed TI because after 72 hrs, TI could not be detected in all samples. On the other hand, natural fermentation was more effective in degrading phytic acid than the LAB fermentation. After 24 hrs fermentation, 33 to 54% reductions in phytic acid were achieved by natural fermentation and by 72 hrs, 85% reduction was noted and in some samples the phytate could not be detected. Whereas, only 18 to 32% reductions were achieved in LFP at 24 hrs and by 72 hrs, 37 to 49% reductions were observed. Variations in the extent of phytic acid degradation were attributed to the type of microorganisms involved in the fermentations, the complexity of the physiological and environmental factors that affect the production and activities of phytases in microorganisms, whether the phytases were intracellular or extracellular and also the specificity of the enzymes with regard to phytic acid {31, 41, 97, 172, 200}.

4.2 Microbial population, diversity and metabolism in natural and lactic acid bacteria fermentations

4.2.1 Microbial population

Lactic acid bacteria involved in natural and LAB accelerated fermentations were characterized using culture-dependent and culture-independent techniques (Paper II). Changes

in microbial populations were investigated on appropriate media. Initial microbial counts of LAB, *Lactobacillus* spp., total aerobic bacteria, spores, yeasts and molds were higher in LFP than in NFP due to the back-slopping. Back-slopping introduced more LAB in the fermenting pastes than the other microorganisms. At 0 hrs, LAB was about 3 $\log_{10}$ cfu/g more in LFP (8.13 – 8.49 $\log_{10}$ cfu/g) than in NFP (4.97 – 5.31 $\log_{10}$ cfu/g), while the other microorganisms were 1 or 2 $\log_{10}$ cfu/g more in LFP. Lactic acid bacteria increased throughout fermentation in NFP while LAB decreased slightly in LFP during 24 – 72 hrs of fermentation from 9.45 – 9.82 $\log_{10}$ cfu/g to 9.24 – 9.33 $\log_{10}$ cfu/g. At the end of the fermentation, LAB population in NFP ranged from 8.71 to 9.62 $\log_{10}$ cfu/g.

Cell counts of LAB, *Lactobacillus* spp., and total aerobic bacteria remained higher in LFP than in NFP during fermentation, while trends in spores, yeasts and mold counts varied. Decreases in spore formers populations between 0 and 24 hrs for all LFP were observed and counts at 72 hrs were lower (5.82 – 6.02 $\log_{10}$ cfu/g) than in all NFP (6.10 – 7.10 $\log_{10}$ cfu/g). Lower spore proliferation rate in LFP was explained in terms of increase in acidity in LFP during 24 hrs of fermentation (pH dropped from 6.4 to 4.0). Spore formers that dominate soybean fermentations are usually *B. subtilis* {39, 187, 188, 194} and they exhibit active growth at pH-range between 5.5 and 8.5 {25}. The growth rates of yeasts and molds were lower in LFP than in NFP between 0 and 24 hrs. Yeast and mold counts increased from 2.0 to 7.0 $\log_{10}$ cfu/g in NFP while the increases were from 3.0 – 4.0 to 4.0 – 4.8 $\log_{10}$ cfu/g in LFP and the differences were significant between these fermentation types. Inhibition of yeast and mold growth could not be explained by increasing acidity alone since yeasts are capable of growing in acidic conditions {177}. Therefore, production of other inhibitory metabolites including CO_2 was suggested.

4.2.2 Microbial composition and diversity in the fermented pastes

A total of 239 Gram-positive and catalase negative isolates were assessed for a number of physiological characteristics which aided in grouping the isolates. Heterofermentative rods able to hydrolyze Arg were dominant (59.4%) followed by heterofermentative rods unable to hydrolyze Arg (23%) and homofermentative cocci that hydrolyzed Arg (14.6%) and lastly homofermentative cocci unable to hydrolyze Arg (2.9%).

Representative isolates 72 (30%) from the groups above were used in further phenotypic characterization. Data obtained from profiling of carbohydrate fermentation by API 50 and API 50 CHL medium, analysis of CO_2 production in MRS broth using an infrared gas

analyzer technique and hydrolysis of Arg was subjected to principal component analysis (PCA) in order to cluster phenotypically similar isolates. Three distinct clusters A, B, and C composed of 4, 8 and 5 species, respectively were obtained. A fourth cluster, D was mostly composed of *Lb. brevis* with diverse sugar fermentation profiles. Principal component 1 (33%) delineated isolates mainly based on fermentation patterns with most of the homofermentative cocci on the positive dimension and heterofermentative rods on the negative dimension.

Heterofermentative rods were further allocated to clusters A and B based on PC 2 (14%). Cluster A was on the positive dimension on which L-arabinose, D-ribose, D-xylose, melibiose, D-raffinose and potassium-5-celuconate loaded highly. Strains in cluster A were typical for obligate heterofermenative *Lactobacillus* spp. fermenting glucose and fructose but unable to ferment amygdaline, mannitol, rhamnose and sorbitol {73} and they included *Lb. brevis, Lb. fermentum, Lb. buchneri,* and *Lb. collonoides.*

Cluster B was dominated by *Weissella confusa* and the identities of other isolates were *Lb. collonoides, Lb. acidophilus, Lb. plantarum, Lb. delbrueckii* subsp. *delbrueckii, Lc. lactis* subsp. *lactis*, *Lb. brevis* and *Leu. mesenteroides*. All isolates fermented glucose, fructose, mannose, esculine and maltose. In addition, the isolates fully or partially fermented potassium gluconate, D-xylose and N-acetylglucosamine.

Cluster D was composed of heterofermentative lactobacilli mostly identified as *Lb. brevis* 1 and they all fermented glucose, fructose, mannose, esculine, cellobiose and maltose. However, they showed wide variations in the fermentation of the other sugars. Two isolates close to cluster D were *Lb. pentosus* and *Lb. plantarum*. The *Lb. pentosus* strain exhibited a similar fermentation profile to the *Lb. plantarum* strain except that the later did not ferment turanose, potassium gluconate and potassium-5-celuconate but fermented starch and melezitose.

Homofermentative cocci (cluster C) were identified as *Pediococcus pentosaceus* and *Lc. lactis* subsp. *lactis* by API and all isolates in this group fermented galactose, glucose, fructose, mannose, N-acetyglucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, trehalose and gentibiose. Five cocci strains could not be identified by API were placed in this cluster. Some lactobacilli clustered with the cocci due to their inability to ferment saccharose and raffinose. Sánchez *et al.* {185} classified isolates as *Lb. brevis* 2 based on their ability to ferment raffinose and saccharose.

Genotypic characterization based on 16*S* rDNA gene using universal primers, 1F and 5R/1492R led to identification of 43 of the 72 isolates as *Lb.* fermentum and *Lb. brevis* (cluster A), *W. confusa* and *W. cibaria* (Cluster B), *P. pentosaseus* (Cluster C) and two isolates from cluster D were identified as *Lb. fermentum*. This study observed mismatches between phenotypic and genotypic characterization which have previously been reported {6, 20, 242}. The mismatches were attributed to poor discriminatory power of API because of atypical fermentation patterns in a number of species. Because most LAB have very similar nutritional requirements and grow under similar environmental conditions {6, 20, 227}. According to Hammes *et al.* {73}, strain-to-strain variability within a species in phenotypic characteristics could be partly explained by encoding of specific properties on plasmids.

Genotyping identified *Bacillus* spp. and *E. faecium*/ *E. durans* in addition to the microorganisms identified by API. *Bacillus* species are important predominant microorganisms that cause proteolysis in alkaline fermented soybeans {1, 38, 39, 98, 174, 194, 230}. *Enterococcus faecium* and *E. durans* have previously been isolated from fermented soybeans {95, 188, 194}.

Denaturing gradient gel electrophoresis, a culture-independent technique was used to determine changes and similarity in microbial diversity during fermentation (Paper II). The V3 region of 16*S* rDNA was amplified using universal primers PRBA338fgc and PRUN518r and the same primer pair but without a GC clamp was used for amplification of excised gel bands prior to sequencing. Denaturing gradient gel electrophoresis revealed microbial succession particularly in NFP in which *Lb. fermentum,* and *Bacillus* spp. were present at the beginning of the fermentations. During 24 hrs of fermentation *Bacillus* spp. disappeared while *W. confusa*/*W. cibaria* and *Lb. linderi* were recovered. By the end of fermentations, *Lb. linderi* had disappeared and *P. pentosaceus* was detected along with *Lb. fermentum* and *W. confusa/W. cibaria*. On the other hand, *W. koreensis, W. confusa/W. cibaria* and *P. pentosaceus* were present from the onset of fermentation in LFP. *Lactobacillus fermentum* was isolated as an additional microorganism after 24 hrs fermentation in LFP, and the microbial composition did not change during further fermentation.

4.2.3 Metabolism of the fermenting microflora

Changes in pH, titratable acidity (Papers I, II and IV), sugars and organic acids (Paper II) gave an insight to the possible metabolism of the microorganisms in the fermenting pastes. Fermentations in NFP and LFP were biased towards lactic acid fermentation as indicated by

the decreases in pH throughout fermentation (Papers I & II). Reductions in pH were more pronounced in LFP than in NFP as evidenced by 28 – 35% reduction in pH in LFP compared to 3 – 12% reduction in pH during 24 hrs. By the end of the fermentations, LFP had undergone 34 to 39% pH reduction while the cumulative percent reduction in pH in NFP ranged from 17 – 23%.

Correspondingly, lactic acid levels were higher in LFP than in NFP and lactic acid was the highest produced organic acid in both types of fermentations. Higher lactic acid production suggested lactic acid as the major end product of fermentation, a characteristic of lactic acid bacteria metabolism {9, 79, 88, 103}. This demonstrated the possibility of soybean solid substrate lactic acid fermentation as opposed to alkaline fermentations that have been intensively investigated. Lactic acid bacteria fermentations were achieved probably because of the fermenting conditions which limited the amount of oxygen {2, 174} since the fermentations were performed in nearly full glass jars that were completely closed.

The production of acetic acid in both NFP and LFP suggested heterolactic fermentation. Heterolactic fermentation results in equimolar amounts of CO_2, lactate, and acetate or ethanol {88}. However, the ratios of lactate/acetate were more than 1 in all fermentations except 90S at 24 hrs. Thus homolactic fermentation also contributed to lactic acid production. Besides, both heterofermentative and homofermentative LAB were isolated in both LFP and NFP. Further, the slower pH reduction and the lower lactic acid production in NFP suggested possibility of co-fermentation with other microorganisms. More acetic acid was produced in 100S and 90S than in 100SBS and 90SBS, suggesting the possibility of *B. subtilis* metabolism in the NFP since acetic acid is a major end product of carbohydrate metabolism in *B. subtilis* {136, 195}. Lastly, the presence of succinic acid confirmed heterofermentation {10}, or the possibility of pyruvate entering alternative pathways {136} which could include the Krebs cycle {19, 195}.

At the beginning of the fermentations, the levels of reducing sugars were higher in LFP due to the back-slopping material which contained some flour from malted grain. Malting increases sugar (glucose, fructose or maltose) content due to amylolytic activities {127}. After 24 hrs fermentation, glucose was nearly used up (from 932 – 1171 mg/kg to 204 – 246 mg/kg) in LFP while pronounced decreases in maltose were observed in 100S (5653 to 402 mg/kg) and in 90S (6250 to 1701 mg/kg). On the other hand, fructose accumulation was observed between 0 and 24 hrs in LFP, 90S and 75S. The rapid depletion of glucose and maltose

coincided with the rapid production of lactic acid by a higher LAB population in LFP. The nearly complete utilization of glucose by 24 hrs could indicate preferential use of glucose as carbon source followed by maltose. The slight accumulations of maltose and glucose in NFP at 24 hrs could have been due to slow metabolism because LAB population was lower. In addition amylase activities could have contributed to the slight accumulation of the sugars. Accumulation of fructose at 24 hrs and its gradual decline during further fermentation in all samples except 100S, suggested fructose was not the preferred carbon source.

At the end of the fermentations, 75SBS had significantly higher fructose content than all samples. Fructose content was also higher in 100SBS and 90SBS than in all NFP. It was suggested that the higher sugar content in LFP was responsible for the browning color in LFP during frying (Appendix 1B) due to Maillard reactions and caramelization.

4.3 Food safety implications of natural and lactic acid bacteria fermentations

Growth of *E. coli* and *B. cereus* in pastes of soybeans and soybean-maize blends during fermentation was investigated (Paper IV). Pastes containing 100% or 90% soybeans were either fermented naturally (100S, 90S) or fermented with *Lb. fermentum* as starter culture (100SC, 90SC) or fermented through back-slopping (100SBS, 90SBS) using a traditional fermented cereal gruel, *thobwa* as an inoculum. A single pathogen was inoculated into the pastes to be fermented naturally, while a single pathogen was simultaneously inoculated with the back-slopping material or with the *Lb. fermentum* starter culture in the other fermentations.

4.3.1 *Escherichia coli* growth during fermentation

In pastes inoculated with *E. coli*, the pH of the back-slopped pastes (BSP) decreased significantly ($p<0.05$) faster than in the other fermentations. After 24 hrs fermentation, pH was 4.44 and 5.0 in 90SBS and 100SBS, respectively, while 90S and 100S had pH values of 6.21 and 6.58, respectively and 90SC and 100SC had pH values of 5.69 and 5.74, respectively. In papers I & II, pH had dropped to 4.36 at 24 hrs and by 72 hrs, the pH had reached 4.01 in 90SBS while in 100SBS, pH dropped to 4.26 by 72 hrs. The results confirmed that back-slopping was still subject to variation in product quality {78}.

Nevertheless, back-slopping significantly inhibited *E. coli* proliferation more than natural and starter culture fermentations. During 24 hrs fermentation, *E. coli* increased from 2.4 to 3.2 – 3.5 $\log_{10}$ cfu/g and remained constant at 3.0 $\log_{10}$ cfu/g during further fermentation. While in

Lb. fermentum fermented pastes (SCP), *E. coli* increased from 2.0 – 2.3 $\log_{10}$ cfu/g to 6.8 – 7.6 $\log_{10}$ cfu/g during 24 hrs and by 72 hrs, the cell count was 7.2 $\log_{10}$ cfu/g. In NFP, *E. coli* increased from 2.3 to 8.8 – 9.2 $\log_{10}$ cfu/g during 24 hrs and by 72 hrs, the count was about 9.1 $\log_{10}$ cfu/g. The relatively faster acidification in BSP compared to SCP and NFP partly explained the higher inhibition of *E. coli* growth. Higher initial LAB populations of 6.0 – 8 $\log_{10}$ cfu/g in BSP and SCP were responsible for comparatively faster acidification in these samples. On the contrary, a low initial LAB count of 2.0 to 2.5 $\log_{10}$ cfu/g was observed in NFP. Although LAB increased to 5.5 – 6.3 $\log_{10}$ cfu/g after 24 hrs fermentation and reached 7.6 – 9.4 $\log_{10}$ cfu/g after 72 hrs, a high initial LAB population is required in order to inhibit enteropathogen growth {183} as observed in BSP and SCP.

The LAB profile of BSP and SCP was almost similar but BSP were more effective in suppressing *E.coli* growth than the single strain culture in SCP. This suggested that the mixture of LAB strains in BSP offered a competitive advantage over the single strain culture in SCP. The results were consistent with Drago *et al.* {45} who reported that mixed lactobacilli strains exerted more inhibitory effect on the growth of *E. coli* and *Salmonella enteritidis* than single culture strains.

4.3.2 *Bacillus cereus* growth and survival during fermentation

In pastes inoculated with *B. cereus*, BSP and SCP showed pH trends similar to those observed in pastes inoculated with *E. coli*. On the other hand, NFP had significantly higher pH values. In 100S, the pH decreased from 6.25 to 6.11 during 48 hrs and thereafter it increased to 6.86. While in 90S, the pH decreased from 6.67 to 5.87 during 24 hrs and it increased to 7.2 after 72 hrs fermentation. The increase in pH may be attributed to *B. cereus* metabolism which is highly proteolytic (like in other *Bacillus* spp.) leading to accumulation of ammonia and subsequent increase in pH {114}.

Higher *B. cereus* population in NFP compared to BSP and SCP also explains the increase in pH in NFP. Viable cells of *B. cereus* in NFP increased from 2.2 to ca. 8.0 $\log_{10}$ cfu/g after 24 hrs and remained relatively unchanged throughout fermentation. On the other hand, *B. cereus* counts decreased from 5.0 $\log_{10}$ cfu/g at 24 hrs to 3.7 $\log_{10}$ cfu/g at 72 hrs in 90SC. This was explained in terms of more acidification due to availability of more fermentable sugars in 90SC than in 100SC. Comparatively, back-slopping resulted in higher *B. cereus* growth inhibition than SCP and NFP. Viable cell counts fluctuated between 2.0 and 3.0 log cfu/g in 100SBS while in 90SBS, the highest counts were 2.4 $\log_{10}$ cfu/g and after 24 hrs, no viable

cells could be detected. This was probably due to high LAB metabolic activities in 90SBS due to presence of more fermentable sugars resulting in more production of metabolites with inhibitory effects.

4.3.3 Preferential pathogen inhibition

It was observed that back-slopping and single strain starter culture fermentations inhibited growth of *B. cereus* more than growth of *E. coli*. However, in this study, only one strain of each pathogen species was tested. Kingamkono *et al.* {101} also observed preferential growth inhibition of *Campylobacter jejuni* over *E. coli* in LAB fermentations. *Bacillus cereus* was also reported more sensitive than enterotoxigenic *E. coli* (ETEC) in *togwa* fermentations {99, 100}. Limited growth inhibition of *E. coli* was attributed to its inducible acid-tolerance response system {100, 117}.

4.3.4 Food safety implications of the types of fermentation

Bacillus cereus causes two types of food poisoning. One is an intoxication called emetic syndrome caused by a preformed heat stable emetic toxin known as cereulide and results in vomiting {24, 49}. The other is an infection requiring live cells or spores to be ingested together with food to cause diarrhea {24}. Heat labile enterotoxins are important in the diarrhea syndrome {24, 59, 68}. The infectious dose in the emetic syndrome is estimated to be around 10^5–10^8 cells/g of ingested food {24, 49, 68, 121}. In the diarrhea syndrome, various infectious dose ranges of $10^4 – 10^9$ cells/g {121}, $10^5 – 10^7$ cells/g {68} and $10^3 – 10^7$ cells ingested {196} have been reported. The critical pH value for *B. cereus* growth inhibition is 5.0 {182, 197} although inhibition at higher pH values of 5.3 to 5.7 have been reported {234}. Cereulide and enterotoxin production are inhibited by low pH values (less than 5.6 in cereulide production) {24}.

Considering that viable cells of *B. cereus* at 72 hrs ranged from 0 to 3 $\log_{10}$ cfu/g in BSP and 3.7 to 5.3 $\log_{10}$ cfu/g in SCP while it was > 5.0 $\log_{10}$ cfu/g in NFP; and pH values at 72 hrs were 5.30 and 5.35 in 90SC and 100SC, respectively; 4.86 and 5.30 in 90SBS and 100SBS, respectively; and 7.2 and 6.86 in 90S and 100S, respectively, BSP could be considered relatively safe with regards to *B. cereus* poisonings. However, because the minimum pH for growth for *B. cereus* is 5.0, then pH values close to 5.0 should be considered borderline.

Escherichia coli is a highly adapted pathogen capable of causing gastroenteritis as well as extraintestinal infections and eight *E. coli* pathovars and their mechanisms of disease have been extensively studied {36, 139}. A diarrhoeagenic *E. coli* strain known as

enteropathogenic *E. coli* (EPEC) is a major cause of infant diarrhea in developing countries {154}. The minimum pH for growth of *E. coli* is 4.4 {15}, although some EHEC strains like *E. coli* O157:H7 have a high degree of acid tolerance {21}. The infectious dose for some Shiga toxin-producing *Escherichia coli* (STEC) strains (O111:H2 and O157:H7) is as low as 1 to 100 cells while it may be higher in some strains {175}. In this study, the final pH values for all fermentations containing *E. coli* were > 4.4 with minimum population of 3 $\log_{10}$ cfu/g in BSP. Thus process optimization of the fermentations would be required to improve safety.

This study confirmed that fermentation alone cannot be considered as a critical control point for elimination of all risks of biological hazards {142}. Thus a combination of prevention of contamination and control through hygiene, fermentation and thermal treatment like ordinary cooking could improve the safety of these foods.

4.4 Sensory properties driving consumer acceptance

In paper III, a study was conducted in order to describe sensory properties of the fermented pastes, to determine consumer acceptance of the pastes and to find out sensory properties that would drive acceptance of the pastes. Ten trained panelists were involved in a descriptive sensory analysis. The fermented pastes (Appendix 1A) were molded and were fried (Appendix 1B) before being presented to the panelists for evaluation. The panelists generated 34 descriptors that were used to rate attributes describing appearance, aroma/odor, taste, and texture. Significant differences were found in 27 descriptors which were used in further analyses.

4.4.1 Sensory properties of the fermented pastes

Principal component analysis was used to determine attributes that characterized the fermented pastes. Principal component analysis categorized the fermented pastes into two groups based on type of fermentation and the differences in the sensory properties were due to type of fermentation. Naturally fermented pastes were characterized by yellow color, fried egg-like appearance, raw soybean odor, fried egg aroma, and higher pH. Sensory properties of LAB fermented pastes included brown color, *chitumbuwa*- and *mandazi*-like appearances (terms and definitions of attributes are explained in Paper III), burnt roasted soybean odor, *chigumuyoyo* and *chitumbuwa* aromas, umami, bitterness, aftertaste, and sourness. High sour intensity was due to high lactic acid and succinic acid contents in LFP. Most amino acids responsible for bitterness and glutamate responsible for umami were higher in NFP than in LFP. However, sensory panelists rated LFP higher in bitterness and with higher umami

intensities than NFP. This contradiction was explained in terms of taste interactions in which glutamate could have suppressed bitterness {119} in NFP and sourness could have enhanced bitterness {145} in LFP.

4.4.2 Consumer acceptance and drivers of liking of the fermented pastes

A total of 150 consumers participated in the consumer acceptance study and 32.4% of the participants had consumed soybean-based products within the past two months from the date of data collection. Soybeans were mostly consumed in porridge (69%), although some of the consumers (17.8%) used texturized soy products locally known as *soya pieces* as relish (side dish), roasted beans as snack, soy flour as a condiment in vegetables and other foods, in addition to using soybeans in porridge.

To understand attributes driving consumer liking, sensory, chemical, and physical data were regressed with consumer acceptance data using partial least square regression analysis. The results indicated that consumers' acceptance was influenced by type of fermentation. Most consumers preferred NFP to LFP. The drivers of liking were identified as strong yellow color, higher pH, fried egg-like appearance and aroma, sweetness, softness, rancid odor, and raw soybean odor. It appeared that the positive impact of higher pH (low sourness intensity), sweetness, and fried egg aroma masked the negative impact of rancid and raw soybean odors. Rancid and raw soybean odors have been documented as deterring consumer acceptance of soybeans {71, 221}. Other consumers preferred LAB fermented pastes which were characterized by brown color, sourness, bitterness, saltiness, umami, burnt roasted soybeans and maize aromas. These attributes were considered drivers of liking of LFP.

Cluster analysis revealed consumer heterogeneity, and four clusters of consumers with the following drivers of liking were identified: Clusters 1 & 3, yellow color, higher pH, raw soybean odor, and fermented aroma; Cluster 2, roasted soybean aroma and *thobwa* aroma; Cluster 4, yellow color, higher pH, raw soybean odor, fermented aroma, sweet taste, fried egg aroma, fried egg-like appearance, rancid odor, and soft texture. The drivers of dislike for most consumers were burnt roasted soybean odor, *chigumuyoyo* aroma, soaked burnt roasted maize aroma, *mandazi* aroma, *chitumbuwa* aroma, *mandazi*- and *chitumbuwa*- like appearances, sourness, umami, bitterness, saltiness, aftertaste and brown color. Umami is the savory taste in fermented soybeans {240}. Its presence among drivers of disliking could have been due to its high correlation with bitterness. Optimization by enhancing drivers of liking while

suppressing the drivers of dislike would improve utilization and acceptance of the fermented pastes of soybeans and soybean-maize blends.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

The following conclusions were drawn from this work:

The study demonstrated the possibility of soybean solid substrate fermentation achieved with lactic acid bacteria naturally present on the raw materials or introduced through back-slopping, although possibility of *B. subtilis* metabolism in addition to LAB was suggested in the case of natural fermentation. The dominant lactic acid bacteria flora in both natural and lactic acid bacteria fermentations were *Weissella cibaria/W. confusa, Lb. fermentum* and *P. pentosaceus*. Denaturing gradient gel electrophoresis revealed succession of microbial communities during natural fermentation in which *Bacillus* spp. and *Lb. linderi* present during early stages of fermentation were succeeded and more acid tolerant organisms like *Lb. fermentum*, *Weissella* spp. and *P. pentosaceus* proliferated and survived during further fermentation. Some species were only recovered by culture-dependent techniques and others were only recovered by DGGE, therefore the use of polyphasic approaches to study microbial diversity in complex systems was recommended.

High organic acid production in lactic acid bacteria fermentations suggested that back-slopping has the potential of producing fermented soybean pastes with reduced risk of foodborne illnesses with regards to *B. cereus*. This conclusion was drawn because of the possibility of reaching pH values of lower than 5.0, a critical value for *B. cereus* growth inhibition. In addition, inhibition of growth of spore formers, yeasts and molds suggested a better microbial safety due to back-slopping. Although soybean pastes naturally fermented with *B. subtilis* have always been considered as safe, failure of both natural and lactic acid bacteria fermentations to reduce pH of the pastes to lower than 4.4, the critical value for *E. coli* growth inhibition, suggested a risk of food poisoning if contamination by *E. coli* should occur during raw material preparation prior to fermentation and even during fermentation under the conditions employed in this study. Therefore application of a thermal processing step like ordinary cooking before consumption was recommended to ensure safety.

Lactic acid bacteria fermentation through back-slopping and natural fermentation improved the nutritional quality of pastes of soybeans and soybean-maize blends through degradation of anti-nutritional factors and increases in soluble protein, increases in some free amino acids and increases in total amino acids including the limiting amino acids cysteine and methionine. The main total amino acids throughout fermentation were Glu, Asp, Leu, Arg, Lys, Ser and

Phe. Proteolytic activities and amino acid metabolism resulted in differences in major free amino acids which were Asp, Glu, Ala, Val, Phe, Leu, and Lys in naturally fermented pastes and Asp, Glu, Gly, Ala, GABA, Leu and Lys in lactic acid bacteria fermented pastes. A comparative advantage of natural fermentation in this study was its higher degradation of phytic acid compared to lactic acid bacteria fermentation.

Sensory properties in the fermented pastes were influenced by type of fermentation and consumers also used type of fermentation to determine their preference patterns. Although there was consumer segmentation, most consumers preferred naturally fermented pastes to lactic acid bacteria fermented pastes. Sensory properties that were drivers of liking included strong intensities of yellow color, pH, sweet taste, egg aroma, egg-like appearance, raw soybean odor, rancid odor, fermented aroma, soft texture and roasted soybean aroma. Therefore, optimization of the desirable properties could increase acceptance of fermented soybean products.

5.2 Future perspectives

Future work should focus on the research gaps that were not addressed in this study and these include:

Identification of other microorganisms (total aerobic bacteria, yeasts and molds and spore formers) that were part of the microflora in the fermented pastes. Next generation sequencing can be used for identification of LAB and other microbial groups in the fermented pastes.

Isolation, identification and characterization of the microflora and its diversity in the fermented cereal gruel, *thobwa*, used as a source of back-slopping material in this study. Although numerous studies have reported that LAB and yeasts are dominant microflora in similar gruels, but geographical influence in microbial diversity has also been reported. Therefore a study in this product is important since the product seems to be a potential source of starter cultures.

Lactic acid bacteria strains that were isolated in this study could be tested for pH lowering ability and could be used in mixed starter culture development studies for fermentation of soybean pastes.

Investigation on technological, functional and sensory properties of *Weissella cibaria/W. confusa, Lb. fermentum* and *P. pentosaceus* strains which were identified as the dominant

species in the microflora, to establish their roles during fermentation and to explore their potential use as starter cultures in fermented pastes of soybeans and soybean-maize blends.

Investigation on the changes in atmospheric gases under the fermenting conditions and the possibility of *B. cereus* enterotoxin or cereulide production during fermentation is recommended.

Investigation on the changes in soy proteins and ammonia production with fermentation time would be important in optimization to overcome the objectionable odor associated with proteolysis of soybean fermentations.

A direct measurement of oligosaccharides should be undertaken to determine the fate of these flatulence causing sugars during natural and lactic acid bacteria fermentations.

To increase utilization of the fermented pastes, optimization is required in which the intensities of drivers of liking would be increased while decreasing the intensities of drivers of disliking.

Determination of volatile compounds associated with soybean aromas/odors would increase an understanding of the consumer preference patterns.

6 APPENDICES

Appendix 1A: Raw fermented pastes of soybeans and soybean-maize blends

Fig. 1: Raw fermented pastes at 72 hrs. A) From left to right: 100S, 90S and 75S; B) From left to right: 100SBS, 90SBS and 75SBS.

Appendix 1B: Fried fermented pastes

Fig. 2: Fried fermented pastes at 72 hrs. A) From left to right: 100S, 90S and 75S; B) From left to right: 100SBS, 90SBS and 75SBS.

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8 ENCLOSED PAPERS I-IV

PAPER I

Food Science & Nutrition

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ORIGINAL RESEARCH

Metabolite changes during natural and lactic acid bacteria fermentations in pastes of soybeans and soybean–maize blends

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Abstract

The effect of natural and lactic acid bacteria (LAB) fermentation processes on metabolite changes in pastes of soybeans and soybean–maize blends was studied. Pastes composed of 100% soybeans, 90% soybeans and 10% maize, and 75% soybeans and 25% maize were naturally fermented (NFP), and were fermented by lactic acid bacteria (LFP). LAB fermentation processes were facilitated through back-slopping using a traditional fermented gruel, *thobwa* as an inoculum. Naturally fermented pastes were designated 100S, 90S, and 75S, while LFP were designated 100SBS, 90SBS, and 75SBS. All samples, except 75SBS, showed highest increase in soluble protein content at 48 h and this was highest in 100S (49%) followed by 90SBS (15%), while increases in 100SBS, 90S, and 75S were about 12%. Significant ($P < 0.05$) increases in total amino acids throughout fermentation were attributed to cysteine in 100S and 90S; and methionine in 100S and 90SBS. A 3.2% increase in sum of total amino acids was observed in 75SBS at 72 h, while decreases up to 7.4% in 100SBS at 48 and 72 h, 6.8% in 100S at 48 h and 4.7% in 75S at 72 h were observed. Increases in free amino acids throughout fermentation were observed in glutamate (NFP and 75SBS), GABA and alanine (LFP). Lactic acid was 2.5- to 3.5-fold higher in LFP than in NFP, and other organic acids detected were acetate and succinate. Maltose levels were the highest among the reducing sugars and were two to four times higher in LFP than in NFP at the beginning of the fermentation, but at 72 h, only fructose levels were significantly ($P < 0.05$) higher in LFP than in NFP. Enzyme activities were higher in LFP at 0 h, but at 72 h, the enzyme activities were higher in NFP. Both fermentation processes improved nutritional quality through increased protein and amino acid solubility and degradation of phytic acid (85% in NFP and 49% in LFP by 72 h).

Introduction

Legumes, cereals, and their blends remain important in the diets of many people in developing countries. Legumes are the main source of protein because animal proteins are expensive. Soybeans contain up to 40% protein (Redondo-Cuenca et al. 2007) and when consumed together with maize, they provide a high-quality protein diet comparable to animal protein (Asgar et al. 2010). Soybeans and maize complement each other in terms of limiting amino acids. Cereals are deficient in lysine, but are rich in cysteine and methionine, whereas legumes are rich in lysine, but deficient in the sulfur-containing amino acids (Palanisamy et al. 2012). Therefore, by combining cereals with legumes, the overall protein quality of the diet is improved.

However, the biological utilization of nutrients from legumes is affected by the presence of antinutritional

factors. Cereals, legumes, and their blends contain phytic acid, trypsin inhibitors, polyphenols, and flatulence causing oligosaccharides such as raffinose and stachyose (Mulimani and Devendra 1998; Sindhu and Khetarpaul 2001; Yoon and Hwang 2008). Trypsin inhibitor reduces digestibility of proteins by inhibiting protease activity of trypsin enzyme (Sindhu and Khetarpaul 2001), while α-galactosides (raffinose and stacchyose) are broken down by intestinal anaerobic microorganisms causing flatulence (Vidal-Valverde et al. 1993). Phytic acid forms complexes with proteins and minerals such as calcium, iron, magnesium, and zinc reducing their biological availability (Yoon et al. 1983; Chitra et al. 1996; Urbano et al. 2000). The presence of antinutritional factors along with disagreeable beany flavor has limited the consumption of soybean as a raw material (Wang et al. 2003). Several processing methods including fermentation reduce levels of antinutritional factors and hence they improve the nutritive value of processed foods (Golbitz 1995; Chitra et al. 1996; Wang and Murphy 1996; Palanisamy et al. 2012). Fermentation also improves flavors and textures of legumes (Deshpande and Salunkhe 2000) and other fermented products in general.

In Malawi, utilization of soybeans is limited to maize–soybean composite flour locally known as *likuni phala* which is used as a weaning food (Kalimbira et al. 2004; Maleta 2006). In an effort to increase utilization and consumption of soybeans by all age groups, solid state fermented pastes of soybeans and soybean–maize blends to be used as a side dish or meat alternative were developed (Ng'ong'ola-Manani et al. 2014). Many studies on solid state fermentation of soybeans and legumes have focused on natural fermentation which favors growth of *Bacillus subtilis* or molds. *Bacillus*-fermented soybean products include soy-*dawadawa* (Dakwa et al. 2005), Nepalese *kinema* (Sarkar and Tamang 1995), Japanese *natto*, Thai *thua-nao* (Dajanta et al. 2012), and Korean *doenjang* (Kim et al. 2010). The main metabolic activity of *B. subtilis* is proteolysis of proteins into amino acids and subsequent production of ammonia (Sarkar and Tamang 1995; Dakwa et al. 2005). High amount of ammonia in the fermented product results in a strong odor which some people find objectionable (Allagheny et al. 1996; Parkouda et al. 2009). On the other hand, lactic acid fermentation processes improve texture, flavor, and shelf life of traditional foods (Steinkraus 1997).

Cereal gruels such as *ogi*, *koko*, *kenkey*, and *mahewu* made from maize and/or sorghum (Sanni 1993), *bushera* from sorghum and millet (Muyanja et al. 2003), *bensaalga* from pearl millet (Songré-Ouattara et al. 2008), and *togwa* from cassava, maize, sorghum, millet, or their blends (Mugula et al. 2003) are fermented by LAB. Like *B. subtilis*, some LAB degrade antinutritional factors like trypsin inhibitor, phytic acid, raffinose, and stachyose (Holzapfel 1997, 2002; Sindhu and Khetarpaul 2001). An additional advantage of lactic acid fermentation is the possibility of involvement of LAB with potential probiotic characteristics (Sindhu and Khetarpaul 2001) in addition to increased safety of the product. In this study, *thobwa*, a Malawian fermented cereal gruel prepared from maize flour and cofermented with malt flour from finger millet was used as a back-slopping material to facilitate LAB fermentation processes in LAB-fermented pastes (LFP).

Lactic acid bacteria (LAB)-fermented pastes were characterized by brown color, sourness, bitterness, saltiness, umami, burnt roasted soybean aroma, and maize aroma (Ng'ong'ola-Manani et al. 2014). Sensory properties that characterized naturally fermented pastes included higher pH, yellow color, fried egg-like appearance and aroma, sweetness, softness, roasted soybean aroma, rancid odor, and raw soybean odor (Ng'ong'ola-Manani et al. 2014). There was consumer segmentation in preference patterns of the fermented pastes and liking was biased toward naturally fermented pastes (Ng'ong'ola-Manani et al. 2014).

The fermented pastes were developed to serve as major sources of protein in maize-based diets, and a report on proximate composition of the pastes would give important nutrition information. Therefore, this study aimed at reporting and comparing metabolites and metabolite changes in pastes of soybeans and soybean–maize blends fermented naturally and by LAB. Particularly, changes in proteins, amino acids, organic acids, sugars, antinutritional factors, and enzyme activities during fermentation were investigated.

Materials and Methods

Preparation of pastes of soybeans and soybean–maize blends

Pastes of soybeans and soybean–maize blends were prepared in the laboratory according to Ng'ong'ola-Manani et al. (2014). Portions of 500 g pastes of soybeans and soybean–maize blends were naturally fermented or LAB fermented through back-slopping using *thobwa*. *Thobwa* was produced by making maize porridge containing 15% (w/v) maize flour and 80% water according to the protocol for *togwa* processed in the southern part of Tanzania (Kitabatake et al. 2003). The porridge was cooled to about 50–60°C before the addition of finger millet (*Eleusine coracana*) malt flour (5%, w/v). The porridge was left to ferment naturally at room temperature (23–28°C) for 18 h before being used as inocula in back-slopped samples. The quality of the *thobwa* was determined through monitoring continuous pH reduction during 18 h of *thobwa* fermentation. The LFP were back slopped with

10% (v/w) of the *thobwa*. The pH of the *thobwa* was around 4.5 with a LAB population of 10^8 cfu/mL.

Naturally fermented pastes (NFP) were designated as 100S, 90S, and 75S according to 100%, 90%, and 75% soybean composition in the pastes, the remaining proportions being maize. Similarly, back-slopped LFP were designated as 100SBS, 90SBS, and 75SBS. All treatments were fermented at 30°C for 72 h. The fermenting pastes were sampled at 0, 24, 48, and 72 h and samples were frozen at −20°C until analysis. Analyses were made from three independent experiments except in amino acids, organic acids, and sugars in which analyses were made from two experiments.

pH, titratable acidity, moisture content, and protein determination

AOAC (1990) methods were used to determine moisture content, pH, and titratable acidity. The pH was measured using a pH meter (WTW pH 525; D. Jurgens and Co., Bremen, Germany) fitted with a glass electrode (WTW SenTix 97T). Total proteins and water-soluble proteins were analyzed as total nitrogen and water soluble nitrogen, respectively by the Kjeldahl method according to Thiex et al. (2002). For total protein, samples were ground in a mortar with a pestle until they turned fine and homogenous, and 0.5 g of the sample was transferred into a digestion flask where 0.8 g $CuSO_4$, 7.0 g of K_2SO_4, and 15 mL H_2SO_4 (98%) were added. The digestion was done on a Labconco microKjeldahl digestor (Model 60300-00; Kansas City) for 3 h. The digested material was distilled using a Kjeltec System 1002 distillation unit (Tecator, Hoganas, Sweden) with 4% boric acid containing a mixed indicator in the receiving flask. Samples for determination of water soluble nitrogen were prepared according to Sarkar and Tamang (1995) by homogenizing 2.0 g of sample with 100 mL of distilled water for 2 min in a Star Lab blender LB 400 (VWR, Fontenay Sous Bois Cedex, France) and centrifuging at 3500*g* for 10 min at 25°C. The supernatant was filtered through a Whatman No. 2 filter paper and the nitrogen content of a known volume was determined by the Kjeldahl method. A conversion factor of 6.25 was used to obtain percentage of protein (Dajanta et al. 2012).

Enzyme activities

Preparation of enzyme extract

Enzyme extracts of the fermenting pastes were prepared according to Dakwa et al. (2005) and Terlabie et al. (2006). Five grams of the sample was ground in 50 mL of 0.1 mol/L potassium hydrogen phosphate (Merck, KGaA, Damstadt, Germany) buffer, pH 6.5 as the extracting buffer. The suspension was washed with petroleum ether (Sigma-Aldrich, St Louis, MO) to extract the oil. The sample was centrifuged (Kokusan H-201 series; Kokusan Enshinki Co. Ltd., Tokyo, Japan) at 3500*g* for 5 min at 4°C. The supernatant constituting the crude enzyme was stored at −20°C until analysis.

Determination of α-amylase and α-galactosidase activities in fermenting pastes

Alpha-amylase activities were determined by the assay method of Bernfeld (1955). Two milliliters of the enzyme extract was mixed with 1 mL of 1% (w/v) starch (Merck) solution and was incubated for 1 h at 40°C. The reaction was stopped by adding 3 mL of dinitrosalisylic acid reagent (DNS; Alfa Aesar, Karlsruhe, Germany) before heating for 5 min. After cooling, the sample mixture was diluted with 18 mL of distilled water and the optical density was measured at 550 nm in a spectrophotometer (Jenway 6300; Bibby Scientific, Staffordshire, UK). A blank was prepared by adding DNS before the starch solution. The amount of reducing sugars formed was calculated from a standard curve prepared with known concentrations of maltose (Merck) according to Bernfeld (1955).

Alpha-galactosidase activities were determined according to Odunfa (1983). About 2 mL of the enzyme extract was mixed with 1 mL of 1% (w/v) melibiose monohydrate (Merck) solution before incubation for 2 h at 40°C. The reaction was stopped by adding 3 mL of DNS (Alfa Aesar) before boiling in a water bath for 5 min. The subsequent steps proceeded as in alpha-amylase determination.

Amino acids

Total amino acids

Total amino acids were determined according to the method of Official Journal of the European Communities (1998). Amino acids were extracted from a weighed (116.5–190.2 mg) well homogenized freeze-dried sample. A closed hydrolysis was done to extract the amino acids, and the procedure for hydrolysis was amino acid dependent. For instance, cysteine and methionine were oxidized to cysteic acid and methionine sulfone, respectively, prior to hydrolysis. Asparagine and glutamine were converted to aspartic acid and glutamic acid before hydrolysis, while tyrosine was analyzed separately from the rest of the amino acids using basic hydrolysis and high-performance liquid chromatography (HPLC)/fluorescence detection. Different optimal times for hydrolysis for each amino acid were used. The pH of the hydrolysates was adjusted to 2.20 using an autotitrator. The hydrolysates were then

run on a Biochrom 30 amino acid analyzer (Biochrom Co, Cambridge, UK), equipped with a sodium high-performance oxidized column (Biochrom). The UV-signals were read after postcolumn derivatization with ninhydrin at 570 and 440 nm using Chromeleon software (Dionex, Sunnyvale, CA). Cysteic acid, methionine sulfone, lysine, threonine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, tyrosine, and valine standards were used in the analysis and were obtained from Sigma-Aldrich.

Free amino acids

Free amino acids were extracted from a 1.00 g freeze-dried homogenized sample which was weighed into a 15-mL centrifuge tube. To each sample, 5 mL of 0.1 mol/L HCl standard solution containing 0.4 μmol/mL L-norvaline and piperidine-4-carboxylic acid was added. The sample and the standard solution were thoroughly mixed on a vortex. The sample mixture was put on ultra sound water bath (Branson 2510, Soest, Netherland) at room temperature for 30 min. Sonication was followed by centrifugation at 3000*g* (Beckman J2-MC; GMI Inc, Ramsey, MN) for 40 min at 4°C. From the supernatant, 1 mL of extract was transferred into a 2-mL Eppendorf tube to which 1 mL of 4% trichloroacetic acid (Merck) was added. The rest of the procedure was done according to Bütikofer and Ardö (1999).

Organic acids and sugars

To 1.0 g of freeze-dried homogenized sample, 5 mL of milliQ water was added and mixed thoroughly. Then 1.00 g of the sample mixture was transferred to another tube to which 2.5 mL of milliQ water, followed by 0.2 mL of 0.5 mol/L H_2SO_4 (Merck) and 8 mL of acetonitrile (Merck) were added. Mixing was done for 30 min on a MultiRS-60 BIOSAN rotator (Nerlien, Oslo, Norway). The rest of the procedure was done according to Narvhus et al. (1998). Organic acids, glucose, fructose, and maltose levels were analyzed by HPLC. The organic acids were detected with a UV detector set at 210 nm and the sugars were determined using a refractive index detector (Perkin Elmer series 200, Norwalk, CT). Organic acids were identified based on comparison of their retention times with standard solutions of citrate, orotic acid, pyruvate, succinate, DL-lactate, uric acid, DL-pyroglutamate, propionate, α-ketoglutaric acid, oxalic acid, acetate, and formate (Merck). Identification of sugars was also based on retention times of standard solutions of maltose, lactose, galactose, fructose, and glucose (Merck). Quantification was done using external calibration curves of mixed standards in deionized water.

Antinutritional factors (phytic acid and trypsin inhibitor)

Phytic acid was extracted from 0.5 g samples in 25 mL of 0.2 N HCl for 3 h with continuous shaking, according to Erdal et al. (2002). The extracts were centrifuged at 3500*g* for 10 min at 4°C and the supernatants were used for analysis. The extracted phytate was assayed according to the method described by Haug and Lantzsch (1983). Trypsin inhibitors were measured by the method of Kakade et al. (1974) as modified by Hamerstrand et al. (1980).

Statistical analysis

Analysis of variance (ANOVA) at $P = 0.05$ was performed in SPSS 15.0 (SPSS Inc., Chicago, IL) and least squares difference test was used to separate means.

Results and Discussion

Proximate composition

The initial pH and titratable acidity were almost the same for all samples, despite LFP being inoculated with a LAB-fermented product (Table 1). The pH for LFP decreased faster than for NFP. The relatively fast drop in pH as in LFP to about 4.0 at 24 h would be desirable to prevent growth of pathogens and spoilage bacteria. The slow drop in pH in NFP indicated cofermentation by LAB and other microorganisms. Nevertheless, the gradual decline in pH in NFP suggested a bias toward LAB fermentation as opposed to alkaline fermentation, reported in natural fermentation processes of soybeans (Sarkar et al. 1994, 2002; Dakwa et al. 2005; Parkouda et al. 2009; Dajanta et al. 2011). The lactic acid fermentation could be attributed to limited oxygen during fermentation in the jars which could have favored growth of microaerophiles while limiting growth of spore formers, eventually reducing ammonia production with no increase in pH (Allagheny et al. 1996; Parkouda et al. 2009). Significant increases in the amount of titratable acidity were observed in all samples (except in 100S) from 0 to 24 h (Table 1) and thereafter continuous increases throughout fermentation were observed, although some of them were not significant. Continuous increases in titratable acidity in alkaline fermentation processes have been reported previously (Sarkar and Tamang 1995).

Moisture content was not affected by fermentation time except in 100S where significant differences were observed between early stages and late stages of fermentation. In 90SBS, differences were observed between 0 and 48 h (Table 1). During fermentation of *kinema*, no appreciable

Table 1. Changes in pH, acidity, moisture content, protein content, and enzyme activities of the pastes during fermentation.

Parameter	Treatment	0 h	24 h	48 h	72 h
pH	100S	6.95 ± 0.13^{a}	6.74 ± 0.20^{a}	5.93 ± 0.50^{b}	5.81 ± 0.59^{b}
	90S	6.98 ± 0.16^{a}	6.15 ± 0.25^{b}	5.80 ± 0.20^{c}	5.36 ± 0.14^{d}
	75S	6.88 ± 0.14^{a}	6.61 ± 0.32^{a}	6.09 ± 0.27^{b}	5.41 ± 0.18^{c}
	100SBS	6.46 ± 0.57^{a}	4.64 ± 0.37^{b}	4.47 ± 0.34^{b}	4.26 ± 0.28^{b}
	90SBS	6.45 ± 0.48^{a}	4.36 ± 0.20^{b}	4.11 ± 0.36^{b}	4.01 ± 0.31^{b}
	75SBS	6.44 ± 0.40^{a}	4.20 ± 0.24^{b}	4.02 ± 0.39^{b}	3.91 ± 0.29^{b}
Titratable acidity (g lactic acid/100 g sample)	100S	0.10 ± 0.05^{a}	0.16 ± 0.03^{a}	0.40 ± 0.23^{b}	0.58 ± 0.31^{b}
	90S	0.10 ± 0.03^{a}	0.25 ± 0.05^{b}	0.28 ± 0.12^{bc}	0.37 ± 0.08^{c}
	75S	0.09 ± 0.02^{a}	0.17 ± 0.07^{ab}	0.27 ± 0.14^{b}	0.50 ± 0.18^{c}
	100SBS	0.16 ± 0.09^{a}	0.44 ± 0.16^{b}	0.48 ± 0.13^{b}	0.56 ± 0.13^{b}
	90SBS	0.16 ± 0.09^{a}	0.46 ± 0.13^{bc}	0.57 ± 0.12^{cd}	0.68 ± 0.16^{d}
	75SBS	0.20 ± 0.09^{a}	0.53 ± 0.15^{bc}	0.64 ± 0.18^{cd}	0.85 ± 0.24^{c}
Moisture (%)	100S	68.20 ± 3.94^{a}	68.06 ± 2.87^{a}	71.01 ± 0.92^{b}	71.05 ± 1.98^{b}
	90S	69.12 ± 3.49^{a}	68.66 ± 3.24^{a}	70.19 ± 1.02^{a}	69.89 ± 1.23^{a}
	75S	66.87 ± 2.04^{a}	66.10 ± 4.24^{a}	66.40 ± 2.98^{a}	66.59 ± 3.89^{a}
	100SBS	71.40 ± 4.57^{a}	70.99 ± 4.56^{a}	70.01 ± 1.44^{a}	70.74 ± 1.30^{a}
	90SBS	68.76 ± 3.05^{a}	70.01 ± 5.45^{ab}	72.28 ± 1.64^{b}	70.56 ± 1.62^{ab}
	75SBS	67.70 ± 4.36^{a}	67.80 ± 4.32^{a}	67.36 ± 3.29^{a}	67.95 ± 2.83^{a}
Total protein (%)	100S	43.94 ± 5.38^{a}	39.74 ± 6.11^{a}	42.83 ± 5.45^{a}	44.74 ± 3.42^{a}
	90S	40.15 ± 3.50^{a}	36.67 ± 2.71^{a}	39.16 ± 2.38^{a}	39.27 ± 4.82^{a}
	75S	26.36 ± 5.0^{a}	27.77 ± 1.97^{a}	26.42 ± 2.72^{a}	27.99 ± 1.44^{a}
	100SBS	42.47 ± 4.96^{a}	42.19 ± 6.44^{a}	41.82 ± 4.40^{a}	34.82 ± 1.53^{a}
	90SBS	33.65 ± 6.68^{a}	29.55 ± 4.22^{a}	35.66 ± 6.63^{a}	36.52 ± 2.58^{a}
	75SBS	26.15 ± 5.22^{a}	27.11 ± 4.69^{a}	28.10 ± 5.92^{a}	24.99 ± 5.16^{a}
Soluble protein (%)	100S	9.72 ± 1.18^{a}	8.52 ± 1.38^{a}	14.49 ± 2.38^{b}	8.14 ± 5.56^{a}
	90S	11.48 ± 3.01^{a}	11.35 ± 4.87^{a}	12.85 ± 2.81^{a}	10.84 ± 3.68^{a}
	75S	8.80 ± 1.10^{a}	10.37 ± 2.26^{a}	9.92 ± 1.72^{a}	11.21 ± 1.36^{a}
	100SBS	10.82 ± 2.44^{ab}	11.00 ± 4.62^{b}	12.12 ± 3.74^{c}	8.28 ± 0.69^{a}
	90SBS	9.45 ± 1.61^{a}	8.84 ± 3.09^{a}	10.90 ± 3.48^{a}	8.85 ± 1.47^{a}
	75SBS	9.64 ± 1.82^{a}	9.81 ± 2.92^{a}	6.96 ± 1.60^{b}	7.19 ± 2.20^{b}
α-Amylase (mg maltose/mL)	100S	0.41 ± 0.15^{a}	0.43 ± 0.16^{a}	1.29 ± 0.14^{b}	1.07 ± 0.56^{ab}
	90S	0.37 ± 0.24^{a}	0.72 ± 0.41^{b}	0.55 ± 0.32^{ab}	1.05 ± 0.33^{b}
	75S	0.83 ± 0.39^{a}	0.70 ± 0.34^{a}	2.20 ± 1.17^{b}	1.52 ± 0.39^{c}
	100SBS	0.77 ± 0.31^{a}	0.58 ± 0.24^{ab}	0.37 ± 0.11^{b}	0.30 ± 0.24^{b}
	90SBS	0.93 ± 0.37^{ab}	0.74 ± 0.55^{a}	0.66 ± 0.21^{a}	1.57 ± 0.66^{b}
	75SBS	1.30 ± 0.86^{ab}	0.98 ± 0.35^{ac}	2.01 ± 1.45^{b}	0.56 ± 0.30^{c}
α-Galactosidase (mg maltose/mL)	100S	0.39 ± 0.22^{a}	1.06 ± 0.22^{b}	1.61 ± 0.87^{b}	1.83 ± 0.82^{b}
	90S	0.77 ± 0.36^{a}	0.99 ± 0.58^{ab}	1.39 ± 0.64^{b}	1.13 ± 0.55^{b}
	75S	1.32 ± 0.71^{a}	1.18 ± 0.42^{a}	2.48 ± 0.91^{b}	1.16 ± 0.69^{a}
	100SBS	1.61 ± 0.72^{a}	1.21 ± 0.29^{ab}	1.21 ± 0.50^{ab}	0.90 ± 0.62^{b}
	90SBS	1.12 ± 0.46^{ab}	1.48 ± 0.59^{b}	1.04 ± 0.72^{a}	0.99 ± 0.38^{a}
	75SBS	1.58 ± 0.29^{a}	1.02 ± 0.39^{ab}	1.75 ± 0.20^{a}	0.50 ± 0.12^{b}

Means in a row not sharing superscripts are significantly different ($P < 0.05$). Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

changes in moisture content were reported (Sarkar and Tamang 1995; Yang et al. 2011). Samples in this study had higher moisture content than in *kinema* (Yang et al. 2011).

Composition of the samples influenced the amount of total protein, with an increased amount of maize resulting in a reduced amount of total protein. Fermentation time had no significant influence on the amount of total protein in all the treatments, although fluctuations were observed (Table 1). Other studies in fermentation of soybeans, pearl millet, and maize concluded that fermentation does not seem to be a viable means of increasing total protein content (Khetarpaul and Chauhan 1989; Mohiedeen et al. 2010; Yang et al. 2011) because no significant changes were observed. Khetarpaul and Chauhan (1989) and Visessanguan et al. (2005) suggested that

decreases in protein content were due to protein degradation by proteolytic activities of microorganisms, while Mohiedeen et al. (2010) attributed the slight gains to protein synthesis during microbial growth.

Nevertheless, there were significant changes ($P < 0.05$) in total soluble protein content at 48 h in 100S (Table 1) and from 24 to 48 h in 100SBS. At 24 h, soluble protein content of 75S increased by 17% and slight increases were observed in 100SBS and 75SBS. On the other hand, 100S had the highest percentage loss (12%) amongst all samples at 24 h but had the highest percentage gain at 48 h. In fact, net increases in soluble protein content from the initial were highest at 48 h and all samples showed soluble protein gains at this time except in 75SBS in which a 27% loss was observed. The highest soluble protein increase was in 100S (49%), followed by 90SBS (15%), while the increases in 100SBS, 90S, and 75S were about 12%. At 72 h, net gain from the initial was only observed in 75S (27%) while soluble protein losses were observed in all the remaining samples. In fermentation of soybeans to produce *kinema*, Sarkar and Tamang (1995) reported a 47% increase in soluble nitrogen between 6 and 9 h of fermentation. Visessanguan et al. (2005) attributed the increases in soluble nitrogen and free amino acids to hydrolysis of soy proteins and suggested the presence of proteolytic activity during fermentation. Sripriya et al. (1997) reported a 10-fold increase in soluble protein during fermentation of finger millet. They attributed the increases to microbial enzyme activity and protein hydrolysis. Increase in soluble protein improves digestibility of soybeans by increasing the amount of protein that could be readily absorbed.

At the beginning of fermentation, higher enzyme activities were observed in LFP because of the back-slopping material, which was made by adding finger millet malt flour to a maize porridge. Malting or sprouting increases activities of starch hydrolyzing enzymes (amylase activities) and galactosidases (Malleshi et al. 1986). At the beginning of fermentation, α-amylase activities were significantly higher in LFPs and 75S than in 100S and 90S (Table 1). After 24 h, different trends in enzyme activities were observed according to the type of fermentation and composition of the paste. There was a lag phase in 100S before a significant increase of α-amylase activity was observed at 48 h. Fluctuations were observed in 90S with significant increases at 24 and 72 h and a significant decrease at 48 h. Trends in α-amylase activities were similar in 75S and 75SBS; decreases at 24 h were followed by significant increases at 48 h which were followed by decreases. The α-amylase activities in these two samples were comparatively higher probably because of the higher starch content. There were continuous decreases in α-amylase and α-galactosidase activities in 100SBS throughout fermentation, while continuous increases in α-amylase and α-galactosidase activities in 100S were observed except at 72 h where a decrease in α-amylase was seen (Table 1). The α-galactosidase activities significantly increased at 48 h in NFP, while in 90SBS, though not significant, an increase was noticed at 24 h. With the exception of the α-amylase activity of 90SBS, α-amylase and α-galactosidase activities were higher in NFP than in LFP at 72 h.

Production of α-amylase and α-galactosidase by *Lactobacillus fermentum* and *Lactobacillus planturum* (Songré-Ouattara et al. 2008) has been documented. In this study, *Lb. fermentum* was among the dominant LAB microflora involved in fermentation (data not shown). In *B. subtilis*-dominated fermentation, increases in α-amylase activities (Dakwa et al. 2005; Terlabie et al. 2006) and degradation of oligosaccharides (Sarkar et al. 1997a) were reported. The importance of high amylase activities and their starch-hydrolyzing capacity in cereal and legume-based foods is the possibility of increasing energy density in fermented foods since dietary bulkiness is reduced and hence more raw material can be used (Mosha and Svanberg 1983; Hansen et al. 1989; Lorri and Svanberg 1993). This could eventually address low energy and nutrient density intake, a nutritional problem recognized in most African countries (Lorri and Svanberg 1993; Maleta 2006). The main oligosaccharides in mature soybeans are stachyose (14–41 g/kg dry weight) and raffinose (1–9 g/kg dry weight). These two flatulence-causing sugars contain both β-fructosidic and α-galactosidic linkages (Lan et al. 2007). Mammals do not synthesize α-galactosidase enzymes required to hydrolyze α-galactosidic linkages (Medic et al. 2014). Therefore, increases in α-galactosidase activities could imply a possible degradation of flatulence-causing oligosaccharides. This could in turn improve the acceptance and utilization of soybeans.

Amino acids

Seventeen total amino acids including cyst(e)ine (Cys), methionine (Met), aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), lysine (Lys), and arginine (Arg) were identified. Fluctuations in amino acids were observed and in most cases the changes were not significant (Table 2). Significant increases ($P < 0.05$) throughout fermentation were only observed in Cys in 100S and 90S, and Met in 90S, while significant increases during 48 h of fermentation were observed in Cys in all LFP and in Met in 75SBS. Significant increases at 48 h followed by decreases at 72 h were observed in Cys in LFP and in Met, Asp, Ser, and Arg in 75SBS. In 100S, significant decreases at 48 h in

Asp, Glu, Pro, Val, Phe, His, Arg were observed, and levels of many amino acids decreased in 100SBS at 48 h.

The sums of the total amino acids were higher in NFP than in LFP except in 75S at 48 and 72 h. Although not significant, slight percent increases in sums of total amino acids were observed in 90SBS and 75SBS (48 h). At 48 and 72 h, 90SBS showed 1.5% and 1.7% increases, respectively. In 75SBS, a 3.2% increase was noted at 48 h. On the other hand, reductions were noted in all NFP at 48 h (from 0.01% in 90S to 6.8% in 100S) and in 100SBS. Higher decreases were noted at 72 h in 75S (4.7%), at 48 h in 100S (6.8%), and at 48 and 72 h in 100SBS (7.4%). In 75S, there were decreases throughout fermentation.

In all samples, Glu was the most abundant amino acid followed by Asp, while Cys and Met were the limiting amino acids. Similar results were reported in fermentation of *kinema* by *B. subtilis* (Sarkar et al. 1997b). In fermentation of *doenjang* by *B. subtilis*, increases in Leu, Phe, Lys, and Ala were up to three times greater after 40 and 100 days of fermentation than the initial levels (Namgung et al. 2010). In *cheonggukjang* fermented for 3 days with *Bacillus* spp., total amino acids significantly ($P < 0.05$) increased between 24 and 48 h (Baek et al. 2010). In their study, Baek et al. (2010) identified Ala, Glu, Phe, and Trp as major amino acids (above one related peak area) during the initial stages of fermentation. In this study, Glu, Asp, Leu, Arg, Lys, Ser, and Phe were considered the main amino acids (>20 g per kg sample) throughout fermentation. In *kinema*, Glu, Asp, Leu, Arg and Lys were major amino acids (Sarkar et al. 1997b), while in *soy-dawadawa*, Glu and Ser were not among the major amino acids (Dakwa et al. 2005).

A total of 21 free amino acids (Table 3) including cyst(e)ine, methionine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, glutamine (Gln), asparagine (Asn), citrulline (Cit), γ-aminobutyric acid (GABA), ornithine (Orn), and tryptophan (Trp) were identified. Fluctuations in free amino acids were also observed. The fluctuations reflected the conversion of peptides to free amino acids and the subsequent utilization of these amino acids. Peptide conversion into free amino acids is a central metabolic activity in LAB (Christensen et al. 1999). Increases throughout fermentation were observed in Glu (all samples), Ala (all LFP), GABA and Lys (100SBS and 90SBS) and Asp (90S). Decreases at 24 h followed by increases at 48 and 72 h were observed in NFP in Ala, Val, Ile, and Leu; and in LFP in Asn and Leu. These changes were also observed in 100S in Asn and Gly and in 100SBS in Val. At the end of the fermentation, the following amino acids were significantly higher than at the beginning of the fermentation: Glu, Ala, Lys in all samples; Leu in NFP; Gln in LFP, 100S and 90S; Thr and GABA in 100SBS and 90SBS; Asn, Cit, and Ile in 100S; Gly in 100S, 90S, and 100SBS; Phe in 100S and 90S; and Val in 90S and 100SBS (Table 3). Sarkar et al. (1997b) reported significant increases in free amino acids during 48 h of fermentation in *kinema*. They also reported net decreases in some amino acids and suggested that the amino acids were metabolized to a greater extent than they were replaced by proteolytic activities. In *cheonggukjang* fermented for 2–3 days, fluctuations in amino acids were also observed and the levels of most amino acids decreased in the early stages of fermentation and increased in the late stages of fermentation (Park et al. 2010). Increases in free amino acids would be desirable to improve digestibility of soybean proteins.

In LFP, His was not detected beyond 24 h while Met was not detected at 24 h but was detected at 48 and/or 72 h. The absence of His and Met during further fermentation suggested degradation of the amino acids. The breakdown of His to the biogenic amine, histamine has received attention due to food safety concerns since histamine can result in food poisoning (Christensen et al. 1999; Fernandez and Zuniga 2006). The physiological roles of His decarboxylation in LAB include regulation of intracellular pH and generation of metabolic energy (Christensen et al. 1999). On the other hand, Met degradation is associated with aroma compounds in cheese (Fernandez and Zuniga 2006). In all samples, Arg decreased between 0 and 24 h and the decreases were more pronounced in LFP. Sarkar et al. (1997b) attributed Arg's pronounced decreases to its preferential uptake by *B. subtilis*. In addition, Arg provides energy in LAB via substrate-level phosphorylation (Christensen et al. 1999). Arg can also be converted to Orn via the arginine-deiminase pathway by several lactobacilli. This pathway contributes to the acid tolerance of lactobacilli (Gänzle et al. 2007).

The main free amino acids at the beginning of the fermentation in NFP and LFP were Asp, Glu, Arg, Ala, Orn, and Asn. At 72 h, major amino acids in NFP were Asp, Glu, Ala, Orn, Val, Ile, Phe, Leu and Lys while major amino acids in LFP were Asp, Glu, Gly, GABA, Val and Lys. Gly was one of the major amino acids in 90S and LFP, Leu in 100SBS, GABA in 100SBS, and 90SBS and Orn in 75SBS. High quantities of Ser were also observed in 100S. GABA, a nonprotein amino acid abundant in nature and present in soybeans (Namgung et al. 2010; Park et al. 2010), significantly increased at 48 and 72 h in 100SBS and 90SBS. GABA is produced by decarboxylation of L-Glu catalyzed by glutamate decarboxylase and has diverse physiological functions in humans including hypotensive effects and regulation of cardiovascular functions (Park and Oh 2007; Park et al. 2010).

Table 2. Changes in levels of total amino acids in naturally fermented pastes during fermentation.

	g per kg sample								
Sample	100S			90S			75S		
Amino acid	0 h	48 h	72 h	0 h	48 h	72 h	0 h	48 h	72 h
Cys	5.96 ± 0.10^{a}	6.76 ± 0.058^{b}	6.82 ± 0.16^{b}	5.61 ± 0.03^{a}	6.04 ± 0.03^{b}	6.11 ± 0.06^{b}	5.01 ± 0.01^{a}	5.29 ± 0.07^{a}	5.21 ± 0.14^{a}
Met	5.65 ± 0.07^{a}	5.69 ± 0.34^{a}	5.88 ± 0.11^{a}	4.85 ± 0.06^{a}	5.27 ± 0.17^{b}	5.21 ± 0.06^{b}	4.64 ± 0.22^{a}	4.56 ± 0.28^{a}	4.48 ± 0.14^{a}
Asp	53.85 ± 0.57^{a}	50.54 ± 0.64^{b}	51.95 ± 1.61^{ab}	45.21 ± 0.12^{ab}	45.80 ± 0.31^{b}	44.26 ± 0.51^{a}	39.45 ± 1.06^{a}	37.99 ± 0.59^{a}	37.29 ± 0.69^{a}
Thr	18.73 ± 0.39^{a}	17.72 ± 0.62^{a}	18.49 ± 0.72^{a}	16.03 ± 0.01^{a}	16.22 ± 0.03^{a}	15.80 ± 0.44^{a}	14.15 ± 0.39^{a}	13.83 ± 0.23^{a}	13.37 ± 0.27^{a}
Ser	24.89 ± 0.13^{a}	23.33 ± 0.34^{a}	24.34 ± 1.03^{a}	21.16 ± 0.05^{a}	21.64 ± 0.12^{a}	20.82 ± 0.46^{a}	18.84 ± 0.50^{a}	18.51 ± 0.06^{a}	18.04 ± 0.35^{a}
Glu	93.29 ± 0.44^{a}	86.30 ± 0.10^{b}	87.69 ± 2.86^{b}	79.56 ± 0.26^{a}	79.01 ± 0.58^{a}	76.83 ± 0.12^{b}	70.41 ± 1.78^{a}	66.36 ± 0.18^{b}	65.85 ± 1.43^{b}
Pro	21.73 ± 0.26^{a}	19.94 ± 0.25^{b}	21.03 ± 0.96^{a}	19.14 ± 0.28^{a}	18.89 ± 0.60^{a}	18.17 ± 0.0^{9}	17.61 ± 0.56^{a}	16.45^{b}	16.74 ± 0.30^{ab}
Gly	17.20 ± 0.07^{a}	16.09 ± 0.01^{a}	16.52 ± 0.64^{a}	14.57 ± 0.05^{a}	14.47 ± 0.09^{a}	14.03 ± 0.01^{b}	12.69 ± 0.21^{a}	12.21 ± 0.03^{a}	12.07 ± 0.19^{b}
Ala	17.71 ± 0.07^{a}	17.03 ± 0.05^{a}	17.84 ± 0.86^{a}	15.74 ± 0.09^{a}	15.76 ± 0.13^{a}	15.18 ± 0.07^{a}	14.10 ± 0.06^{a}	13.91 ± 0.17^{a}	13.84 ± 0.07^{a}
Val	20.79 ± 0.48^{a}	18.91 ± 0.21^{b}	20.71 ± 0.85^{a}	18.07 ± 0.03^{a}	17.84 ± 0.14^{a}	16.96 ± 0.22^{b}	15.48 ± 0.36^{a}	15.10 ± 0.13^{a}	14.93 ± 0.15^{a}
Ile	21.63 ± 0.70^{a}	20.40 ± 0.16^{a}	21.59 ± 1.38^{a}	18.87 ± 0.28^{a}	18.47 ± 0.27^{ab}	17.82 ± 0.07^{b}	15.91 ± 0.46^{a}	15.66 ± 0.02^{a}	15.33 ± 0.53^{a}
Leu	36.13 ± 0.57^{a}	34.07 ± 0.37^{a}	35.99 ± 1.77^{a}	31.91 ± 0.14^{a}	31.47 ± 0.05^{b}	30.52 ± 0.12^{c}	28.59 ± 0.56^{a}	27.83 ± 0.19^{a}	27.65 ± 1.06^{a}
Tyr	17.00 ± 0.26^{a}	16 ± 0.13^{a}	16.44 ± 0.86^{a}	14.50 ± 0.45^{a}	14.67 ± 0.19^{a}	14.32 ± 0.04^{a}	12.70 ± 0.15^{a}	12.40 ± 0.02^{a}	12.26 ± 0.28^{a}
Phe	24.83 ± 0.07^{a}	22.47 ± 0.37^{b}	23.57 ± 1.04^{ab}	20.81 ± 1.13^{a}	20.76 ± 0.48^{a}	20.37 ± 0.12^{a}	18.22 ± 0.56^{a}	17.28 ± 0.03^{a}	17.25 ± 0.07^{a}
His	14.18 ± 0.18^{a}	13.15 ± 0.05^{b}	13.47 ± 0.55^{ab}	12.11 ± 0.02^{a}	12.05 ± 0.14^{a}	11.70 ± 0.10^{b}	10.65 ± 0.20^{a}	10.29 ± 0.06^{a}	10.11 ± 0.21^{a}
Lys	29.45 ± 0.06^{a}	28.4 ± 0.62^{a}	28.76 ± 0.83^{a}	25.09 ± 0.29^{a}	25.22 ± 0.22^{a}	24.41 ± 0.19^{b}	21.50 ± 0.38^{a}	20.81 ± 0.03^{a}	20.76 ± 0.54^{a}
Arg	35.88 ± 0.19^{a}	30.64 ± 1.77^{b}	32.78 ± 1.48^{ab}	29.67 ± 0.01^{a}	29.24 ± 0.12^{a}	29.51 ± 2.04^{a}	25.54 ± 0.61^{a}	24.36 ± 0.06^{ab}	23.80 ± 0.86^{b}
SumAA	458.9 ± 3.39^{a}	427.45 ± 2.19^{b}	443.8 ± 17.68^{ab}	392.85 ± 1.91^{a}	392.8 ± 2.55^{a}	382 ± 4.10^{b}	345.5 ± 7.92^{a}	332.85 ± 0.92^{a}	328.95 ± 6.86^{a}
	(g per kg sample)								
Sample	100SBS			90SBS			75SBS		
Amino acid	0 h	48 h	72 h	0 h	48 h	72 h	0 h	48 h	72 h
Cys	5.95 ± 0.11^{a}	6.43 ± 0.18^{b}	6.36 ± 0.15^{ab}	5.35 ± 0.24^{a}	6.11 ± 0.06^{b}	6.02 ± 0.09^{b}	4.66 ± 0.09^{a}	5.46 ± 0.02^{b}	5.28 ± 0.01^{b}
Met	5.41 ± 0.38^{a}	5.56 ± 0.20^{a}	5.53 ± 0.11^{a}	4.90 ± 0.09^{a}	5.02 ± 0.19^{a}	5.13 ± 0.28^{a}	4.04 ± 0.15^{a}	4.55 ± 0.03^{b}	4.45 ± 0.10^{b}
Asp	51.88 ± 1.13^{a}	47.92 ± 0.08^{b}	47.86 ± 0.33^{b}	43.02 ± 0.63^{a}	44.19 ± 0.46^{a}	44.36 ± 0.78^{a}	37.2 ± 0.14^{a}	38.83 ± 0.75^{b}	37.07 ± 0.60^{a}
Thr	18.28 ± 0.78^{a}	16.98 ± 0.22^{a}	17.07 ± 0.19^{a}	15.32 ± 0.34^{a}	15.72 ± 0.39^{a}	15.83 ± 0.28^{a}	13.28 ± 0.23^{a}	14.21 ± 0.15^{a}	13.40 ± 0.50^{a}
Ser	24.14 ± 0.78^{a}	22.48 ± 0.14^{b}	22.49 ± 0.17^{b}	20.02 ± 0.51^{a}	20.85 ± 0.24^{ab}	21.18 ± 0.23^{b}	17.63 ± 0.27^{a}	18.82 ± 0.11^{b}	17.67 ± 0.54^{a}
Glu	89.87 ± 1.42^{a}	81.80 ± 1.11^{b}	80.99 ± 0.28^{b}	75.94 ± 1.05^{a}	76.52 ± 0.23^{a}	75.97 ± 1.11^{a}	68.06 ± 1.42^{a}	68.98 ± 1.07^{a}	68.25 ± 1.41^{a}
Pro	21.27 ± 1.08^{a}	19.87 ± 0.70^{a}	19.02 ± 0.38^{a}	18.40 ± 0.62^{a}	18.43 ± 0.01^{a}	18.53 ± 0.18^{a}	17.03 ± 0.23^{a}	16.87 ± 0.07^{a}	17.29 ± 0.48^{a}
Gly	16.38 ± 0.24^{a}	15.14 ± 0.01^{b}	15.31 ± 0.04^{b}	13.81 ± 0.11^{a}	14.12 ± 0.12^{a}	14.04 ± 0.22^{a}	12.32 ± 0.24^{a}	12.62 ± 0.30^{a}	12.17 ± 0.12^{a}
Ala	17.14 ± 0.34^{a}	16.17 ± 0.16^{b}	16.80 ± 0.32^{ab}	15.28 ± 0.11^{a}	15.59 ± 0.08^{a}	15.55 ± 0.09^{a}	13.80 ± 0.34^{a}	14.53 ± 0.31^{a}	14.15 ± 0.13^{a}
Val	20.01 ± 0.47^{a}	18.23 ± 0.19^{b}	19.03 ± 0.29^{ab}	17.17 ± 0.08^{a}	16.81 ± 0.13^{b}	16.94 ± 0.12^{ab}	15.18 ± 0.27^{a}	15.52 ± 0.35^{a}	14.76 ± 0.08^{a}
Isoleu	20.91 ± 0.32^{a}	19.59 ± 0.41^{a}	20.35 ± 0.54^{a}	17.84^{a}	17.58 ± 0.34^{a}	18.07 ± 0.19^{a}	15.45 ± 0.13^{a}	16.09 ± 0.22^{a}	15.53 ± 0.29^{a}
Leu	34.83 ± 0.67^{a}	32.82 ± 1.02^{a}	33.68 ± 0.82^{a}	30.91 ± 0.14^{a}	30.49 ± 0.39^{a}	30.83 ± 0.20^{a}	28.09 ± 0.37^{a}	28.24 ± 0.69^{a}	28.11 ± 0.70^{a}
Tyr	16.40 ± 0.03^{a}	15.43 ± 0.20^{b}	14.69 ± 0.11^{c}	13.89 ± 0.31^{a}	14.47 ± 0.28^{a}	14.29 ± 0.21^{a}	12.41 ± 0.05^{a}	12.79 ± 0.10^{a}	12.44 ± 0.10^{a}

(*Continued*)

Table 2. Continued.

	(g per kg sample)									
Sample	100SBS			90SBS			75SBS			
Amino acid	0 h	48 h	72 h	0 h	48 h	72 h	0 h	48 h	72 h	
Phe	23.22 ± 0.13^{a}	21.67 ± 0.04^{b}	21.32 ± 0.22^{b}	19.76 ± 0.05^{a}	20.36 ± 0.32^{a}	20.26 ± 0.35^{a}	17.87 ± 0.045^{ab}	18.03 ± 0.12^{b}	17.55 ± 0.14^{a}	
His	13.66 ± 0.20^{a}	12.39 ± 0.01^{b}	12.48 ± 0.05^{b}	11.57 ± 0.05^{a}	11.65 ± 0.03^{a}	11.59 ± 0.14^{a}	10.36 ± 0.22^{a}	10.53 ± 0.09^{a}	10.30 ± 0.08^{a}	
Lys	28.19 ± 0.36^{a}	26.55 ± 0.05^{b}	26.55 ± 0.15^{b}	23.46 ± 0.12^{a}	24.33 ± 0.04^{b}	24.42 ± 0.19^{b}	20.34 ± 0.23^{a}	21.32 ± 0.71^{a}	20.48 ± 0.41^{a}	
Arg	34.47 ± 0.62^{a}	30.30 ± 0.10^{b}	30.38 ± 0.11^{b}	28.26 ± 0.52^{a}	28.22 ± 0.05^{a}	28.09 ± 0.83^{a}	25.11 ± 0.54^{ab}	26 ± 2.78^{b}	23.53 ± 0.31^{a}	
SumAA	442 ± 8.77^{a}	409.3 ± 3.11^{b}	409.9 ± 0.99^{b}	374.85 ± 3.46^{a}	380.45 ± 3.04^{a}	381.1 ± 4.81^{a}	332.8 ± 3.54^{a}	343.4 ± 7.78^{a}	332.45 ± 0.21^{a}	

Means in a row and within a sample not sharing superscripts are significantly different ($P < 0.05$). Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

Amino acids play important roles in aroma and taste development in food (Dajanta et al. 2011) as they are involved in Maillard reactions and Strecker degradation (Park et al. 2010). For instance, Orn is a precursor for a key flavor compound of wheat bread crust that intensifies the roasty note of the crust odor (Gänzle et al. 2007). During fermentation, Orn was highest at 24 h in most samples and by 72 h, the highest content was in 75SBS. Glu elicits the savory taste sensation of umami in humans (Zhao et al. 2003). At the end of the fermentation, Glu was three to six times greater in NFP and about three times greater in LFP than at the start of the fermentation and Glu was highest in 100S. Glu was reported as the most abundant amino acid in soybean paste during ripening and storage (Namgung et al. 2010). Amino acids associated with bitterness were high in 100S (Val and Leu) and 90S (Ile, Leu and Phe). Amino acids associated with sweetness such as Gly, Ala, and Lys were mostly high in LFP and were highest in 100SBS, while other sweet amino acids such as Ser and Ala were high in 100S. The higher levels of total and free Asp and Glu in NFP would suggest that NFP would have higher umami intensities, while the higher levels of amino acids associated with sweetness in LFP would suggest high sweetness intensities in LFP. On the contrary, sensory perception by descriptive panel rated LFP higher in umami intensities and NFP higher in sweetness intensities. This was explained in terms of interaction effects with other tastants including organic acids that were responsible for high sourness intensities in LFP (Ng'ong'ola-Manani et al. 2014).

Organic acids and sugars

Citric, orotic, succinic, DL-lactic, uric, DL-pyroglutamic, propionic, α-ketoglutaric, oxalic, acetic and formic acids, and pyruvate were analyzed in the samples. However, detectable levels were only found in lactic, succinic, and acetic acids (Fig 1). More lactic acid was produced in both NFP and LFP compared to acetic and succinic acids. Higher lactic acid productions implied lactic acid as the major end product of fermentation, a characteristic of LAB metabolism (Kandler 1983; Axelsson 1998; Klein et al. 1998; Holzapfel et al. 2001). Lactic acid in LFP was 2.5 to 3.5-fold higher than in NFP (Fig 1A). Significantly high lactic acid production in LFP could mean higher LAB numbers in LFP resulting in dominant LAB metabolism compared to NFP. Alternatively, mixed fermentation processes of LAB and other microflora could be suggested for NFP. At 72 h, production of lactic acid was five to 16-fold and 19- to 30-fold higher than of succinic acid in NFP and LFP, respectively, while at the same time, lactic acid was one to twofold and 10- to 11-fold higher than acetic acid in NFP and LFP, respectively. The presence of

Table 3. Changes in levels of free amino acids in naturally fermented pastes during fermentation.

	(μmol/mL)											
Sample	100S				90S				75S			
Amino acid	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Asp	0.58 ± 0.04^{a}	0.36 ± 0.33^{a}	0.55 ± 0.5^{a}	0.79 ± 0.50^{a}	0.56 ± 0.014^{a}	0.64 ± 0.36^{a}	0.84 ± 0.08^{a}	1.23 ± 1.05^{a}	0.61 ± 0.02^{a}	0.71 ± 0.46^{a}	0.85 ± 0.71^{a}	0.78 ± 0.37^{a}
Glu	0.8 ± 0.03^{a}	1.12 ± 0.45^{a}	2.92 ± 1.43^{ab}	4.84 ± 0.48^{b}	0.87 ± 0.02^{a}	0.92 ± 0.35^{a}	2.16 ± 0.91^{b}	3.71 ± 2.64^{b}	0.82 ± 0.01^{a}	0.99 ± 0.13^{a}	1.36 ± 0.09^{ab}	2.38 ± 0.72^{b}
Asn	0.6^{a}	0.03^{b}	0.13b^{c}	0.2 ± 0.08^{c}	0.51 ± 0.01^{a}	0.17 ± 0.03^{ab}	0.09 ± 0.08^{b}	0.15 ± 0.15^{ab}	0.63 ± 0.03^{a}	0.19 ± 0.14^{b}	0.05 ± 0.03^{b}	0.12 ± 0.09^{b}
Ser	0.12 ± 0.01^{a}	0.35 ± 0.01^{b}	0.65 ± 0.09^{c}	0.63 ± 0.07^{c}	0.10 + 0.01^{a}	0.11 ± 0.06^{a}	0.27 ± 0.22^{a}	0.29 ± 0.3^{a}	0.1^{a}	0.1 ± 0.02^{a}	0.22 ± 0.09^{a}	0.3 ± 0.17^{a}
Gln	0.01^{a}	0.03 ± 0.01^{a}	0.06 ± 0.04^{ab}	0.12 ± 0.03^{b}	0.01^{a}	0.04 ± 0.01^{a}	0.06 ± 0.03^{ab}	0.13 ± 0.12^{b}	0.01^{a}	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.07 ± 0.04^{a}
His	0.29 ± 0.04^{a}	0.09 ± 0.03^{b}	0.06 ± 0.02^{b}	0.38^{c}	0.19 ± 0.01^{a}	0.12 ± 0.01^{a}	0.12 ± 0.09^{a}	0.24^{a}	0.16 ± 0.02^{a}	0.15 ± 0.02^{a}	0.14 ± 0.06^{a}	0.07 ± 0.06^{a}
Gly	0.22^{ab}	0.15 ± 0.08^{b}	0.25 ± 0.13^{ab}	0.47 ± 0.09^{c}	0.20^{a}	0.19 ± 0.04^{a}	0.41 ± 0.18^{b}	0.57 ± 0.30^{b}	0.23 ± 0.01^{a}	0.18 ± 0.04^{a}	0.18 ± 0.08^{a}	0.29 ± 0.16^{a}
Thr	0.08 ± 0.01^{a}	0.03 ± 0.01^{a}	0.10 ± 0.11^{a}	0.13 ± 0.08^{a}	0.08 ± 0.01^{a}	0.03 ± 0.01^{a}	0.12 ± 0.06^{a}	0.20 ± 0.24^{a}	0.08 ± 0.01^{a}	0.04 ± 0.01^{b}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}
Cit	0.01^{a}	0.19^{b}	0.13 ± 0.16^{b}	0.37^{c}	0.01^{a}	0.06 ± 0.07^{a}	0.07 ± 0.06^{a}	0.07 ± 0.07^{a}	n.d.	0.07	0.10 ± 0.06	0.06 ± 0.01
Arg	5.82 ± 0.01^{a}	1.45 ± 1.49^{b}	0.05 ± 0.02^{c}	0.06^{c}	3.16 ± 0.02^{a}	1.94 ± 1.46^{ab}	0.11 ± 0.12^{b}	0.07 ± 0.01^{b}	2.82 ± 0.04^{a}	1.31 ± 0.24^{b}	0.04 ± 0.01^{c}	0.04 ± 0.02^{c}
Ala	0.67^{ac}	0.35 ± 0.11^{ab}	1.79 ± 0.66^{c}	3.63 ± 0.46^{d}	0.59 ± 0.01^{a}	0.30 ± 0.18^{a}	1.15 ± 1.03^{b}	2.63 ± 2.32^{c}	0.64 ± 0.01^{a}	0.37 ± 0.12^{a}	0.61 ± 0.37^{a}	1.43 ± 0.41^{b}
GABA	0.13 ± 0.14^{a}	0.14 ± 0.02^{a}	0.13 ± 0.04^{a}	0.12^{a}	0.11^{a}	0.10^{a}	0.12 ± 0.01^{a}	0.23 ± 0.13^{a}	0.15 ± 0.01^{a}	0.13 ± 0.01^{a}	0.13 ± 0.01^{a}	0.21 ± 0.09^{a}
Tyr	0.11 ± 0.01^{a}	0.15 ± 0.15^{a}	0.12 ± 0.11^{a}	0.07 ± 0.06^{a}	0.12^{a}	0.10^{a}	0.19 ± 0.23^{a}	0.18 ± 0.03^{a}	0.14 ± 0.01^{a}	0.11 ± 0.11^{a}	0.13 ± 0.16^{a}	0.08 ± 0.06^{a}
Val	0.17 ± 0.01^{a}	0.06^{a}	0.57 ± 0.11^{ab}	1.0 ± 0.37^{b}	0.13 ± 0.01^{a}	0.05 ± 0.05^{a}	0.42 ± 0.52^{ab}	0.89 ± 1.17^{b}	0.13 ± 0.01^{a}	0.05 ± 0.03^{a}	0.21 ± 0.24^{a}	0.50 ± 0.43^{a}
Met	0.10 ± 0.01^{a}	0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.02^{a}	0.07^{a}	0.05^{a}	0.05^{a}	0.05^{a}	0.06	0.03	0.02	n.d.
Ile	0.16 ± 0.01^{a}	0.01^{a}	0.26 ± 0.13^{ab}	0.55 ± 0.19^{b}	0.12^{a}	0.04 ± 0.04^{a}	0.21 ± 0.26^{a}	0.65 ± 0.88^{a}	0.10 ± 0.01^{a}	0.05 ± 0.04^{a}	0.15 ± 0.17^{a}	0.32 ± 0.26^{a}
Phe	0.2 ± 0.03^{a}	0.25 ± 0.17^{a}	1.13 ± 0.31^{b}	1.66 ± 1.12^{b}	0.2^{a}	0.15^{a}	0.84 ± 1.06^{b}	1.61 ± 2.15^{b}	0.15 ± 0.01^{a}	0.12 ± 0.11^{a}	0.44 ± 0.56^{a}	0.67 ± 0.71^{a}
Trp	0.29 ± 0.04^{a}	0.27 ± 0.11^{a}	0.33 ± 0.07^{a}	0.33 ± 0.05^{a}	0.28 ± 0.021^{a}	0.29 ± 0.01^{a}	0.25 ± 0.06^{a}	0.24 ± 0.04^{a}	0.22 ± 0.01^{a}	0.24 ± 0.05^{a}	0.25 ± 0.01^{a}	0.21^{a}
Leu	0.20 ± 0.01^{a}	0.09 ± 0.04^{a}	1.06 ± 0.21^{ab}	2.19 ± 1.0^{b}	0.16 + 0.01^{a}	0.05 ± 0.05^{a}	1.12 ± 1.47^{b}	2.59 ± 3.49^{b}	0.13^{a}	0.06 ± 0.03^{a}	0.42 ± 0.52^{a}	1.11 ± 0.95^{b}
Orn	1.05 ± 0.20^{ab}	1.91 ± 0.61^{a}	0.82 ± 0.67^{ab}	0.42 ± 0.34^{b}	0.72 ± 0.08^{a}	0.75 ± 0.21^{a}	1.01 ± 0.12^{a}	0.25 ± 0.08^{b}	0.61 ± 0.09^{a}	0.83 ± 0.24^{a}	1.38 ± 0.25^{b}	0.56 ± 0.16^{a}
Lys	0.33 ± 0.01^{a}	0.35 ± 0.06^{ab}	0.58 ± 0.13^{b}	0.95 ± 0.08^{c}	0.31 ± 0.10^{a}	0.25 ± 0.01^{a}	0.65 ± 0.45^{ab}	0.92 ± 0.65^{b}	0.3^{a}	0.37 ± 0.12^{a}	0.34 ± 0.01^{a}	0.74 ± 0.30^{a}
	(μmol/mL)											
Sample	100SBS				90SBS				75SBS			
Amino acid	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Asp	0.55 ± 0.04^{a}	0.99 ± 0.41^{a}	0.74 ± 0.13^{a}	0.78 ± 0.12^{a}	0.66 ± 0.11^{a}	0.84 ± 0.3^{a}	1.05 ± 0.03^{a}	0.90 ± 0.04^{a}	0.89 ± 0.12^{a}	1.13 ± 0.39^{a}	0.83 ± 0.11^{a}	0.72 ± 0.14^{a}
Glu	0.87 ± 0.01^{a}	2.79 ± 0.1^{b}	2.33 ± 0.54^{b}	2.55 ± 0.38^{b}	0.98 ± 0.1^{a}	2.56 ± 0.1^{b}	3.37 ± 0.1^{c}	3.07 ± 0.35^{c}	0.98 ± 0.06^{a}	2.37 ± 0.05^{ab}	2.94 ± 0.42^{b}	3.14 ± 1.30^{b}
Asn	0.50 ± 0.05^{a}	0.15 ± 0.03^{a}	0.25 ± 0.21^{a}	0.36 ± 0.19^{a}	0.50 ± 0.08^{a}	0.12 ± 0.01^{b}	0.33 ± 0.10^{ab}	0.35 ± 0.10^{a}	0.85 ± 0.11^{a}	0.11 ± 0.01^{b}	0.21 ± 0.04^{b}	0.25 ± 0.14^{b}
Ser	0.14^{a}	0.09 ± 0.05^{a}	0.11 ± 0.01^{a}	0.18 ± 0.05^{a}	0.18 ± 0.01^{a}	0.08 ± 0.04^{b}	0.12 ± 0.04^{ab}	0.19 ± 0.01^{a}	0.20 ± 0.01^{a}	0.07 ± 0.02^{b}	0.11^{ab}	0.18 ± 0.01^{a}
Gln	0.01^{a}	0.12 ± 0.01^{b}	0.22 ± 0.03^{c}	0.33 ± 0.06^{d}	0.02^{a}	0.16 ± 0.03^{ab}	0.42 ± 0.16^{b}	0.5 ± 0.21^{b}	0.03 ± 0.01^{a}	0.18 ± 0.01^{b}	0.39 ± 0.03^{c}	0.43 ± 0.08^{c}
His	0.14 ± 0.08	n.d.	n.d.	n.d.	0.11 ± 0.06	n.d.	n.d.	n.d.	0.17 ± 0.09	n.d.	n.d.	n.d.
Gly	0.25 ± 0.01^{a}	0.46 ± 0.03^{ab}	0.79 ± 0.14^{ab}	1.07 ± 0.47^{b}	0.27 ± 0.01^{a}	0.51 ± 0.02^{ab}	0.75 ± 0.18^{b}	1.06 ± 0.06^{c}	0.32 ± 0.02^{a}	0.49 ± 0.01^{b}	0.71 ± 0.08^{c}	0.73 ± 0.01^{c}
Thr	0.07 ± 0.02^{a}	0.13 ± 0.06^{a}	0.11 ± 0.02^{a}	0.24 ± 0.01^{b}	0.10 ± 0.05^{a}	0.11 ± 0.04^{a}	0.29 ± 0.09^{b}	0.29 ± 0.05^{b}	0.11 ± 0.04^{a}	0.13 ± 0.04^{a}	0.19 ± 0.09^{a}	0.31 ± 0.11^{a}
Cit	0.01^{a}	0.28 ± 0.36^{a}	0.13 ± 0.15^{a}	0.04 ± 0.02^{a}	0.01^{a}	0.29 ± 0.36^{a}	0.27 ± 0.36^{a}	0.15 ± 0.18^{a}	0.02 ± 0.01^{a}	0.37 ± 0.39^{a}	0.31 ± 0.38^{a}	0.19 ± 0.23^{a}
Arg	5.09 ± 0.03^{a}	0.06 ± 0.04^{b}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}	3.12 ± 0.02^{a}	0.06 ± 0.04^{b}	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	4.20 ± 0.05^{a}	0.08 ± 0.05^{b}	0.09 ± 0.06^{b}	0.10 ± 0.04^{b}
Ala	0.70 ± 0.01^{a}	1.34 ± 0.43^{a}	2.67 ± 0.66^{ab}	3.84 ± 1.53^{b}	0.72 ± 0.02^{a}	1.25 ± 0.34^{ab}	2.32 ± 0.5^{bc}	3.27 ± 0.66^{c}	0.89 ± 0.01^{a}	1.19 ± 0.19^{ab}	2.00 ± 0.53^{ab}	2.54 ± 0.81^{b}
GABA	0.17 ± 0.01^{a}	0.51 ± 0.42^{a}	2.49 ± 2.77^{b}	3.75 ± 3.75^{b}	0.21 + 0.03^{a}	0.35 + 0.13^{a}	1.07 ± 0.86^{b}	2.20 ± 1.96^{b}	0.27 ± 0.04^{a}	0.31 ± 0.06^{a}	0.41 ± 0.03^{a}	0.43 ± 0.15^{a}

(*Continued*)

Table 3. Continued.

	(μmol/mL)											
Sample	100SBS				90SBS			75SBS				
Amino acid	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Tyr	0.13 ± 0.01^{a}	0.06 ± 0.02^{ab}	0.03^{b}	0.06 ± 0.03^{ab}	0.15^{a}	$0.04 + 0.02^{b}$	0.03 ± 0.01^{b}	0.04 ± 0.01^{b}	0.16^{a}	0.03 ± 0.02^{b}	0.02 ± 0.01^{b}	0.04 ± 0.03^{b}
Val	0.20 ± 0.01^{ab}	0.05 ± 0.01^{a}	0.25 ± 0.21^{ab}	0.53 ± 0.26^{b}	0.21 ± 0.01^{a}	0.03 ± 0.01^{a}	0.13 ± 0.13^{a}	0.26 ± 0.18^{a}	0.24 ± 0.01^{a}	0.02^{b}	0.06 ± 0.03^{b}	0.18 ± 0.12^{ab}
Met	0.10 ± 0.01	n.d.	0.03	$0.04 + 0.03$	0.09	n.d.	n.d.	0.02	0.09	n.d.	n.d.	0.01
Ile	0.14 ± 0.02^{a}	0.10 ± 0.01^{a}	0.03 ± 0.03^{a}	0.13 ± 0.07^{a}	$0.12 + 0.01^{a}$	0.11 ± 0.01^{a}	0.07 ± 0.07^{a}	0.04 ± 0.03^{a}	0.12^{a}	0.11 ± 0.01^{a}	0.12 ± 0.02^{a}	0.10 ± 0.08^{a}
Phe	0.20^{a}	0.14 ± 0.08^{a}	0.20 ± 0.15^{a}	0.38 ± 0.25^{a}	0.20 ± 0.02^{a}	0.10 ± 0.05^{a}	0.15 ± 0.08^{a}	0.22 ± 0.15^{a}	0.18 ± 0.01^{a}	0.09 ± 0.06^{a}	0.08 ± 0.01^{a}	0.17 ± 0.12^{a}
Trp	0.29 ± 0.02^{a}	0.26 ± 0.07^{a}	0.16 ± 0.08^{a}	0.13 ± 0.07^{a}	0.27 ± 0.01^{a}	0.22 ± 0.03^{ab}	0.16 ± 0.07^{ab}	0.12 ± 0.04^{b}	0.23 ± 0.01^{a}	0.19 ± 0.01^{a}	0.12 ± 0.06^{a}	0.09 ± 0.08^{a}
Leu	0.22 ± 0.01^{a}	0.07 ± 0.05^{a}	0.4 ± 0.05^{a}	0.96 ± 0.87^{a}	0.23 ± 0.01^{a}	0.05 ± 0.06^{a}	0.22 ± 0.28^{a}	0.42 ± 0.49^{a}	0.22 ± 0.01^{a}	0.03 ± 0.01^{a}	0.08 ± 0.09^{a}	0.28 ± 0.33^{a}
Orn	0.98 ± 0.06^{a}	1.63 ± 1.98^{a}	0.21 ± 0.03^{a}	0.25 ± 0.01^{a}	0.74^{a}	1.01 ± 1.09^{a}	0.71 ± 0.71^{a}	0.35 ± 0.16^{a}	$0.84 + 0.01^{a}$	2.77 ± 0.01^{b}	0.99 ± 0.94^{a}	0.62 ± 0.53^{a}
Lys	0.30 ± 0.10^{a}	0.67 ± 0.04^{a}	1.00 ± 0.5^{ab}	1.49 ± 0.77^{b}	0.28 ± 0.06^{a}	0.50 ± 0.08^{a}	0.98 ± 0.09^{b}	1.47 ± 0.26^{c}	0.35 ± 0.01^{a}	0.57 ± 0.03^{ab}	0.84 ± 0.16^{b}	0.78 ± 0.23^{b}

Means in a row and within a sample not sharing superscripts are significantly different ($P < 0.05$). n.d., not detected. Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

acetic acid suggested heterofermentation in both LFP and NFP. Heterofermentative LAB produce acetic acid, ethanol, and CO_2 in addition to lactic acid as products of fermentation (Kandler 1983). Heterofermentative and homofermentative LAB were identified in both the fermentation processes, and the former were dominant (data not shown).

At 24 h, there were no significant differences in acetic acid production in all samples except 75S (Fig 1C) which had a significantly ($P < 0.05$) lower acetic acid level. At 48 h, 90S and 100S contained more acetic acid than all LFP and 75S. At the end of the fermentation, highest acetic acid amount was produced in 100S followed by 90S and 75S although contents in 90S and 75S and all LFP were not significantly different ($P > 0.05$). High acetic acid production in NFP could be attributed to natural fermentation probably by *Bacillus* spp. because acetic acid is a major product of carbohydrate metabolism in *B. subtilis* (Moat et al. 2002). The presence of succinic acid confirmed heterofermentation (Axelsson 2004) and could also mean that pyruvate entered alternative pathways (Moat et al. 2002). No particular trend in succinic acid was observable except that there was a continual increase in production throughout fermentation in 75SBS, 90SBS, 90S, and 100S, while increases were followed by sharp decreases at 48 h in 75S and 100SBS (Fig 1B).

Organic acids in fermented soybean pastes like *doenjang* are used as quality indicators. They affect the flavor of the pastes through increases in acidity and development of unpleasant odors. Lactic and succinic acids for instance are related to sourness (Namgung et al. 2010). Likewise, the sourness intensities of LFP were higher than those of NFP (Ng'ong'ola-Manani et al. 2014). Acetic acid is considered to provide an unpleasant flavor in fermented soy foods (Namgung et al. 2010).

Soybeans contain about 9.94% carbohydrates in the form of polysaccharides and sugars. Fermentable sugars such as glucose and galactose ranging from 3.29 to 4.44/100 g and 2.91 to 3.36/100 g, respectively, were reported as part of the total dietary fiber (Redondo-Cuenca et al. 2007). Iheanacho (2010) reported 5.13% of maltose and 14.05% of fructose in soybeans. In this study, there were more sugars in LFP than in NFP at 0 h (Fig 2) probably because of the back-slopping material which had been previously fermented and was made using malt flour. Malting increases sugar (glucose, fructose, or maltose) content due to amylolytic activities (Malleshi et al. 1986). Fermentation leads to increases and decreases in sugar content in cereal-based products (Palanisamy et al. 2012). Rapid decreases in maltose in 100SBS and 90SBS (Fig 2A) could be due to its utilization as energy source and subsequent conversion into organic acids and other metabolites. The catabolism of maltose begins with

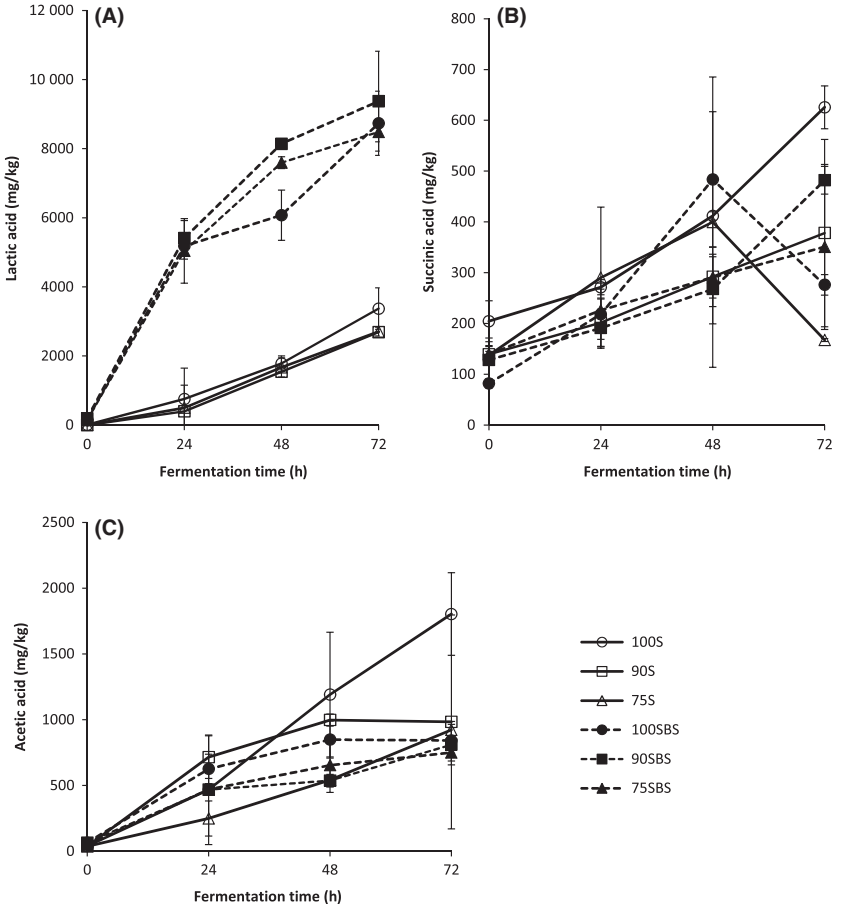

Figure 1. Changes in organic acids during fermentation. Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

its phosphorylatic cleavage catalyzed by maltose phosphorylase, yielding glucose and glucose-1-phosphate (Axelsson 2004; Gänzle et al. 2007). Homofermentative and heterofermentative LAB convert glucose-1-phosphate to glucose-6-phosphate, which is further metabolized via glycolysis to lactic acid or via phosphogluconate pathway to yield lactic acid, carbon dioxide, and ethanol/acetic acid, respectively. Glucose can also be phosphorylated by homofermentative LAB and follow the glycolytic pathway or it can be converted to glucose-6-phosphate and follow the phosphogluconate pathway by heterofermentative LAB (Vogel et al. 1999; De Vuyst et al. 2002; Axelsson 2004; Gänzle et al. 2007).

Rapid decreases in glucose in LFP between 0 and 24 h (Fig 2B) could be due to its utilization in the generation of energy via glycolysis or the phosphogluconate pathway (Vogel et al. 1999; Axelsson 2004) to support growth of a higher microbial population in LFP at the beginning of the fermentation. Although fructose content was higher throughout fermentation in LFP than in NFP (Fig 2C), it followed similar trends. Increases between 0 and 24 h in all samples, except 100S, and thereafter gradual decreases throughout fermentation in all samples, except 75SBS, were observed. The presence and increases of fructose could have been due to accumulation as a result of metabolism of other sugars like sucrose while decreases could have been due to utilization as carbon source or bioconversion of the sugar. Fructose accumulation can also be explained in terms of preferential carbohydrate utilization of LAB (Gänzle et al. 2007). All microorganisms important in foods can metabolize glucose, but vary greatly in their ability to utilize other fermentable sugars including fructose (Ray 2003). Fructose and glucose can be released from sucrose fermentation which starts with the cleavage of the sugar by sucrose hydrolase into the two monosaccharide units. The two sugars then enter the major fermentation pathways (Axelsson 2004). Heterofermentative LAB can assimilate fructose via the

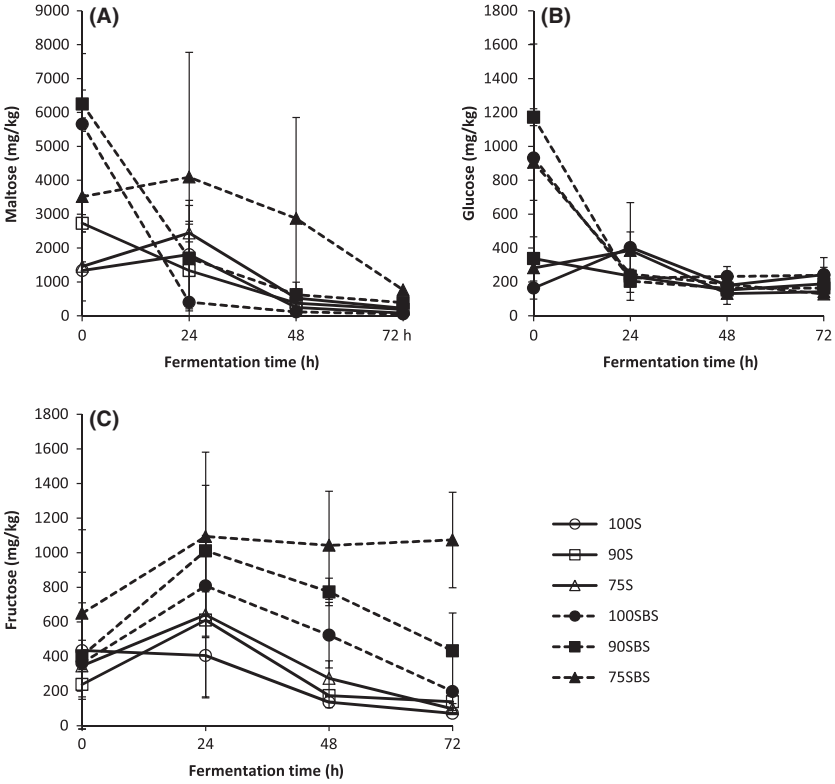

Figure 2. Changes in sugars during fermentation. Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

6-phosphogluconate/phosphoketolase pathway for hexose (Wisselink et al. 2002). Fructose may also be used as an alternative electron acceptor in LAB fermentation processes to increase LAB energy yield, resulting in its reduction to mannitol (Chen et al. 1983; Kandler 1983; Vogel et al. 1999; Gänzle et al. 2007). By 72 h, there were no significant differences in maltose and glucose levels between LFP and NFP and these two sugars were nearly all used up, while there were significant differences in the fructose content.

Antinutritional factors

Phytic acid

The levels of phytic acid content at the beginning of the fermentation processes varied among the pastes and did not seem to be influenced by sample composition. However, after 48 and 72 h, significant reductions were observed in all samples and levels of degradation depended on the type of fermentation. Overall, natural fermentation was more effective in reducing phytic acid levels (Table 4). A 33–54% reduction was achieved by natural fermentation at 24 and by 72 h, 85% reduction was noted while in some samples the phytate could not be detected. On the contrary, only 18–32% reduction was achieved in LFP at 24 h and 37–49% reduction was achieved by 72 h. *Bacillus subtilis* (Shimizo 1992; Kerovuo et al. 1998) and LAB species (Songré-Ouattara et al. 2008; Khodaii et al. 2013) with phytase activities have been reported previously.

The differences in the extent of phytic acid degradation between LFP and NFP can probably be explained in terms of the complexity of the physiological and environmental factors that affect the production and activity of phytases; and also in terms of the types of microflora in the pastes. Phytase activities in *Bacillus* spp. are optimal at a wide pH range of 4.5–8.5 (Shimizo 1992; Kim et al. 1998; Choi et al. 2001). In sourdough LAB, pH 4.0 was optimum for phytase activity and the activity rapidly decreased at pH 3.5 or pH 4.5 (De Angelis et al. 2003). Palacios et al. (2005) reported an optimum pH of 5.0 and 50% retention of optimum phytase activity at pH 4.5 and 5.5, while only 20% retention at pH 4.0 and 6.0 were reported for various LAB strains. Extracellular phytase activities in *Bacillus* spp. are known (Shimizo 1992; Kim et al. 1998;

Table 4. Phytic acid content at different times of fermentation of the pastes.

	Phytic acid (g/100 g sample dry matter)			
Sample	0 h	24 h	48 h	72 h
100S	0.314 ± 0.037^{a}	0.183 ± 0.112^{ab}	n.d.	n.d.
90S	0.274 ± 0.036^{a}	0.125 ± 0.094^{b}	0.132 ± 0.087^{ab}	0.040 ± 0.215^{c}
75S	0.319 ± 0.063^{a}	0.213 ± 0.05^{b}	0.079 ± 0.06^{bc}	n.d.
100SBS	0.322 ± 0.019^{a}	0.226 ± 0.072^{b}	0.199 ± 0.053^{c}	0.202 ± 0.048^{bc}
90SBS	0.234 ± 0.080^{a}	0.191 ± 0.128^{a}	0.194 ± 0.043^{a}	0.138 ± 0.062^{b}
75SBS	0.276 ± 0.047^{a}	0.186 ± 0.030^{b}	0.160 ± 0.010^{bc}	0.141 ± 0.025^{c}

Means not sharing superscript letter(s) are significantly different ($P < 0.05$) within a row. n.d., not detected. Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

Choi et al. 2001) while in LAB only intracellular activities have been detected (De Angelis et al. 2003; Palacios et al. 2005). Further, Palacios et al. (2005) purified and characterized an acid phosphatase (produced by LAB strains) with broad specificity that hydrolyzed monophosphorylated substrates and also phytic acid. This could suggest the possibility of phytic acid degradation activity by LAB due to nonspecific acid phosphatase with residual activity on phytic acid (Haros et al. 2008). On the contrary, enzymes with high specificity for sodium phytate have been isolated and purified from *Bacillus* spp. (Shimizo 1992; Kim et al. 1998). Finally, the synthesis of phytase in lactobacilli strains responded to limiting concentrations of carbon source (Palacios et al. 2005). Nevertheless, phytic acid degradation in both LFP and NFP fermentation processes is essential to improve bioavailability of minerals such as Ca and Zn (Kim et al. 2010).

Table 5. Trypsin inhibitor at different times of fermentation of the pastes.

	Trypsin inhibitor (mg/g sample dry matter)			
Sample	0 h	24 h	48 h	72 h
100S	n.d.	0.002 ± 0.15	n.d.	n.d.
90S	0.156 ± 0.08	n.d.	n.d.	n.d.
75S	0.013 ± 0.17	n.d.	n.d.	n.d.
100SBS	0.160 ± 0.20	0.293 ± 0.43	n.d.	n.d.
90SBS	0.069 ± 0.09	0.112 ± 0.33	n.d.	n.d.
75SBS	0.169 ± 0.10	n.d.	n.d.	n.d.

Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize. n.d., not detected.

Trypsin inhibitor

In this study, heating during paste preparation was the most effective way of reducing trypsin inhibitor. This was in agreement with results reported by Egounlety and Aworh (2003). The content of trypsin inhibitor in raw soybeans was 19 mg/g sample, but after boiling, trypsin inhibitor could not be detected in 100S while the highest trypsin inhibitor at 0 h was 0.169 mg/g sample signifying a 99% reduction (Table 5). Higher levels of trypsin inhibitor in LFP could be due to the back-slopping material which was made using finger millet malt that was added after cooling the porridge to 50–60°C. Although reductions were observed in both types of fermentation processes, fluctuations were observed in 100SBS and 90SBS in which marked increases were observed at 24 h. Higher trypsin inhibitor levels at 24 h in 100SBS and 90SBS could be due to release of bound trypsin inhibitors. Wang et al. (1972) and Egounlety and Aworh (2003) reported increases in levels of trypsin-inhibiting activities of heated soybeans fermented with *Rhizopus oligosporus*. According to Wang et al. (1972), various proteases produced by the mold were responsible for releasing bound trypsin inhibitor from the soybean substrate. Release of bound trypsin inhibitors by gastric digestion has also been suggested (Wang et al. 1972).

Conclusions

LAB fermentation and natural fermentation improved the nutritional quality of pastes of soybeans and soybean–maize blends through increases in soluble protein, increases in some total and free amino acids, and degradation of antinutritional factors. Increases in α-amylase activities in NFP and 75SBS could suggest an increased starch digestibility and possibility of reduced dietary bulkiness providing room for increasing energy density. Both types of fermentation processes resulted in nonsignificant changes in most of the total amino acids, although the fermentation processes increased the levels of the sulfur-

containing amino acids, cysteine, and methionine, which are limiting in legumes. In this study, Glu, Asp, Leu, Arg, Lys, Ser, and Phe were considered the main total amino acids throughout fermentation. Amino acid metabolism and proteolytic activities in the fermentation processes resulted in differences in major free amino acids. In NFP, these were Asp, Glu, Ala, Val, Phe, Leu, and Lys, while in LFP, these were Asp, Glu, Gly, Ala, GABA, Leu, and Lys. The free amino acids together with the organic acids would influence the taste of the pastes. High lactic acid production in LFP could mean an increased shelf life, a better microbial safety, and an increased sour taste. A comparative advantage of natural fermentation over lactic acid fermentation in this study was the higher degradation of the antinutrient, phytic acid in natural fermentation.

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Conflict of Interest

None declared.

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PAPER II

Identification and characterization of lactic acid bacteria involved in natural and lactic acid bacteria fermentations of pastes of soybeans and soybean-maize blends using culture-dependent techniques and denaturing gradient gel electrophoresis

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Abstract

Pastes of soybeans and soybean-maize blends were fermented without inoculum (naturally) and with inoculum through back-slopping using lactic acid bacteria (LAB) fermented cereal gruel, *thobwa*. LAB involved in the fermentations were characterized using culture-dependent and culture-independent analyses. Decreases in pH from 6.4 to 3.9 – 4.2 and from 6.9 to 5.4 – 5.8 after 72 h were observed in LAB fermented pastes (LFP) and in naturally fermented pastes (NFP), respectively. LAB increased from 5.0 to 8.7 – 9.6 $\log_{10}$ cfu/g in NFP and from 8.1 to 9.3 $\log_{10}$ cfu/g in LFP. LAB in both fermentations were heterofermentative lactobacilli (82.4%) and homofermentative cocci (17.6%), of which 44.7% and 42.9% were exopolysaccharide producers, respectively. Principal component analysis based on carbohydrate fermentation, CO_2 production and arginine hydrolysis showed four clusters dominated by *Lactobacillus fermentum*, *Weissella confusa*, *Lactobacillus brevis* 1 and *Pediococcus pentosaceus*, respectively. Sequencing of 16S rDNA gene confirmed *Lb. fermentum, W. confusa/W. cibaria*, and *P. pentosaceus* as identities of species in three clusters. Denaturing gradient gel electrophoresis (DGGE) confirmed these species as the dominant microbiota. DGGE showed higher similarity in microbial profiles of LFP throughout fermentation and low similarity in NFP during early and late stages of fermentation.

Key words: soybean pastes, lactic acid bacteria, fermentation, back-slopping, culture-dependent techniques, DGGE.

1. Introduction

Fermented soybean (*Glycine max*) foods are the subject of worldwide interest these days because of their health-related beneficial properties (Esaki et al., 1999; Peng et al., 2003; Park et al., 2003; Yang et al., 2011) in addition to nutritional benefits. Fermented soybean pastes are consumed widely in Far East Asia and are commonly known as *dajiang* in China, *miso* in Japan, and *doenjang* in Korea (Kim et al., 2009; Kim et al., 2010). Fermented soybean pastes are fermented foods with soybeans as an essential ingredient; they may be semi-solid and/or contain partially intact soybeans with optional ingredients like grains and are fermented by naturally occurring or cultivated microorganisms (Kim et al., 2010).

In many developing countries, legumes contribute a significant proportion of protein (Odunfa, 1983). Although soybeans have the highest protein content among legumes (Redondo-Cuenca et al., 2007), their utilization in Malawi is limited to maize-soybean blend flour used as a weaning food (CYE Consult, 2009; Kalimbira et al., 2004; Maleta, 2006). In an effort to increase consumption of soybeans by all age groups, solid-state fermented pastes of soybeans and soybean-maize blends to be used as side dishes or meat alternatives were developed (Ng'ong'ola-Manani et al., 2014).

In Africa, most food fermentations are spontaneous by lactic acid bacteria (LAB) and yeasts (Axelsson, 1998; Lei and Jakobsen, 2004; Mukisa et al., 2012; Oyewole, 1997). However, solid state fermentations of soybeans favor alkaline fermentation by *Bacillus subtilis* (Parkouda et al., 2009), a proteolytic organism that produces ammonia (Dakwa et al., 2005; Sarkar and Tamang, 1995) resulting in a strong odor which some people find objectionable (Allagheny et al., 1996; Parkouda et al., 2009). On the contrary, LAB are weakly proteolytic and do not lead to accumulation of metabolic products that are organoleptically unpleasant (Narvhus and Axelsson, 2003). LAB fermentations also improve flavor, texture, keeping quality and safety of many traditional foods (Parkouda et al., 2009; Steinkraus, 1997).

Natural fermentations and LAB fermentations of pastes of soybeans and soybean-maize blends were studied. LAB fermentations were facilitated through back-slopping using traditional fermented cereal gruel called *thobwa*. The preparation of *thobwa* is similar to that of gruels of Tanzania and Uganda (Kitabatake et al., 2003; Muyanja et al., 2003). Predominant microorganisms in these gruels are LAB and yeasts (Mugula et al., 2003; Muyanja et al., 2003; Odunfa and Adeyele, 1985). Back-slopping involves addition of material from a previous fermentation to facilitate the initiation of a new fermentation.

Back-slopping results in selection of best adapted strains, some of which may be suitable for use as starter cultures (Holzapfel, 2002; Leroy and De Vuyst, 2004). Back-slopping is still practiced in fermentations of sourdoughs and sauerkraut (Ali, 2010; De Vuyst and Neysens, 2005; Lattanzi et al., 2013; Scheirlinck et al., 2008). Back-slopping can be achieved by repetitive use of 5-10% (w/w) inoculation of a previously fermented batch as a starter (Nout, 1991).

Culture-dependent techniques were complemented by the culture-independent denaturing gradient gel electrophoresis (DGGE) method. Culture-dependent techniques are limited to isolation and cultivation of strains onto suitable substrates (Chen et al., 2008; Temmerman et al., 2004). One of their limitations is failure to provide a true reflection of microbial diversity in complex ecosystems (Muyzer, 1999; Muyzer and Smalla, 1998; Temmerman et al., 2004). Lately, DGGE has been successfully and widely used to study microbial diversity of different foods (Feligini et al., 2012; Kim et al., 2009; Miambi et al., 2003; Kim et al., 2010; Mukisa et al., 2012). During DGGE, there is sequence-dependent separation of DNA fragments of the same length based on decreased electrophoretic mobility of partially melted double-stranded DNA molecule in polyacrylamide gels containing linear gradient of DNA denaturants (Muyzer and Small, 1998; Muyzer, 1999; Temmerman et al., 2004). Separation of a PCR amplified mixture of DNA fragments of a hypervariable region such as 16*S* rDNA generates a genetic fingerprint of a community (Walter et al., 2001; Meroth et al., 2003; Muyzer and Small, 1998; Muyzer, 1999; Temmerman et al., 2004). The community members are identified by sequencing of excised gel bands (Ferris et al., 1996). DGGE allows simultaneous analysis of multiple samples; therefore, it has been successfully used for investigating microbial community dynamics during food fermentations (ben Omar and Ampe, 2000; Meroth et al., 2003; Ampe et al., 2001).

Therefore, this study aimed at characterizing LAB involved in natural and LAB accelerated fermentations of pastes of soybeans and soybean-maize blends using culture-dependent and culture-independent methods. In addition, the changes in bacteria diversity during fermentation were investigated.

2. Materials and Methods

2.1 Preparation of fermented gruel (Thobwa)

Thobwa was produced by making maize porridge containing 15% (w/v) maize flour and 80% water according to the protocol for *togwa* processed in southern part of Tanzania (Kitabatake et al., 2003). The porridge was cooled to about 50 - 60 ^{o}C before addition of finger millet (*Eleusine coracana*) malt flour (5%, w/v). The porridge was left to ferment naturally at room temperature (23 – 28 ^{o}C) for 18 h before being used as inocula in back-slopped samples.

2.2 Preparation of soybean and soybean-maize blend pastes

Pastes of soybeans and soybean-maize blends were prepared in the laboratory according to Ng'ong'ola-Manani et al (2014). Soybeans (Nasoko, variety code 427/6/7) were sorted, washed and boiled for 30 min and dehulled by rubbing between palms in cold water, washed again and then boiled for one hour (Dakwa et al. 2005). Maize (DK8071) was boiled for two hours (to soften it) before being ground together with soybeans into a paste. Grinding was done for 10 to 15 min in a Waring Commercial blender (800ES, Torrington, USA) which was sterilized by boiling for 5 min. Sterile water (100 ml) was added to 500 g material during the grinding to make pastes. Lactic acid bacteria fermentation was facilitated by addition of *thobwa*. The LAB fermented pastes (LFP) were back-slopped using 10% (v/w) *thobwa*. The pH of the *thobwa* was around 4.5 with a LAB population of 10^{8} cfu/ml. Naturally fermented pastes (NFP) were made similarly but without adding the *thobwa*. Pastes compositions were determined based on preliminary laboratory trials whereby pastes containing 100%, 75% and 50% soybeans (the remaining proportions being maize) were studied. The preliminary study showed no significant differences in pH reduction and microbial loads (total aerobic counts and lactic acid bacteria counts) of pastes containing 75% and 50% soybeans. Thus for this study, pastes were prepared according to the following compositions: pastes of soybeans only; pastes of soybeans and maize blends containing 90% and 75% soybeans, respectively. Naturally fermented pastes were designated as 100S, 90S and 75S according to 100%, 90% and 75% soybean composition in the pastes, the remaining proportions being maize. Similarly, back-slopped (BS) LAB fermented pastes were designated 100SBS, 90SBS and 75SBS. Portions of 500 g for all treatments were fermented at 30 ^{o}C for 72 h in glass jars. The fermenting pastes were sampled at 0, 24, 48 and 72 h. Three independent experiments were made.

2.3 Determination of pH and titratable acidity

AOAC (1990), methods were used to determine pH and titratable acidity. The pH was measured using a pH meter (WTW pH 525, D. Jurgens and Co., Bremen, Germany) fitted with a glass electrode (WTW SenTix 97T).

2.4 Enumeration of bacteria, yeasts and molds

From each sample, 10 g was transferred aseptically into a sterile stomacher bag before adding 90 mL sterile diluent containing 0.1% buffered peptone water (Mast DM494D, Mast Group Ltd, Merseyside,UK) and homogenising in a VWR Star-blenderTM LB400 (VWR, France) for 120 s. From the homogenate, appropriate ten-fold dilutions were taken for enumeration of aerobic mesophiles on Plate Count Agar (PCA, Merck, Darmstadt, Germany), lactic acid bacteria were enumerated on De Man, Rogosa and Sharpe Agar (MRS, Merck), and lactococci were enumerated on M17 agar (Merck), all after incubation at 30 ^{o}C for 48 h. Lactobacilli were enumerated on *Lactobacillus* selective agar (LBS agar BBL^{TM}, Becton, Dickinson and Company, Le Pont de Claix, France) incubated in anaerobic jars containing AnaeroGenTM sachet (Oxoid AN35A, Oxoid Ltd, Basingstoke, Hampshire, England) at 30 ^{o}C for 4 days. *Bacillus* were isolated on Brain Heart Infusion agar (BHI agar, Merck) by spore plating in which the homogenate was heated to 80 ^{o}C for 10 min according to Harrigan (1998). Yeasts and molds were enumerated on Rose Bengal Chloramphenicol agar (RBC agar, Oxoid) containing 100 mg chloramphenicol (Chloramphenicol Supplement SR0078E, Oxoid) per litre incubated at 25 ^{o}C for 3 – 5 days. Coliforms were enumerated on Violet Red Bile agar with lactose (VRBA, Merck) at 37 ^{o}C for 24 hours.

2.5 Isolation of lactic acid bacteria

Five colonies each were randomly picked from the fermenting pastes isolated on MRS, M17 and LBS agar for purification at all sampling times. A total of 360 isolates were screened and only Gram-positive and catalase negative organisms were selected for further characterization. Eventually, 239 isolates were subcultured to purity at least twice on MRS. The pure cultures were frozen and stored at -20 ^{o}C in MRS medium containing 15% (v/v) glycerol.

2.6 Phenotypic characterization of lactic acid bacteria

Gram's staining and catalase reaction (3% H_2O_2) were carried out on the isolates. A total of 239 Gram-positive and catalase negative isolates were characterized morphologically by light microscopy, CO_2 production from glucose, growth in MRS broth containing 7% and 10% salt, growth at 4 ^oC, 15 ^oC and 45 ^oC, growth at pH 3.9 and hydrolysis of arginine according to Schillinger and Lucke (1987) and Samelis et al. (1994). Production of dextran (slime) from sucrose was determined on MRS agar in which glucose was replaced by 5% (w/v) sucrose (Schillinger and Lucke, 1987; Samelis et al., 1994). Amylolytic activity was determined on MRS agar in which glucose was replaced with 2% (w/v) soluble starch (Oguntoyinbo, 2007). A single line streak of each pure isolate was made on MRS-starch agar before incubation at 30 ^oC for 48 h. Enzymatic activity was indicated as clear zones on the plates after flooding with iodine solution (Dakwa et al., 2005). Isolates were grouped according to similarities of the physiological characteristics. Representative isolates, 72 (30%) from the different groups were assessed for CO_2 production in MRS broth after incubation at 30 ^oC for 24 h, using an infrared gas analyzer (ADC 225 MK3, The Analytical Development Hertfordshire, UK) connected to a Chromatopac (C-R3A, Shimadzu Analytical Instruments, Kyoto, Japan) according to Narvhus et al. (1992). Carbohydrate fermentation patterns of the representative isolates were determined using API 50 CH gallery with API 50 CHL medium (BioMérieux, Marcy L'Etoile, France) according to manufacturer's specifications. Anaerobic conditions in the inoculated tubes were obtained by overlaying with sterile paraffin oil. The results were recorded after 24 h and 48 h and were interpreted using apiweb (BioMérieux).

2.7 16*S* rDNA sequence analysis of pure isolates

Genomic DNA from 43 out of the 72 isolates was extracted from cells harvested from overnight cultures. DNA was extracted using GenEluteTM Bacterial Genomic DNA kit (Sigma-Aldrich, St Louis, Missouri) according to manufacturer's instructions with some modifications. Bead beating was introduced after incubation with proteinase K. The lysis solution (400 µl) contained 45 mg/ml lysozyme (Sigma, Aldrich), 250 U/ml mutanolysin (Sigma, Aldrich) and 0.5 g of acid-washed glass beads (Sigma, Aldrich). The cells were lysed in a cell disrupter (FastPrep® FP 120 BIO101, ThermoSavant) for 20 s at 6 m/s. The reaction mixture was then centrifuged at 16, 000 x *g* for 3 min and the liquid was transferred

into a new Eppendorf tube. Remaining glass beads were removed by giving the mixture a short spin. Subsequent DNA recovery was done according to the manufacturer's instructions. The quality and concentration of the DNA were determined using a nanoDrop spectrophotometer (ND-1000 spectrophotometer, V3.2, Wilmington, USA).

Universal primers, 1F (5′ GAGTTTGATCCTGGCTCAG 3′) and 5R/1492R (5′ GGTTACCTTGTTACGACTT 3′) were used to amplify the 16S rDNA gene. The PCR reactions were performed in a final volume of 50 µl containing 5 µl 10x PCR buffer (Finnzymes Oy, Espoo, Finland), 1 µl (10 mM) dNTP mixture (Finnzymes), 0.5 µl 1F primer (100 pmol/µl), 0.5 µl 5R primer (100 pmol/µl), 0.5 µl (2 U/µl) DyNAzyme™ II DNA polymerase (Finnzymes), 3 µl template DNA and 39.5 µl milliQ-water. The samples were amplified in a DNA Thermocycler (MJ research PTC-200 Inc., California, USA) with an initial denaturation step at 94 ^{o}C for 3 min, followed by 29 cycles of denaturation (94 ^{o}C, 3 s), annealing (55 ^{o}C, 3 s), extension (72 ^{o}C, 3 min) and final extension (72 ^{o}C, 10 min). The sizes of the amplicons were checked by electrophoresis on 1% agarose gels. Prior to sequencing, PCR products were purified using QIAquickR PCR Purification kit (Qiagen, Hilden, Germany) according to manufacturers' instructions. The PCR products were sent to GATC Biotech (Konstanz, Germany) for sequencing. Sequences were edited in BioEdit software (Ibs Biosciences, Carlsbad, CA) and were compared in Genbank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast).

2.8 Total DNA extraction from the pastes

Fermented pastes (10 g) were suspended in 90 ml of 0.9% NaCl and homogenized in a stomacher blender (Seward Laboratory blender, STOMACHER 400) for 2 min. Avoiding debris, 30 ml of the mixture was collected and centrifuged at 800 x *g* for 5 min at 4 ^{o}C. Ten ml of the supernatant was transferred into a new centrifuge tube and the contents were centrifuged at 12, 000 x *g* for 15 min at 4 ^{o}C. The supernatant was discarded and the pellet was suspended with 1 ml of 0.9% NaCl to wash the cells. The cells were centrifuged at 16, 000 x *g* for 2 min and were washed two times. DNA extraction was done according to the procedure described for pure isolates with minor modifications. To increase the quality of community DNA extracted from starchy foods, 20 µl of 10% hexadecyltrimethyl-ammonium bromide (CTAB) (Ampe et al., 1998), was added during incubation with proteinase K. The extracted DNA was used as template for PCR amplification for DGGE analysis.

2.9 DGGE analysis and band identification

Universal primers PRBA338fgc (5' C GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG ACT CCT ACG GGA GGC AGC AG'3) and PRUN518r (5'ATT ACC GCG GCT GCT GG '3) targeting the V3 region of 16S rDNA and suited for DGGE were used (Øvreås et al., 1997). PCR was performed in a final volume of 50 µl containing 5 µl 10X DreamTaqTM Green Buffer (Fermentas GmbH, Leon-Rot, Germany), 8 µl dNTP-mix (1.25 mM (Finnzymes), 1.0 µl forward primer (5 pmol/µl) 1.0 µl reverse primer (5 pmol/µl), 0.5 µl formamide, 0.5 µl bovine serum abumin (1mg/ml), 0.25 µl DreamTaqTM DNA polymerase (5U/µl) (Fermentas), 3 µl template DNA and 30.75 µl milliQ-water. The samples were amplified in a thermal cycler (BIO-RAD C1000™, Bio Rad Laboratories Inc., USA). Amplification conditions included an initial denaturation at 94 °C for 5 min followed by 34 cycles of denaturation (94 °C, 30 s), annealing (53 °C, 60 s), extension (72 °C, 60 s) and final extension (72 °C, 10 min). Gel electrophoresis, band excision and DNA elution were done according to Porcellato et al. (2012) except that the gels contained 20% to 50% urea-formamide as denaturants. The eluted DNA was reamplified using the same primer set but without the GC-clamp on the forward primer. The PCR products were purified using QIAquickR PCR Purification kit (Qiagen) according to manufacturers' instructions and were sent to GATC Biotech (Germany) for sequencing. Sequences were searched in Genbank using the BLAST algorithm.

Cluster analysis of DGGE fingerprints was performed in GelCompar II software version 6.5. The fingerprints were processed according to the software's manual. Similarity between DGGE profiles was calculated according to Dice's similarity coefficients and a dendrogram was constructed using unweighted pair group method with arithmetic average (UPMGA).

2.10 Statistical analysis

Analysis of variance (ANOVA) at p=0.05 was performed in SPSS 15.0 (SPSS Inc., Chicago, Illinois., USA) to find out if type of fermentation and duration of fermentation had significant effects on pH, titratable acidity and microbial counts among the samples. Principal component analysis was done to group isolates based on carbohydrate fermentation profile from API, CO_2 production, and arginine hydrolysis. A matrix was generated based on colors of the results of the API reading after 48 h in which 1 was definitely negative and 5 was definitely positive; strains able to produce >1000 mg/kg of CO_2 were considered heterofermentative and positive, those that produced less were

homofermentative and negative; strains able to hydrolyze arginine were positive and those unable were negative. Carbohydrates that were not fermented by all isolates, or were fermented by one or two isolates were not included in the matrix. Principal component analysis was done in UnscramblerX 10.2 (CAMO Software AS, Oslo, Norway).

3. Results

3.1 pH and titratable acidity

The initial pH (6.44 – 6.46 for lactic acid bacteria fermented pastes (LFP) and 6.88 – 6.95 for naturally fermented pastes, NFP) and titratable acidity (0.16 - 0.20 for LFP and 0.09 - 0.10 for NFP) were not significantly different, despite LFP being inoculated with *thobwa* (Fig. 1). The pH for LFP decreased faster than for NFP (Fig 1a). After 24 h, the pH for LFP was between 4.2 and 4.6 while that of NFP was between 6.1 and 6.7. In 100S and 75S, the pH did not change significantly ($p>0.05$) from 0 h to 24 h, but the decreases were significant from 24 h to 48 h. Further, from 48 h to 72 h, the change was significant in 75S only. On the other hand, there were significant decreases in pH in 90S throughout fermentation. In all LFP, there were significant ($p<0.05$) decreases in pH from 0 h to 24 h, and thereafter, there were non-significant decreases throughout fermentation. Correspondingly, the percent reduction in pH was higher in LFP than in NFP. At 24 h, the pH was reduced between 28% and 35% in LFP and this was 3 to 9 times higher than in NFP. After 72 h, the cumulative percent reduction in LFP ranged from 34 to 39% while it was 17 to 23% in NFP. The influence of maize in pH reduction was more pronounced in LFP from 24 h until further fermentation. Significant increases in titratable acidity were observed in all samples between 0 and 24 h. At 24 h, increases were about 3 fold in LFP and about 2 fold in NFP. Changes from 24 h to 48 h were only significant in 100S, while changes from 48 to 72 h were significant in 75SBS, 75S and 100S (Fig 1b).

3.2 Microbial counts

There were higher microbial counts in LFP (due to back-slopping) than in NFP throughout the fermentation except where mentioned. Coliforms were not detected in any of the samples.

3.2.1 Aerobic mesophilic bacteria and spore counts

Although the initial aerobic mesophilic bacteria counts were slightly higher in LFP (4.58 – 7.14 $\log_{10}$ cfu/g, Fig 2a) than in NFP (4.98 – 5.58 $\log_{10}$ cfu/g, Fig 2a), significant ($p<0.05$) differences were only observed between 100SBS and 90S; and 100SBS and 75S. The growth rates were highest between 0 and 24 h in all the samples. After 24 h, the total counts ranged between 8.99 – 9.36 $\log_{10}$ cfu/g in NFP and between 9.23-10.57 $\log_{10}$ cfu/g in LFP and the differences were significant in pair wise comparisons of all the samples except 100SBS and 75SBS. After 48 h, counts for all LFP were significantly higher ($p<0.05$) than counts for NFP, but there were no significant differences within fermentation type (Fig 2a).

While LFP had significantly high initial spore counts (4.41 – 4.45 $\log_{10}$ cfu/g, Fig 2b) than NFP (2.46 – 3.30 $\log_{10}$ cfu/g, Fig 2b), decreases in spore population between 0 and 24 h for all LFP (Fig 2b) were observed and counts at 72 h were lower than in all NFP. At 72 h, spore counts ranged from 6.10 - 7.10 $\log_{10}$ cfu/g in NFP and 5.82 – 6.02 $\log_{10}$ cfu/g in LFP.

3.2.2 Lactic acid bacteria counts

Lactic acid bacteria counts in LFP (8.13-8.49 $\log_{10}$ cfu/g) were 3 $\log_{10}$ cfu/g higher than in NFP (4.97-5.31 $\log_{10}$ cfu/g) at 0 h. Comparatively, back-slopping introduced more LAB than other types of microorganisms (aerobic mesophilic bacteria, spore and yeast and molds counts were 1-2 $\log_{10}$ cfu/g higher in the LFP than in NFP). There were significant differences ($p<0.05$) in counts among the NFP and the LFP at 0 and 24 h while at 48 h, only 100SBS was different from all NFP (Fig 2c). Lactic acid bacteria continued to increase throughout fermentation in NFP while LAB decreased slightly in LFP during fermentation period from 24 h to 72 h (9.45-9.82 $\log_{10}$ cfu/g to 9.24-9.33 $\log_{10}$ cfu/g). There were no significant differences ($p>0.05$) among samples within fermentation type. A similar trend was observed with *Lactobacillus* counts (Fig 2d) except that 100S was different ($p<0.05$) from all NFP at 24 and 72h.

3.2.3 Yeasts and molds counts

There was lower proliferation of yeasts and molds in LFP than NFP. Counts increased from 2 to 7 $\log_{10}$ cfu/g between 0 and 24 h in NFP, while increases were from 3 - 4 to 4 - 4.8 $\log_{10}$ cfu/g in LFP. There were significant differences ($p<0.05$) when all NFP were compared to all LFP at 0 and 24 h. At 72 h, differences were significant between 100S and the other NFP and between all LFP and 90S, and all LFP and 75S (Fig 2e).

3.3 Phenotypic characterization of lactic acid bacteria isolates

Among the 239 isolates, 197 (82.4%) were rods and 42 (17.6%) were cocci. The rods were put in Group I, which was subdivided into two subgroups A and B. Group IA was composed of 142 (59.4%) heterofermentative arginine positive isolates while Group IB was composed of 55 (23%) heterofermentative arginine negative isolates. Homofermentative cocci formed Group II and were subdivided into Group IIC composed of 35 (14.6%) arginine positive isolates and Group IID composed of 7 (2.9%) isolates not able to hydrolyze arginine. Many isolates in both groups were able to grow at high temperature, high salt content and low pH (Table 1).

A total of 72 (30%) representative isolates were identified using carbohydrate fermentation profiles. The dominant species of Group I were *Lactobacillus brevis* 1 and *Weissella confusa* while *Pediococcus pentosaceus* dominated Group II. All strains could not ferment erythritol, D-arabinose, D-adonitol, MDX, L-serbose, D-ulcitol, unositol, MDM, MDG, inuline, D-melezitose, xylitol, lyxose, D-fucose, L-fucose and L-arabitol. All cocci strains could not ferment amidon, glycogen and D-arabitol while all rods could not ferment D-sorbitol and L-xylose. Turanose was fermented by one coccus and two Lactobacilli, glycerol was fermented by one *Lactobacillus* and so were D-arabitol and glycogen while two Lactobacilli utilized amidon.

When Principal component analysis of the API results, CO_2 production and arginine hydrolysis was considered, three distinct clusters A, B and C with 4, 8 and 5 species, respectively, were obtained (Fig 3A). Cluster D was mainly composed of *Lb. brevis* with diverse sugar fermentation profiles. Principal component 1 was mainly responsible for grouping of isolates according to type of fermentation, homofermentative cocci (cluster C) on the right and heterofermentative lactobacilli on the left (clusters A and B). Cluster C was dominated by *P. pentosaceus* strains, all of which utilized galactose, glucose, fructose, mannose, N-acetyglucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, trehalose and gentibiose. These sugars loaded highly on the positive dimension of PC1 (Fig 3B). Five cocci strains (17, 232, 330, 53b, 238) with assimilation patterns like *Lb. brevis* 1, clustered in this group and were tentatively identified as *P. pentosaceus*.

Clusters A and B (Fig 3A) were composed of strains that produced high amounts of CO_2 (>1000 mg/kg) and CO_2 production loaded highly on the negative dimension of PC1 (Fig 3B). The separation of the two clusters however, was based on PC2, on which L-arabinose,

D-ribose, D-xylose, melibiose, D-raffinose and potassium-5-celuconate loaded highly on the positive dimension (Fig 3B). In cluster A, all *Lb. brevis* 3 strains utilized L-arabinose, D-xylose and D-ribose while all *Lb. fermentum* strains fermented the mentioned sugars plus D-raffinose. Further, all strains of the two species showed partial fermentation of potassium-5-celuconate.

Cluster B was dominated by *W. confusa* which were arginine positive (with a few exceptions). All isolates in cluster B fermented glucose, fructose, mannose, esculine and maltose. The isolates also fully or partially fermented potassium gluconate, D-xylose and N-acetylglucosamine. Some isolates also utilized amygdaline (105), arbutine (105), and trehalose (105, 205).

Most isolates identified as *Lb. brevis* 1 grouped into cluster D and were all arginine positive and heterofermentative. They all utilized glucose, fructose, mannose, esculine, cellobiose and maltose. With exception of the strains in parenthesis, fermentation or partial fermentation of the following sugars was observed: L-arabinose (106s), ribose (172), D-xylose, N-acetylglucosamine, amygdaline (225c), arbutine, salicine, melibiose (106b, 172), saccharose (106b, 140b, 371a), trehalose (172), gentibiose (172R), and potassium gluconate (172R). Partial fermentation of potassium-5-celuconate was done by 181s, 181b, 182s, and 324w.

Lactobacillus pentosus 127R was placed close to cluster D, since it was heterofermentative and arginine positive. Apart from utilizing all sugars used by the cocci, this isolate also fermented melibiose, saccharose, raffinose, turanose, lactose (Fig 3B) and partially fermented mannitol, amygdaline, potassium gluconate and potassium-5-celuconate. The isolate was close to the heterofermentative, arginine positive *Lb. plantarum* 273 on the principal component analysis map (Fig 3A), indicating similarity in fermentation profiles except that the later did not ferment turanose, potassium gluconate and potassium-5-celuconate but fermented starch and melezitose.

Almost all species identified by API were present in both LFP and NFP from the onset and throughout the fermentation period. API identification showed that *Lb. brevis* 1 (19.2%), *W. confusa* (17.8%) and *P. pentosaceus* (13.7%) were the dominant species. Table 2 shows the distribution of strains based on API identification. At 0h, *Lb. buchneri* and *P. pentosaceus* were identified from both NFP and LFP while *Lb. fermentum* and *W. confusa* were only identified in LFP and *Lb. plantarum* 1 was only identified in NFP. At 24h more species

appeared, *Lb. brevis* 1, *Lb. brevis* 3, *Lactococcus. lactis*, *P. pentosaceus* and *W. confusa* were identified in both NFP and LFP, *Lb. delbrueckii*, *Lb. fermentum,* and *P. damnosus* were only identified in NFP while *Lb. buchneri* and *Leu. mesenteroides* were identified in LFP only. At 48 h, new species were *Lb. acidophilus* and *Lb. collinoides* in NFP, *Lb. pentosus* and *Lb. plantarum* 1 and 2 in LFP. At 72 h, *Lb. acidophilus* and *L.brevis* 1 were identified in LFP.

3.4 Identification of isolates by 16*S* rDNA sequencing

Among the 72 strains characterized by API, 43 representative strains were identified using 16S rDNA gene sequencing. *Bacillus* spp. and *Enterococcus faecium* were identified in addition to the species identified by API. The dominant species of Group I (Table 1) were found to be *W. cibaria* and *Lb. fermentum* while *P. pentosaceus* was the only species identified in Group II (Table 1). Subgroup A (Table 1) was composed of *Lb. buchneri*, *Lb. brevis 3, Lb.collinoides* and *Lb. fermentum*, according to API identification, however, all strains were identified as *Lb. fermentum* by 16*S* rDNA except two *Lb. brevis* 3 strains which were identified as *Lb. brevis* (Table 3). Most strains in subgroup B (Table 1) were identified as *W. cibaria*, except one strain of *Lb. delbrueckii* ssp. *delbrueckii* which was identified as *W. confusa*. All cocci in subgroup C (Table 1) were identified as *P. pentosaceus* including the five strains (17, 232, 330, 53b, 238) with fermentation profiles similar to *Lb. brevis* 1.

Principal component analysis grouped isolates with similar fermentation patterns into clusters with dominant strains representing the correct classification at species level when compared with 16S rDNA. For instance cluster A was dominated by *Lb. fermentum* and 7 of 9 strains 16S rDNA sequenced from this cluster were *Lb. fermentum*. Sixteen isolates representing 7 species and grouped in a *Weissella* dominated cluster (cluster B) were identified as *W. confusa* or *W. cibaria* by 16S rDNA sequencing. Further, all 10 strains sequenced from cluster C were confirmed as *P. pentosaceus*, although there were three lactobacilli identified as *Lb. brevis* 1 which were clearly misplaced into this cluster. These lactobacilli could not ferment saccharose and raffinose as the other *Lb. brevis* 1, hence their placement in cluster C. Two *Lb. brevis* 1 strains in cluster D were identified as *Lb. fermentum* by 16S rDNA sequencing.

3.5 Bacteria community based on DGGE analysis

Culture-independent techniques based on amplification of the V3 region of the 16S rDNA gene were used to identify bacteria diversity of the fermenting pastes throughout fermentation. Figure 4 shows changes in the microflora of NFP and LFP during fermentation. *Weissella cibaria/W. confusa* were the dominant organisms from the beginning (0 h in LFP and 24 h in NFP) of the fermentation to the end as shown by the intensity of their bands (Fig. 4 bands 4, 6 and 11; Table 4). Although *Lactobacillus fermentum* was present at 0 h in 100S (Fig. 4 band 13), but in other NFP this species appeared at 48 h while in all LFP, the species appeared at 24 h (Fig.4 band 3).

At 0 h, *Lb. fermentum* (bands 7 and 13), *Bacillus* spp. (band 14) and uncultured bacteria (band 15) were recovered in NFP (Fig. 4; Table 4) while in LFP uncultured *Weissella* (band 1), *W. koreensis* (band 2), *W. confusa/W. cibaria* (bands 6 and 11), and *P. pentosaceus* (band 5) were recovered (Fig. 4 lanes 100SBS 1, 90SBS 1, 75SBS 1; Table 4). At 24 h, uncultured *Weissella* (band 1), *W. confusa/W. cibaria* (bands 4, 6, 11) and *Lb. linderi* (band 12) appeared while *Bacillus* (band 14) disappeared in NFP (Fig. 4 lanes 100S 2, 90S 2, 75S 2; Table 4). In LFP, an additional species at 24 h was *Lb. fermentum* (band 3). At 48 h, all microorganisms (except *Bacillus*, band 14) present at 0 and 24 h were recovered in NFP (Fig 4. lanes 100S 3, 90S 3, and 75S 3; Table 4) as well as in LFP (lanes 100SBS 3, 90SBS 3 and 75SBS 3), although LFP showed *P. pentosaceus* as weak bands (band 5) and no *Lb. linderi* (band 12). At 72 h, the microflora of NFP (Fig. 4 lanes 100S 4, 90S 4, and 75S 4; Table 4) and LFP (Fig. 4 lanes 100SBS 4, 90SBS 4, 75SBS 4; Table 4) were similar; *Lb. linderi* (band 12) had disappeared in NFP (100S) while *P. pentosaceus* (band 5) was detected in both fermentation types as weak bands.

3.6 Bacterial profile of the samples

Cluster analysis of the DGGE profiles showed low similarity in microbial communities between NFP and LFP during early stages of fermentation. Three distinct clusters and outer groupings were formed, most LFP clustered separately from NFP. All 75SBS and 90SBS samples plus 100SBS 72h formed one cluster with 70% similarity, remaining 100SBS samples clustered together with 75S 48h and 72h (78% similarity), while 100S and 90S also clustered together (68% similarity). Outer groupings were formed by all NFP samples at 0 h and 75S and 90S at 24 h (Fig 5).

4. Discussion

The study has shown the possibility of solid state LAB fermentation of soybeans. The continual decrease in pH throughout fermentation indicated a bias towards LAB fermentation in both NFP and LFP. Although a relatively fast drop in pH to about 4.0 by 24 h, as observed in LFP, would be desirable to prevent growth of pathogens. The slow decrease in pH in NFP suggested co-fermentation between LAB and proteolytic organisms which probably released ammonia resulting in neutralization of the medium. Nevertheless, the gradual pH decline suggested a bias towards lactic fermentation as opposed to alkaline fermentation as reported in most natural fermentations of soybeans (Chukeatirote et al., 2010; Dajanta et al., 2011; Dakwa et al., 2005; Parkouda et al., 2009; Sarkar et al., 1993; Sarkar et al., 1994; Sarkar et al., 2002). This lactic fermentation could be attributed to limited oxygen during fermentation in the jars which could have favored growth of microaerophiles while limiting growth of spore formers, eventually reducing ammonia production and less increase in pH (Allagheny et al., 1996; Parkouda et al., 2009). In addition, soybeans and maize contain fermentable sugars such as sucrose, fructose, glucose and maltose (Medic et al., 2014; Ferguson et al., 1979). Maize contains 4.78 – 18% sucrose, 0.58 – 2.73% fructose, 0.19 – 2.20% glucose and 0.02 – 0.7% maltose (Ferguson et al., 1979) while soybeans contain 1.1 – 7.4% sucrose, 0.03 – 2.5% fructose, 0.03 – 2.4% glucose and 0.3 – 0.5% maltose (Medic et al., 2014). The sugars are converted to lactic acid and other metabolites during LAB growth (Mensah, 1997; Steinkraus, 1997). The higher sugar content in maize explains the higher percent pH reduction in maize containing samples. Further, since the samples were cooked and ground hence most of the nutrients and sugars were released and available for growth even by the fastidious LAB.

In NFP, LAB competed as the dominant flora increasing almost two folds (5 to 9 $\log_{10}$ cfu/g) during 72 h of fermentation. On the other hand, LAB was dominant from the onset in LFP (ca. 8.0 $\log_{10}$ cfu/g) and by 72 h, LAB was 9.0 $\log_{10}$ cfu/g. Dakwa et al. (2005) reported an initial LAB count of 6.1×10^3 cfu/g which increased to 1.4×10^6 cfu/g during 72 h of fermentation in soy – *dawadawa*. Lactic acid bacteria dominated in both LFP and NFP because the fermentations were done in nearly full closed glass jars which limited the amount of oxygen (Allagheny et al., 1996) and favored proliferation of microaerophilic LAB. Higher *Lactobacillus* counts in most pastes of soybean-maize blends were observed probably due to availability of more fermentable sugars in the blends. While high *Lactobacillus* counts in 100SBS was due to the back-slopping.

Different growth trends were observed in yeast and mold populations and in spore counts. All the initial counts were higher in LFP than in NFP probably due to the back-slopping, but as fermentation progressed; spore numbers and yeast and mold counts in LFP were lower than in NFP suggesting inhibition. Low spore proliferation in LFP could be due to an increase in acidity in LFP as the pH dropped from 6.4 to ca. 4.0 during 24 h fermentation. Spore formers that dominate soybean fermentations are mostly *B. subtilis* (Dakwa et al., 2005; Sarkar et al., 2002; Sarkar et al., 1994), and they exhibit active growth at pH-range between 5.5 and 8.5 (Chantawannakul et al., 2002). Inhibition of spore formers could improve safety of the fermented products because some pathogenic sporulating organisms like *Clostridium* spp. have been implicated in foodborne disease outbreaks associated with fermented foods including pastes of soybeans (Motarjemi, 2002). Unlike *Bacillus* spp., yeasts proliferate even in acidic conditions causing spoilage of low acid foods (Praphailong and Fleet, 1997). Therefore, low yeast and mold proliferation in LFP could suggest an inhibitory effect other than acidity by LAB. Production of CO_2 by heterofermentative LAB may have inhibited growth of obligately aerobic yeasts which have an exclusive oxidative metabolism (Rosenfeld and Beauvoit, 2003). In addition, the possibility of production of bacteriocins active against yeasts may be suggested because LAB producing such bacteriocins have been reported (Atanassova et al., 2003).

Phenotypic identification showed *Lb. brevis* 1 as the dominant species. However, *Lb. brevis* 1 strains varied in their sugar fermentation profiles, hence were placed in all clusters. Their spreading in different clusters suggested differences in fermentation patterns within the *Lb. brevis* group. All *Lb. brevis* not included in cluster A were atypical because they fermented amygdaline and most also fermented trehalose (Kandler and Weiss, 1986). Strains in cluster A were typical for obligate heterofermentative *Lactobacillus* spp. fermenting glucose and fructose, and unable to ferment amygdaline, mannitol, rhamnose and sorbitol (Hammes et al., 1992). Although strains 106b, 140b, and 371a were heterofermentative rods identified as *Lb. brevis* 1 by API, they were clustered together with *P. pentosaceus* in cluster C because of their inability to ferment saccharose and raffinose. Sánchez et al. (2000) classified isolates as *Lb. brevis* 2 based on their ability to ferment raffinose and saccharose.

This study agrees with studies of Boyd et al. (2005), Østlie et al. (2004.) and Andrighetto et al. (1998) in which phenotypic identification based on API 50CH mismatched genotypic identification of some *Lactobacillus* spp. The mismatches were attributed to poor discriminatory power of API because of atypical fermentation patterns in a number of

species, particularly in *Lb. brevis* 1 and *P. pentosaceus*. Most isolates identified as *Lb. brevis* by API were identified as *Lb. fermentum* by 16S rDNA sequencing. In addition, some cocci with fermentation patterns similar to *Lb. brevis* 1 on API, were identified as *P. pentosaceus* by 16S rDNA gene sequencing. Physiological and biochemical criteria used for strain identification are often ambiguous because most of the *Lactobacillus* species have very similar nutritional requirements and grow under similar environmental conditions (Andrighetto et al., 1998). Thus, phenotypic heterogeneity among *Lactobacillus* spp. account for discrepancies between genotypic and phenotypic identification (Boyd et al., 2005; Vandamme et al., 1996). The strain–to–strain variability within a species in phenotypic characteristics may be partly explained by encoding of specific properties located on plasmids (Hammes et al., 1992).

Genotyping revealed that seven species were involved in the fermentation of the pastes including *E. faecium/E. durans* and *Bacillus* spp. *Bacillus* spp. are important predominant microorganisms that cause proteolysis in alkaline fermented soybeans (Dakwa et al., 2005; Sarkar et al., 1994; Steinkraus, 2002). *Enterococcus faecium* have also been isolated in soybean fermentations (Kim et al., 2009; Sarkar and Tamang, 1995; Sarkar et al., 1994). Although some *Enterococcus faecalis/E. faecium* strains are used as probiotics (Franz et al., 2003; Lund and Edlund, 2001) and others produce bacteriocins with activity against food spoilage and pathogenic bacteria (Criado et al., 2006), they are also known nosocomial pathogens with resistance to a variety of antibiotics (Banwo et al., 2013; Yoon et al., 2008; Yousif et al., 2005). Therefore, *E. faecalis/E. faecium* strains unintentionally found in food raise a food safety concern.

Lactobacillus fermentum, P. pentosaceus and *W. confusa* have been reported in lactic acid fermentations of cereal based products of Africa (Kalui et al., 2010; Mukisa et al., 2012; Oyewole, 1997). These organisms were dominant species in naturally fermented millet porridge (Lei and Jakobsen, 2004). Their presence in lactic acid bacteria fermented pastes could be attributed to the back-slopping material which was maize and finger millet based. Their presence in naturally fermented pastes however, suggested they were contaminants from the environment. Although studies on soybean fermentation have focused on *Bacillus* spp., many LAB species have been isolated. For instance, *Leu. mesenteroides, Tetragenococcus halophilus,* and *E. faecium* were observed as the dominant bacteria species in Korean *deonjang,* while *Lb. sakei, P. pentosaceus, Lb. plantarum, W. confusa* and other *Lactobacillus* spp. were also detected (Kim et al., 2009). *Pediococcus pentosaceus, Lb.*

plantarum, Lb. delbrueckii ssp. *delbrueckii, Lb. vaccinostercus, Lb. collinoides, Lb. panis, Lb. pentosus, Lb. fructivorans, E. faecium, E. durans, E. avium* and *E. feacalis* were isolated in a Chinese fermented soybean paste (Cui et al., 2012). Further, Kim et al. (2010) isolated *W. cibaria, Lb. citreum, Leu. pseudomesenteroides* and *P. pentosaceus* in Japanese fermented soybean pastes.

The dominance of *Weissella* spp., *Lb. fermentum,* and *P. pentosaceus* probably suggests they have important roles in lactic acid fermentation of soybeans hence could be explored as starter cultures. Their roles could be organoleptic, contributing to the flavor and texture of the pastes. For instance, acidification of food by both homofermentative and heterofermentative LAB imparts a tangy lactic acid taste (Leroy and De Vuyst, 2004). In addition, LAB produces aromatic and volatile compounds from bioconversion, that contributes to typical flavor of certain fermented products when pyruvate enters alternative pathways leading to generation of acetate, ethanol, diacetyl, and acetaldehyde (Leroy and De Vuyst, 2004). Another important characteristic of the isolates was their ability to produce exopolysaccharides (EPS). The use of EPS-forming starter cultures to improve rheological properties of dairy products is well known (Ruas-Madiedo et al., 2002; Leroy and De Vuyst, 2004). In sourdoughs, EPS influence the viscoelastic properties of the dough and has beneficial effects that improve dough rheology, bread texture and shelf life of the bread (Galle and Arendt, 2014; Tieking et al., 2003). Application of EPS in the bakery industry includes beneficial effect on bread volume and staling (Leroy and De Vuyst, 2004). Thus, EPS in the fermented soybean pastes could influence organoleptic properties as texture improvers responsible for cohesion of the matrix. Therefore, a better understanding of the structure–function relationship of EPS in soybean fermented products is crucial to expand their technological applications. Further research is required to determine the roles of the LAB in fermented soybean pastes since functional or technological properties of the LAB were not investigated in this study.

Diversity of microorganisms identified with DGGE was almost similar as identified with culture-dependent techniques, except that *Lb. brevis* and *E. faecium* were not recovered with DGGE while two other species, *Lb. linderi* and *W. koreensis* were detected. Although DGGE gives a better reflection of the actual diversity in complex ecosystems, failure to recover species representing less than 1% of the total community has been reported as its limitation (Miambi et al., 2003). The cell numbers of *Lb. brevis* and *E. faecium/E. durans* could have been lower than the DGGE threshold detection limit. A 10^4 cfu/ml detection

limit of PCR-DGGE has been indicated as one of the limitations of PCR-DGGE techniques (Cocolin et al., 2004). Other reasons for failure to recover the species could include choice of PCR primers, as universal primers targeting V3 or V6-V8 allow only the dominant flora to be visualized when analyzing ecosystem with high bacteria diversity; the possibility of species giving identical band positions (Temmerman et al., 2004); and biases in DNA extraction and selective PCR amplification (Miambi et al., 2003; Muyzer, 1999; Muyzer & Smalla, 1998). These results are in agreement with Miambi et al. (2003) who recommended a combination of culture-dependent and culture-independent techniques to obtain a more detailed view of microbial communities.

Nevertheless, DGGE confirmed that the dominant microorganisms were *Weissella* species and *Lb. fermentum* as observed by the intensity of their bands. *Weissella cibaria/W. confusa* and *Lb. fermentum* were represented by multiple bands (4, 6, 8, and 11) and (3, 7 and 13), respectively. Multiple banding patterns have previously been observed (Kim et al., 2009; Kim et al., 2010) and are attributed to sequence heterogeneity between multiple copies of the 16S rDNA of any given strain (Nübel et al., 1996). DGGE also revealed low similarity in microbial composition at the beginning of fermentation between NFP and LFP. Microbial diversity remained relatively unchanged in LFP from onset to termination because of the back-slopping, while microbial succession was observed in NFP. Thus, most LFP samples had similar microbial profiles at different fermentation stages, forming one cluster. In contrast, microbial profiles for NFP were different during early and late fermentation stages. At 24 h, *Bacillus* had been succeeded probably due to acidification of the environment and colonization of *W. confusa/W. cibaria* in NFP. Microorganisms capable of flourishing in acid environments were abundant at the end of the fermentations.

Conclusions

The study demonstrated the possibility of soybean solid state fermentation achieved with lactic acid bacteria in both naturally fermented and back-slopped pastes. However, co-fermentation was suggested for natural fermentation. The advantage of back-slopping was the possibility of inhibition of unwanted microorganisms such as spore formers which may be pathogenic. Although, successions were observed in naturally fermented pastes; and bacterial diversity in back-slopped samples remained relatively unchanged throughout fermentation, but common species were dominant microflora in both types of fermentation. These were *Weissella cibaria/W. confusa, Lb. fermentum* and *P. pentosaceus*. The study

therefore recommends further investigations on technological, functional and sensory properties of the dominant microflora to establish their roles during fermentation and to explore their potential use as starter cultures in fermented soybeans and soybean-maize products. Because some species were only recovered by culture-dependent techniques while others were only recovered by PCR-DGGE, this study confirms the need for polyphasic approaches to study microbial diversity of complex systems.

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Table 1: Biochemical and physiological characteristics of lactic acid bacteria strains isolated during fermentation

Group		I (Rods)		II (Cocci)	
Characteristics		A (n= 142)	B (n= 55)	C (n=35)	D (n=7)
CO_2 production from glucose		+[a]	+	-[b]	-
Hydrolysis of arginine		+	-	+	-
Growth at	4 ^oC	70 +[c]	28 +	20 +	5 +
	15 ^oC	120 +	47 +	33 +	+
	45 ^oC	141 +	52 +	+	+
	pH 3.9	138 +	54 +	+	+
Growth in	7% NaCl	136 +	46 +	+	6 +
	10% NaCl	93 +	32 +	24 +	3 +
Exopolysaccharide production		54 +	34 +	14 +	4 +
Amylolytic activity		3 w+[d]	8 w+	3 w+	1 w+

[a]+, all isolates positive
[b]-, all isolates negative
[c]Number then +, e.g. (70 +), 70 isolates positive
[d]Number then w+, e.g. (3 w+), 3 isolates weakly positive

Table 2: Distribution of isolates according to source of paste and fermentation time.

Lactic acid bacteria identity based on API	Frequency (%)	Fermentation time (hrs)	Source[a]					
			100S	90S	75S	100SBS	90SBS	75SBS
Lactobacillus buchneri	2	0		+				+
Lactobacillus fermentum 2	1	0						+
Lactobacillus plantarum 1	1	0	+					
Pediococcus pentosaceus 1	4	0		+		++	+	
Pediococcus pentosaceus 2	2	0				+	+	
Weissella confusa	2	0				++		
Lactobacillus brevis 1	4	24			+++		+	
Lactobacillus brevis 3	5	24	+	+		+	+	+
Lactobacillus buchneri	1	24				+		
Lactobacillus delbrueckii	1	24	+					
Lactobacillus fermentum 2	2	24	+		+			
Lactococcus lactis ssp. *lactis 1*	2	24	+				+	
Leuconostoc mesenteroides	1	24					+	
Pediococcus damnosus 2	1	24		+				
Pediococcus pentosaceus 1	4	24		++			++	
Weissella confusa	7	24	+++	+	++			+
Lactobacillus acidophilus	2	48	+		+			
Lactobacillus brevis 1	9	48	+++		++	+++	+	
Lactobacillus buchneri	1	48				+		
Lactobacillus collinoides	2	48	+		+			
Lactobacillus delbrueckii	2	48	+		+			
Lactobacillus fermentum 2	2	48	++					

Table 2 continued: Distribution of isolates according to source of paste and fermentation time

Lactic acid bacteria identity based on API	**Frequency (%)**	**Fermentation time (hrs)**	**Source[a]**					
			100S	90S	75S	100SBS	90SBS	75SBS
Lactococcus lactis ssp. *lactis* 1	2	48	+			+		
Lactobacillus pentosus	1	48				+		
Lactobacillus plantarum 1	2	48				+	+	
Lactobacillus plantarum 2	1	48				+		
Pediococcus pentosaceus 1	2	48	+	+				
Pediococcus pentosaceus 2	1	48				+		
Weissella confusa	4	48	++		+	+		
Lactobacillus acidophilus	1	72				+		
Lactobacillus brevis 1	1	72				+		
Lactobacillus brevis 1	4	24			+++		+	

[a]Naturally fermented pastes (NFP) are 100S, 90S and 75S and lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS.

Table 3. Comparison of identification by phenotyping (API) and 16S rDNA sequencing of the LAB isolates

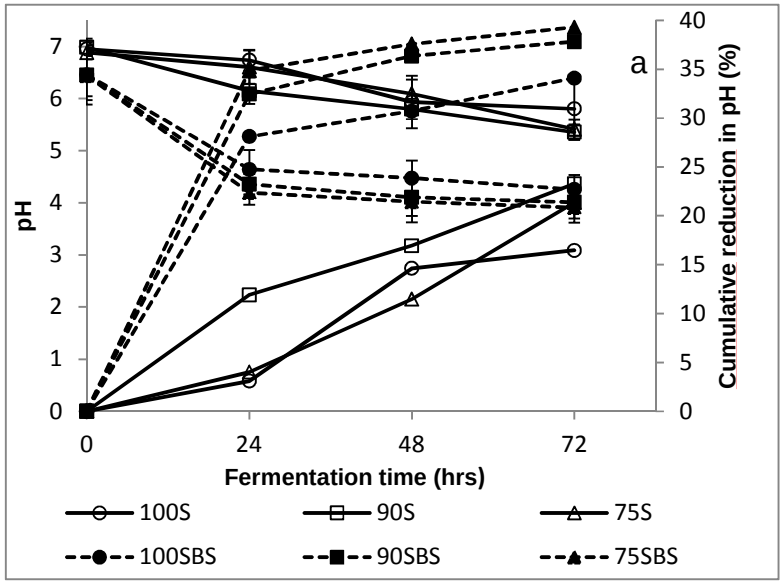
No of Strains (Isolate identity number)	Fermentation type	API Identification[a]	16 S rDNA Identification[b]
2 (189, 369)	Heterofermentative	*Lactobacillus acidophilus 1*	*W. cibaria*
2 (314, 127)	Heterofermentative	*Lactobacillus brevis 1*	*Lb. fermentum*
2(172, 122)	Heterofermentative	*Lactobacillus brevis 1*	*W. cibaria*
2(116(1), 152)	Heterofermentative	*Lactobacillus brevis 3*	*Lb. brevis*
2(336, 342b)	Heterofermentative	*Lactobacillus buchneri*	*Lb. fermentum*
1(317)	Heterofermentative	*Lactobacillus collinoides*	*Lb. fermentum*
1(188b)	Heterofermentative	*Lactobacillus collinoides*	*W. cibaria*
1(187,)	Heterofermentative	*Lactobacillus delbrueckii* ssp. *delbrueckii*	*W. cibaria*
1(76)	Heterofermentative	*Lactobacillus delbrueckii* ssp. *delbrueckii*	*W. confusa*
2(109T, 297)	Heterofermentative	*Lactobacillus fermentum 2*	*Lb. fermentum*
1(221a)	Heterofermentative	*Lactobacillus plantarum 2*	*W. cibaria*
2(5)	Homofermentative	*Lactococcus lactis* ssp. *lactis 1*	*E. faecium strain D-TSB-8/ E. durans*
1(53s)	Heterofermentative	*Leuconostoc mesenteroides* ssp. *mesenteroides/dextranicum 2*	*W. cibaria*
1(301)	Homofermentative	*Pediococcus damnosus 2*	*P. pentosaceus*
3(280, 120, 315)	Homofermentative	*Pediococcus pentosaceus*	*P. pentosaceus*
1(125)	Heterofermentative	*Weissella confusa*	*Bacillus* spp. *JS-37*
2(184, 205)	Heterofermentative	*Weissella confusa*	*W. cibaria*
5(107, 71, 67, 105, 218)	Heterofermentative	*Weissella confusa*	*W. confusa*

[a] Only isolates with API percent identity >90% are presented.

[b]Similarity for 16S rDNA was between 98 and 100%, except in *Bacillus* spp. where % similarity was 81; E-values for all isolates were < 0.0

Table 4: Sequencing results of representative DGGE bands from the bacterial DGGE fingerprint in Fig 4.

Band No.	Closest relative (NCBI accession number)	Identity (%)
1	Uncultured *Weissella* sp. isolate DGGE gel band D (HM359077.1)	100
2	*Weissella koreensis* (CP002899.1)	94
3,7	*Lactobacillus fermentum* (JX393057.1)	96-100
4, 6,8,11	*Weissella cibaria/W.confusa* (JX041943.1, JX0411934.1)	100
5	*Pediococcus pentosaceus* (JQ806718.1)	98
9,10	Sequences could not generate readable results	
12	*Lactobacillus linderi* (NR029308.1)	91
13	*Lactobacillus fermentum* (JX202610.1)	93
14	*Bacillus* sp. Hb-0511 (GQ487541.1)	100
15	Uncultured bacterium isolate DGGE gel band C15 (HM115944.1)	100



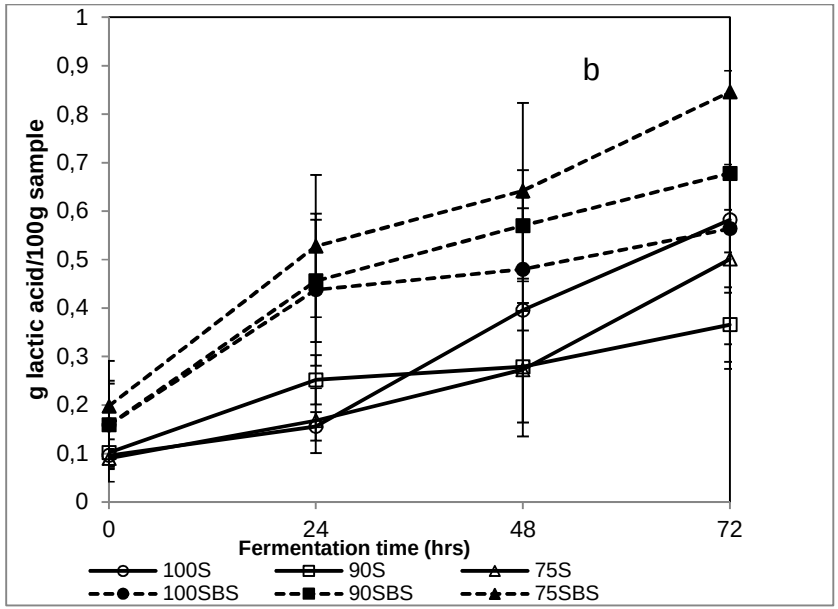


Fig 1a: Changes in pH and cumulative percent reduction in pH during fermentation. Naturally fermented pastes (NFP) are 100S, 90S and 75S. Lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS. Pastes are designated according to 100%, 90% and 75% soybean composition, the remaining proportions being maize.

Fig 1b: Changes in titratable acidity during fermentation**.** Naturally fermented pastes (NFP) are 100S, 90S and 75S. Lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS. Pastes are designated according to 100%, 90% and 75% soybean composition, the remaining proportions being maize.

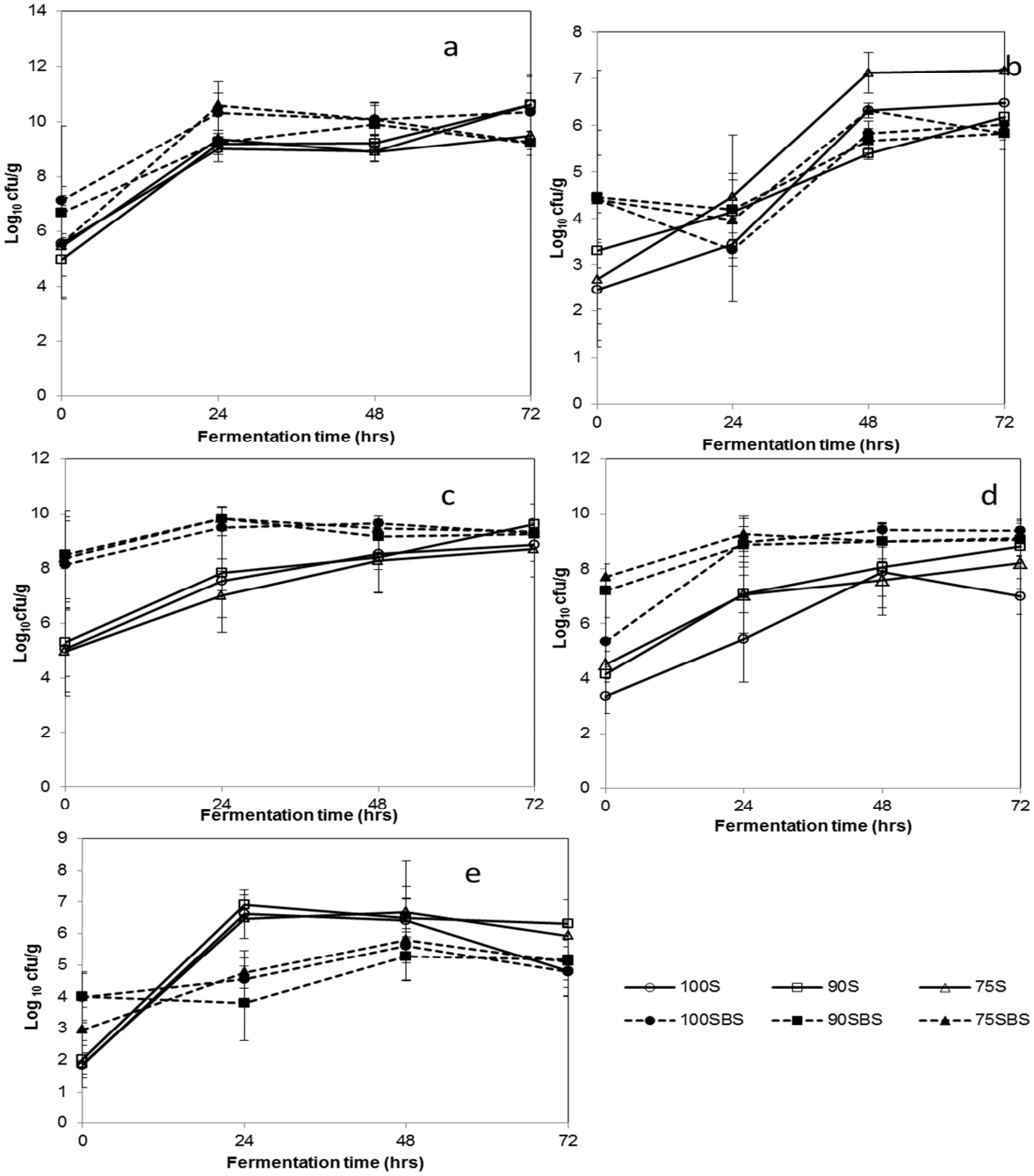


Fig 2: Extent of microbial growth during fermentation at 30 ^{o}C. a) Aerobic mesophillic counts; b) Bacteria spore counts; c) Lactic acid bacteria counts; d) *Lactobacillus* counts; e) Yeasts and molds count. **N**aturally fermented pastes (NFP) are 100S, 90S and 75S. Lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS. Pastes are designated according to 100%, 90% and 75% soybean composition, the remaining proportions being maize.

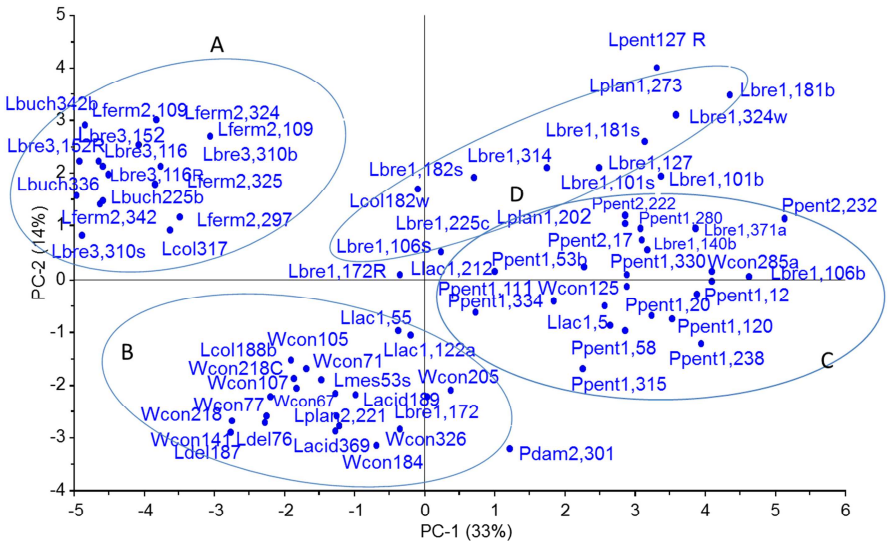


Fig.3A

Fig 3a: Principal component analysis scores plot showing clusters of the isolates formed according to their sugar fermentation profiles, carbon dioxide production and arginine hydrolysis. Lacid, *Lactobacillus acidophilus*; Lbre1, *Lb. brevis* 1; Lbre3, *Lb. brevis* 3; Lbuch, *Lb. buchneri*; Lcol, *Lb. collinoides*; Ldel, *Lb. delbrueckii*; Lferm2, *Lb. fermentum* 2; Lpent, *Lb. pentosus*; Lplan1, *Lb. plantarum* 1; L.plan2, *Lb. plantarum* 2; Llac1, *Lactococcus lactis* ssp. *lactis* 1; Lmes, *Leuconostoc mesenteroides*; Pdam2, *Pediococcus damnosus* 2; Ppent1, *P. pentosaceus* 1; Ppent2, *P. pentosaceus* 2; Wcon, *Weissella confusa*.

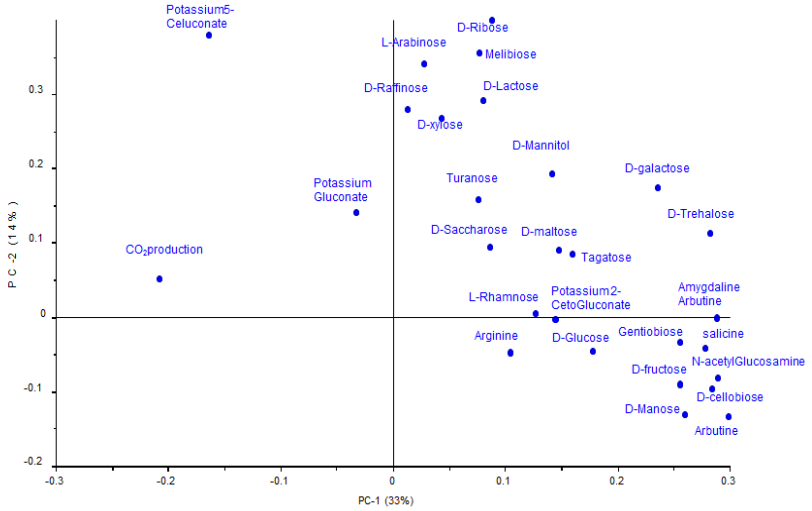


Fig 3b: Principal component analysis loadings plot showing the fermented sugars that categorized the isolates into different groups.

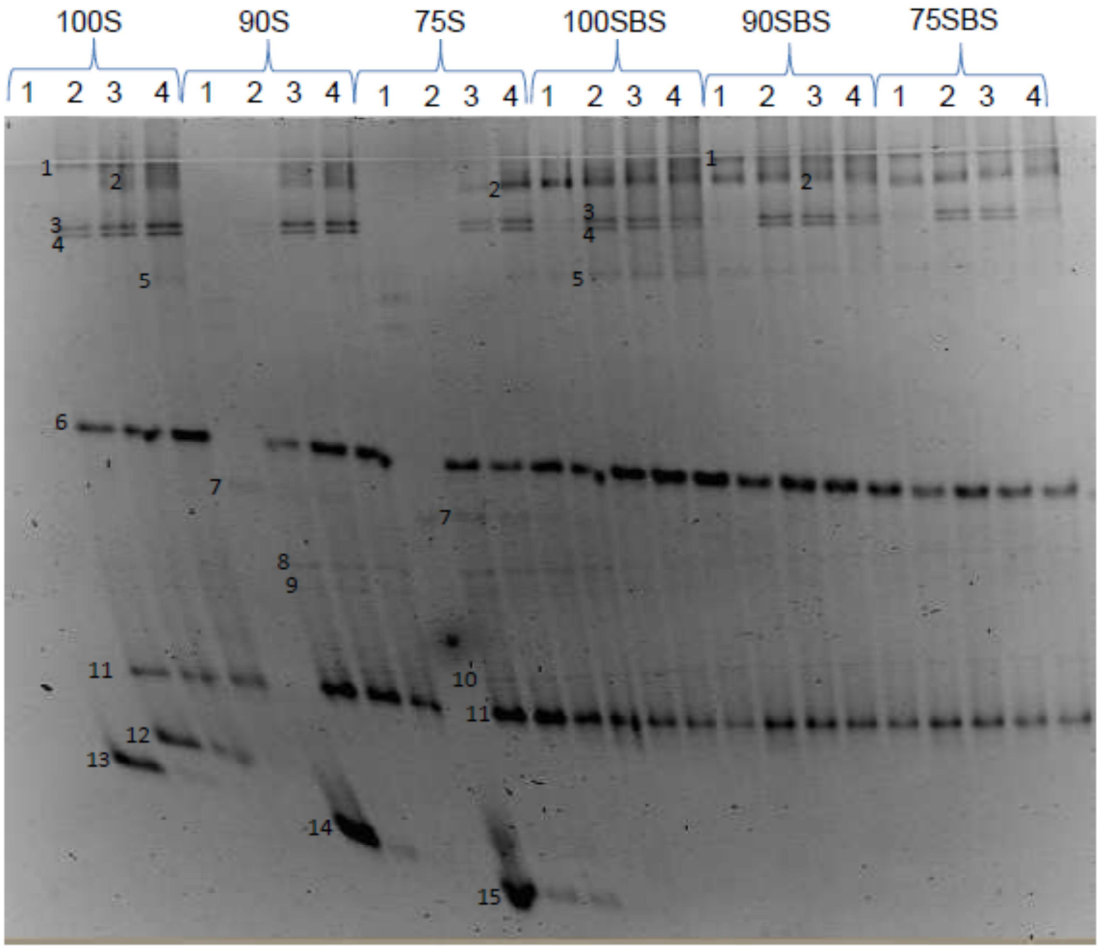


Fig 4: DGGE analysis of V3 16S rDNA gene fragments amplified from fermented soybeans and soybean-maize blend pastes generated by PRUN518r and PRBA338fgc primers. Lanes 1, 0h; lanes 2, 24 h; Lanes 3, 48h; Lanes 4, 72 h. A 20-50% denaturing gradient was used. Results of the DNA sequence analyses of the bands are summarized in Table 4. **N**aturally fermented pastes (NFP) are 100S, 90S and 75S; and lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS. Pastes are designated according to 100%, 90% and 75% soybean composition, the remaining proportions being maize.

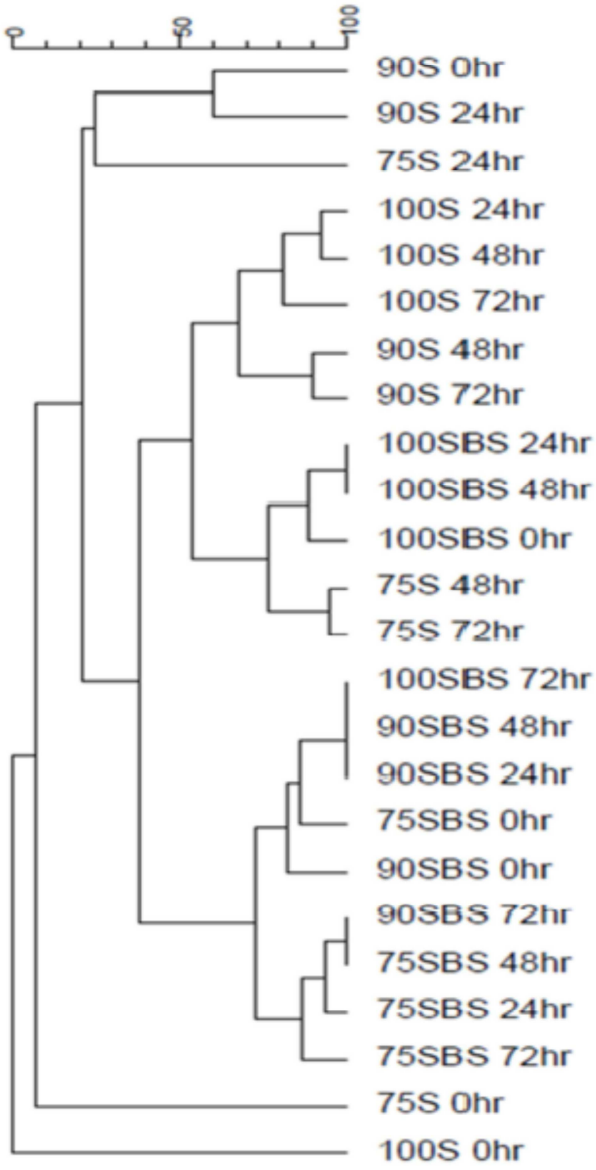


Fig 5: Cluster analysis of bacteria DGGE fingerprints showing similarity in microbial profiles during fermentation of the pastes. Dendrogram was generated by an UPMGA cluster analysis based on DGGE bands. Scale shown is Dice's coefficient of similarity. **N**aturally fermented pastes (NFP) are 100S, 90S and 75S; and lactic acid bacteria fermented pastes (LFP) are 100SBS, 90SBS and 75SBS. Pastes are designated according to 100%, 90% and 75% soybean composition, the remaining proportions being maize.

PAPER III

Food Science & Nutrition

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ORIGINAL RESEARCH

Sensory evaluation and consumer acceptance of naturally and lactic acid bacteria-fermented pastes of soybeans and soybean–maize blends

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Keywords
Drivers of liking, lactic acid bacteria fermentation, natural fermentation, preference mapping, soybean pastes

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Abstract

Fermented pastes of soybeans and soybean–maize blends were evaluated to determine sensory properties driving consumer liking. Pastes composed of 100% soybeans, 90% soybeans and 10% maize, and 75% soybeans and 25% maize were naturally fermented (NFP), and lactic acid bacteria fermented (LFP). Lactic acid bacteria fermentation was achieved through backslopping using a fermented cereal gruel, *thobwa*. Ten trained panelists evaluated intensities of 34 descriptors, of which 27 were significantly different ($P < 0.05$). The LFP were strong in brown color, sourness, umami, roasted soybean- and maize-associated aromas, and sogginess while NFP had high intensities of yellow color, pH, raw soybean, and rancid odors, fried egg, and fermented aromas and softness. Although there was consumer ($n = 150$) heterogeneity in preference, external preference mapping showed that most consumers preferred NFP. Drivers of liking of NFP samples were softness, pH, fermented aroma, sweetness, fried egg aroma, fried egg-like appearance, raw soybean, and rancid odors. Optimization of the desirable properties of the pastes would increase utilization and acceptance of fermented soybeans.

Introduction

Diets of most rural Malawian households are poorly diversified and are predominantly maize-based. Maize contributes to over 60% of energy, total iron, zinc, riboflavin, and about half of protein consumption, when animal-source foods are scarce (Ecker and Qaim 2011). Yet, maize has low protein content (9.42%) and is limited in micronutrients (Nuss and Tanumihardjo 2010). Such maize-based diets increase the risk of various types of malnutrition. In Malawi, the prevalence of chronic malnutrition among under-5 children is high, that is 47% (National Statistics Office and ICF Macro 2011), and micronutrient deficiencies were reported among under-5 children, women, and men (National Statistics Office and Macro 2005). Malnutrition in Malawi is attributed to insufficient energy and nutrient intake, among other factors (Maleta 2006). Animal-source foods provide good

quantities of protein and other nutrients, but they are expensive. This calls for alternative low-cost source of nutrient-dense food that can be consumed by adults and children.

Legumes, including soybeans (*Glycine max*), provide good quantities of protein, riboflavin, calcium, and iron (Messina 1999). Soybeans have been used in the prevention and treatment of protein energy malnutrition in young children, and in improving the nutritional status of communities. Therefore, soybean is a good alternative to expensive animal-source proteins (United Nations Industrial Development Organization 2003). In Malawi, soybean is produced mainly as a cash crop with limited household-based consumption (CYE Consult 2009; Tinsley 2009). Production increased over the past 5 years and in 2010, 73,000 tonnes of soybeans were produced. Most of the soybeans (63,000 tonnes) were used within the country. However, the demand for production is driven by the poultry feed industry (Markets and Economic Research Centre of the National Agricultural Marketing Council 2011) while there is limited demand from the corn–soy blend industry (Tinsley 2009). Unfortunately, there is no statistics indicating the percent consumption of both industries. Nevertheless, various reports show that direct human consumption of soybeans in Malawian households is through enriched maize flour containing up to 20% soybean flour (Katona-Apte 1993; Kalimbira et al. 2004; Maleta 2006; CYE Consult 2009; Tinsley 2009). The enriched flour locally known as *Likuni Phala* is used as a weaning food in children (Kalimbira et al. 2004; Maleta 2006; CYE Consult 2009) and is also distributed by nongovernmental organizations for school feeding programs, for hospitals, orphanages, and refugee camp usage (Katona-Apte 1993; Tinsley 2009). Consumption of maize together with soybeans provide high-quality protein diet comparable to diets containing animal protein (Asgar et al. 2010), because limiting amino acids in maize are complemented by those found in soybeans (Siegel and Fawcett 1976; FAO 1992).

Despite the nutritional benefits, household soybean utilization in Malawi is still minimal due to limited knowledge in processing (Coulibaly et al. 2009). Processing is required to eliminate antinutritional factors and the undesirable characteristic "beany" taste. Various processing methods such as boiling, steaming, roasting, germination, fermentation, and milling improve soybean utilization (Siegel and Fawcett 1976; Anderson and Wolf 1995; Golbitz 1995; Wang and Murphy 1996). Use of fermented soybean products in Asia is widely documented (Sarkar et al. 1994; Kwon et al. 2010; Dajanta et al. 2012; Park et al. 2012).

In order to increase direct household consumption of soybeans in Malawian diets, pastes of fermented soybeans and soybean–maize blends were developed as an alternative low-cost source of protein. The pastes were naturally fermented or lactic acid bacteria (LAB) fermented through backslopping using a traditional fermented cereal gruel, *thobwa*. The developed pastes were to be used as side dishes, such as in *kinema* (Sarkar et al. 1994) and other similar products of the Orient. Most soybean-fermented products are naturally fermented by *Bacillus subtilis* (Steinkraus 1997), a proteolytic microorganism that produces ammonia during fermentation (Sarkar and Tamang 1995; Dakwa et al. 2005). High amounts of ammonia result in strong odor, which some people find objectionable (Allagheny et al. 1996; Parkouda et al. 2009). LAB fermentations, on the other hand, improve flavor of traditional foods (Steinkraus 1997).

The developed products were new to Malawian consumers; therefore, it was important to obtain consumer feedback for improvement of the products. Preference mapping (PREFMAP) techniques were used to find out the potential of the developed products for future use and to determine the sensory properties driving consumer preferences. PREFMAP techniques have been widely used in different food products (Helgesen et al. 1997; Lawlor and Delahunty 2000; Guinard et al. 2001; Thompson et al. 2004) to understand sensory attributes that drive consumer acceptability (Murray and Delahunty 2000; Thompson et al. 2004; van Kleef et al. 2006; Dooley et al. 2010; Resano et al. 2010). Thus, the objectives of this study were to describe sensory properties of the fermented pastes, to determine consumer acceptance of the pastes, and to find out sensory properties that drive acceptance of the pastes.

Material and Methods

Preparation of pastes of soybeans and soybean–maize blends

Pastes of soybeans and soybean–maize blends were prepared in the laboratory. Soybeans (Nasoko, variety code 427/6/7) were sorted, washed, and boiled for 30 min and dehulled by rubbing between palms in cold water, washed again, and then boiled for 1 h (Dakwa et al. 2005). Maize (DK8071) was boiled for 2 h (to make it soft) before being ground together with soybeans into a paste. Grinding was done for 10–15 min in a Waring Commercial blender (800ES; Waring, Torrington, CT), which was sterilized by boiling for 5 min. Sterile water (100 mL) was added during the grinding to make the pastes. LAB fermentation was facilitated by the addition of fermented maize and finger millet (*Eleusine coracana*) gruel (*thobwa*). The preparation of *thobwa* was according to Kitabatake et al. (2003). Pastes for LAB fermentation (LFP) were backslopped (BS) using 10% (v/w) *thobwa*. The pH of the *thobwa* was around 4.5 with a LAB population of

10^8 cfu/mL. Naturally fermented pastes (NFP) were made by similar treatments but without adding the fermented gruel. Paste composition was determined based on preliminary laboratory trials whereby pastes containing 100%, 75%, and 50% soybeans (the remaining proportions being maize) were studied. The preliminary study showed no significant differences in pH reduction and microbial loads (total aerobic count and LAB count) in pastes containing 75% and 50% soybeans. Thus for the study, pastes were prepared according to the following compositions: pastes of soybeans only; pastes of soybean and maize blends containing 90% and 75% soybeans. NFP were designated as 100S, 90S, and 75S according to 100%, 90%, and 75% soybean composition in the pastes, the remaining proportions being maize. Similarly, BS LAB-fermented pastes were designated 100SBS, 90SBS, and 75SBS. Portions of 500 g for all treatments were fermented at 30°C for 72 h in glass jars.

Analyses of chemical and physical properties

Titratable acidity (g lactic acid/100 g sample) and pH were determined according to AOAC (1990). The pH was measured using a pH meter (WTW pH 525; D. Jurgens and Co., Bremen, Germany) fitted with a glass electrode (WTW SenTix 97T). Amino acids were extracted from freeze-dried homogenized samples and were determined using High-performance liquid chromatography according to Bütikofer and Ardö (1999). Salt content was determined using a Sherwood MK II Chloride Analyzer (Model 926; Sherwood Scientific Ltd., Cambridge, U.K.) according to the manufacturer's operating instructions. Freeze-dried samples (1.00 g) were mixed with 20 mL of distilled water. The mixtures were heated to 55°C for 30 min and were filtered before chloride analysis. Viscoelastic properties of the samples were analyzed using a Physica MCR301 rheometer (Paar Physica, Antony Paar, Germany) fitted with a 50-mm plate/plate geometry, PP50. The temperature was kept at 20°C by the Peltier control of the bottom plate. The sample was placed on the bottom plate and gently compressed. The gap was ~3 mm, and a constant normal force of 5 N was applied to the sample while testing took place. Amplitude sweeps were then done in oscillation at a frequency of 1 Hz varying the amplitude from 0.01% to 110% strain.

Descriptive sensory analysis

Panel selection and training

Ten people interested in sensory evaluation of the fermented pastes were recruited among Nutrition and Food Science students in the Department of Home Economics and Human Nutrition; and staff members at Lilongwe University of Agriculture and Natural Resources, Bunda College campus. Panelists with ability to discriminate five tastes (salty, sweet, sour, umami, and bitter) in a solution system were selected by conducting five sets of directional paired comparison tests. Four men and six women in the age range of 20–32 years were selected as panelists. Consensus training as explained by Lawless and Heymann (1998) was conducted. Panelists were exposed to soybean-fermented pastes to be tested in the descriptive analysis sessions. Through consensus, panelists generated terms (descriptors) and definitions to describe the sensory differences among the samples. Panelists also decided on words to anchor the descriptive terms and some reference standards to be used. Trial evaluations were performed to enable decision on panelists' reproducibility. Thirty-four descriptors/attributes describing appearance, aroma/odor, taste, and texture were generated. The descriptors, their meanings, and the reference standards used are presented in Table 1. Four training sessions per week were held for 1.5 months and each session lasted ~1 h 30 min.

Sample preparation and presentation

Maize starch (1%, w/w) was added to the fermented pastes to prevent crumbling during frying. The pastes were molded into rounds ca. 5 g each, and were fried in heated (180–195°C) soybean oil for 3–5 min. Fresh oil was used for each sample. One hour before sensory evaluation, four pieces of 5 g of each fried sample were transferred to a separate glass serving container before covering with aluminum foil. Each sample was coded with a three-digit random number and the samples were presented in random order to the panelists for evaluation. The temperature of the samples at the time of evaluation was room temperature (around 25°C).

Descriptive analysis procedure

Ten panelists were trained to rate attribute intensities of the six products using a 15-point unstructured line scale labeled with either "none, weak, or least" as point 1 and "very strong" as point 15. Each panelist evaluated the products individually. Products were evaluated in three sessions and all products were served at each session, hence the sessions acted as replicates. Tap water was provided to panelists to rinse their palate before and between tasting. The evaluation was conducted in a well-ventilated laboratory fitted with fluorescent lights. The temperature in the evaluation room was between 23°C and 25°C.

Table 1. Descriptors and definitions used to explain sensory characteristics of the fermented pastes.

Descriptors	Abbreviations	Meanings of the descriptors	Reference/standards used
Appearance			
Brown	Brown	Intensity of brown color of the fried pastes	Color wheel
Yellow	Yellow	Intensity of the yellow color of the fried pastes	Color wheel
Fried egg-like	EggL	Appearance associated with fried egg	Fried egg
Chitumbuwa-like	ChituL	Appearance associated with a local snack, *chitumbuwa,* made from deep frying maize flour batter	*Chitumbuwa*
Mandazi-like	MandL	Appearance associated with local fritters, *mandazi*, made from deep frying wheat flour batter	*Mandazi*
Aroma/odors			
Raw soybean odor	RawS	Characteristic soybean odor strong in soymilk made from raw soybeans hydrated in cold water	Raw soymilk
Roasted soybean aroma	RoastS	Aroma associated with roasted soybean	Crushed roasted soybean
Burnt roasted soybean odor	BRoastS	Odor associated with burnt roasted soybean	Crushed burnt roasted soybean
Roasted maize aroma	RoastM	Aroma associated with roasted dried maize	Crushed roasted maize
Burnt roasted maize odor	BRoastM	Odor associated with burnt roasted dried maize	Crushed burnt roasted maize
Soaked burnt roasted maize odor	SBRoastM	Odor associated with soaked burnt roasted dried maize	Soaked burnt roasted maize
Chitumbuwa aroma	ChituA	Aroma associated with a local snack, *chitumbuwa,* made from deep frying maize flour batter	*Chitumbuwa*
Mandazi aroma	MandA	Aroma associated with local fritters, *mandazi,* made from deep frying bread flour batter	*Mandazi*
Chigumuyoyo aroma	Chigumu	Aroma associated with a local snack, *chigumuyoyo,* made from baking maize flour batter	*Chigumuyoyo*
Fried egg aroma	EggA	Aroma associated with fried egg	Fried egg
Fermented aroma	FermA	Aroma associated with fermented cereals	Sugar solution (20%) fermented for 24 h by 1.5 g yeast.
Matsukwa odor	*Matsukwa*	Odor associated with water for soaking degermed maize	Water from degermed maize soaked for 2 days
Kondoole aroma	*Kondoole*	Aroma associated with fermented cassava, *kondoole*	Fermented cassava
Thobwa aroma	*Thobwa*	Aroma associated with a local fermented beverage "*thobwa"*	*Thobwa*
Chambiko aroma	*Chambiko*	Aroma associated with fermented milk, *chambiko*	Commercially available *Chambiko'*
Fermented beans aroma	FBeans	Aroma associated with fermented kidney beans	Cooked beans fermented for 24 h
Mafuta a chiwisi odor	*Chiwisi*	Odor associated with partially heated cooking oil	Soybean cooking oil heated at 100°C for 2 min

Table 1. Continued.

Descriptors	Abbreviations	Meanings of the descriptors	Reference/standards used
Rancid odor	Rancid	Odor associated with rancid oil	Soybean cooking oil reused more than three times
Texture			
Softness	Soft	Amount of force necessary to compress the sample when pressed between fingers	No standard
Easiness to break	Brittle	How easy it was to break the sample (brittle)	Toasted bread, intensity = 15
Surface roughness	Rough	Irregularities on the surface or not a smooth surface	Custard pudding = 1 intensity
Graininess	Grainy	Size of the grains seen inside the sample when broken	Maize and soy grains; whole = 15 and half = 7 intensity
Sogginess	Soggy	Tendency of the sample to absorb oil as observed by pressing the sample between white paper	Comparison of amount of oil absorbed on white paper
Taste			
Sourness	Sour	Taste sensation typical of organic acids	Citric acid; 0.05% = 2 intensity, 0.2% = 15 intensity
Sweetness	Sweet	Taste sensation typical of sucrose solution	Sucrose solution; 2% = 2 intensity and 16% = 15 intensity
Saltiness	Salty	Taste sensation typical of sodium chloride	Sodium chloride; 0.2% = 2 intensity and 1.5% = 15 intensity
Bitterness	Bitter	Taste sensation typical of caffeine and quinine	0.01% quinine sulfate solution
Umami	Umami	Taste sensation typical of monosodium glutamate (MSG)	MSG solution; 0.3% = 3 intensity, 0.7% = 7 intensity
Aftertaste	AfterT	Taste lingering on tongue after sample is removed	Similar to unripe banana taste

Consumer acceptability test

A total of 150 consumers interested in participating in the study were recruited from three villages that participated in a nutrition, health, and agriculture project in Lungwena extension planning area, Mangochi, Malawi. Products were prepared and presented in the same way as in the descriptive analysis except that 1% (w/w) of salt was added prior to frying. Salt was added based on consumer recommendations during a questionnaire pretesting. Products were presented one at a time in a random order. The samples were coded with three-digit random numbers and served in a central location.

Consumers evaluated acceptance on taste, smell, color, smoothness, and overall acceptance of the six products using a 7-point facial hedonic scale. On the scale, point 1 referred to dislike extremely and 7 referred to like extremely, 4 was neither like nor dislike and was in the middle. Consumers were instructed on how to use the scale. Consumers were instructed to sniff and taste a sample. They were also allowed to re-taste and change their

previous scores, if needed. Tap water was provided to consumers to rinse their palate before and between tasting.

Statistical analysis

During training, panelists' reproducibility was determined using analysis of variance (ANOVA) at $P = 0.05$. Scores of each panelist were compared with the rest of the panelists for each sample. When significant differences were found, Duncan's test was performed to identify panelists that differed and the specific descriptors they scored differently. Panelists who were not reproducible were assisted to improve performance. Panel consensus was checked using profile plots generated from PanelCheck. At the end of the descriptive analysis, PanelCheck was used to assess panelists' consensus and discrimination ability of the attributes (Tomic et al. 2010). Mean intensity scores of the descriptors were compared using three-way ANOVA and least square difference test ($P = 0.05$) as post hoc, with panelists, replicates, and products as factors. Correlations between sensory attributes were also obtained.

To understand sensory attributes that characterized the products, principal component analysis (PCA) was performed. In order to identify attributes driving consumer liking, external PREFMAP was obtained by performing a partial least squares regression (PLSR) analysis. Mean intensity scores of attributes that were significantly different ($P < 0.05$) on product effect and mean values of chemical and physical properties were used in PCA and PLSR. Data in PCA and PLSR were centered, full cross-validated, and standardized. Sensory data and data on chemical and physical properties of the pastes were used as explanatory variables (X matrix) while means of overall consumer acceptance data were used as response variables (Y matrix) (Helgesen et al. 1997; Resano et al. 2010).

To identify consumer subgroups sharing common preference patterns, hierarchical cluster analysis using complete linkage and squared Euclidian distance was performed on consumer overall acceptance data. Means of overall acceptance obtained for each cluster and data on sensory, chemical, and physical properties of the pastes were used to obtain a PREFMAP of the clusters. The sensory, chemical, and physical properties data provided the X matrix while means of overall acceptance of clusters provided the Y matrix. Demographic information of the subgroups obtained through cross-tabulations provided an understanding of cluster compositions.

ANOVA, cluster analysis, cross-tabulations, and correlations were performed in SPSS 15.0 (SPSS Inc., Chicago, IL) while PCA and PLSR were performed in UnscramblerX 10.2 (CAMO Software, AS, Norway).

Results and Discussion

Chemical and physical properties of the pastes

There were significant differences ($P < 0.05$) in pH of the samples between the NFP and the LFP. LAB-fermented pastes had lower pH values ranging from 3.91 to 4.26 compared to NFP that had pH values ranging from 5.36 to 5.81 (Table 2). There was an agreement between lactic acid content, presented as titratable acidity, and pH levels in the pastes and the sensory perception of sourness. Lactic acid contents were higher in LFP than in NFP and so were the perceived sourness intensities. On the contrary, the amino acid contents did not agree with umami, bitterness, and sweetness taste perceptions. Amino acids in their free state (as L, D, and DL) contribute to bitter, sweet, and umami tastes in most foods. In this study, amino acids responsible for bitterness and umami were generally high in NFP while those responsible for sweetness were high in LFP (Table 2). However, perceived intensities of these tastes by descriptive sensory panel (Table 3) differed from the expectation from the chemical analyses. Panelists rated LFP high in bitterness and umami while NFP were rated high in sweetness. Descriptive sensory perception of bitterness was high in LFP probably because of interactions of the bitter compounds and the other tastants in the fermented pastes. According to Mukai et al. (2007), mixtures of bitter and sweet tastes resulted in variable effects at low intensity/concentration, while mixtures at moderate and high intensity/concentrations were mutually suppressive. In LFP, mixtures of sweet and bitter tastes were at low concentrations resulting in enhancement of bitter taste. While in NFP, the concentrations of sweet tastes were moderate and the overall concentrations of bitter tastes were high, resulting in suppression of bitterness. Furthermore, bitterness in LFP could have been enhanced due to interactions between sour and bitter compounds at low concentrations (Mukai et al. 2007). On the other hand, bitterness in NFP could have been reduced by aspartic and glutamic acids. Although there were no significant differences in aspartic acid contents among all samples, 90S had the highest content. Furthermore, glutamic acid content was highest in 100S and the content was significantly different between 100S and the rest of the pastes except 90S (Table 2). Thus overall, the amino acids imparting umami flavor were higher in NFP. Aspartic and glutamic acids were reported to be effective in reducing bitterness of solutions comprising bitter amino acids in low

Table 2. Physical and chemical analyses of the fermented pastes.

Parameter[1]	Taste	100S	100SBS	90S	90SBS	75S	75SBS
pH		5.81 ± 0.59^{a}	4.26 ± 0.28^{c}	5.36 ± 0.14^{b}	4.01 ± 0.31^{c}	5.41 ± 0.18^{b}	3.91 ± 0.29^{c}
Titratable acidity (TA)	Sourness	0.58 ± 0.31^{a}	0.56 ± 0.13^{a}	0.37 ± 0.08^{b}	0.68 ± 0.16^{ac}	0.50 ± 0.18^{a}	0.85 ± 0.24^{c}
Histidine (His)	Bitterness	0.38	n.d	0.24	n.d	0.07 ± 0.05	n.d
Arginine (Arg)	Bitterness	0.06^{ab}	0.07 ± 0.01^{ab}	0.07 ± 0.06^{ab}	0.05 ± 0.01^{ab}	0.04 ± 0.02^{a}	0.10 ± 0.03^{b}
Tyrosine (Tyr)	Bitterness	0.07 ± 0.05^{ab}	0.06 ± 0.02^{ab}	0.18 ± 0.19^{a}	0.04 ± 0.01^{b}	0.08 ± 0.06^{ab}	0.04 ± 0.02^{b}
Valine (Val)	Bitterness	1.00 ± 0.31^{a}	0.53 ± 0.21^{ab}	0.89 ± 0.95^{a}	0.26 ± 0.14^{b}	0.50 ± 0.35^{ab}	0.18 ± 0.10^{b}
Methionine (Met)	Bitterness	0.03 ± 0.02^{a}	0.04 ± 0.02^{a}	0.05^{a}	0.02^{a}	n.d	0.01^{a}
Isoleucine (Iso)	Bitterness	0.55 ± 0.16^{ac}	0.13 ± 0.06^{a}	0.65 ± 0.72^{c}	0.04 ± 0.03^{b}	0.32 ± 0.21^{ac}	0.1 ± 0.07^{a}
Phenylalanine (Phe)	Bitterness	2.19 ± 0.81^{ab}	0.96 ± 0.71^{ab}	2.59 ± 2.85^{b}	0.42 ± 0.40^{a}	1.11 ± 0.77^{ab}	0.28 ± 0.27^{a}
Leucine (Leu)	Bitterness	1.66 ± 0.91^{a}	0.38 ± 0.20^{b}	1.61 ± 1.76^{a}	0.22 ± 0.12^{c}	0.67 ± 0.58^{ab}	0.17 ± 0.10^{c}
Aspartate (Asp)	Umami	0.79 ± 0.40^{a}	0.78 ± 0.10^{a}	1.23 ± 0.86^{a}	0.90 ± 0.03^{a}	0.78 ± 0.31^{a}	0.72 ± 0.12^{a}
Glutamate (Glu)	Umami	4.84 ± 0.39^{a}	2.55 ± 0.31^{b}	3.71 ± 2.16^{ab}	3.07 ± 0.26^{b}	2.38 ± 0.59^{b}	3.14 ± 1.06^{b}
Serine (Ser)	Sweetness	0.63 ± 0.06^{a}	0.18 ± 0.04^{b}	0.29 ± 0.25^{b}	0.19 ± 0.01^{b}	0.3 ± 0.14^{b}	0.18 ± 0.01^{b}
Glycine (Gly)	Sweetness	0.47 ± 0.08^{ac}	1.07 ± 0.38^{b}	0.57 ± 0.25^{ac}	1.06 ± 0.05^{b}	0.29 ± 0.13^{c}	0.73 ± 0.01^{a}
Alanine (Ala)	Sweetness	3.63 ± 0.38^{a}	3.84 ± 1.25^{a}	2.63 ± 1.90^{ab}	3.27 ± 0.54^{a}	1.43 ± 0.34^{b}	2.54 ± 0.66^{a}
Lysine (Lys)	Sweetness	0.95 ± 0.06^{abc}	1.49 ± 0.63^{a}	0.92 ± 0.53b^{c}	1.47 ± 0.21^{ab}	0.74 ± 0.24^{c}	0.78 ± 0.19^{c}
Salinity	Saltiness	240 ± 32.66^{a}	262.5 ± 23.63^{a}	228.75 ± 49.39^{a}	241.25 ± 33.26^{a}	245 ± 19.15^{a}	272.5 ± 22.55^{a}

Means not sharing a superscript within a row are significantly different ($P < 0.05$). Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

[1]Units of measurement: titratable acidity (g lactic acid/100 g sample), amino acids (μmol/g), salinity (mg/L).

concentrations (Lindqvist 2010). Apart from amino acids, bitterness in soybeans is also influenced by bitter isoflavone glucosides, which are hydrolyzed during fermentation to bitter isoflavone aglycones (Drewnowski and Gomez-Carneros 2000). Salt content ranged from 228 to 272 mg/L (0.037–0.046%) and was low compared to other fermented soybean pastes, which can contain up to 14% salt (Kim et al. 2010). Salt was mainly due to chlorides naturally present in plants. Although saltiness was rated high in LFP, there were no significant differences ($P > 0.05$) in salinity among the samples. This study agrees with the suggestion that the interaction between tastes is not a fixed action depending on the intensity/concentration of each taste, but rather an enhancing or inhibitory effect, changing with the combined pattern of intensity and concentration (Mukai et al. 2007).

All the samples behaved as viscoelastic solids. Tests in normal rotation were not done as the samples slipped on the rheometer surfaces before yield occurred. The reason for the slimy sample surface was probably due to the presence of exopolysaccharides produced by some LAB. There were no significant differences in relative stiffness between NFP and LFP.

Descriptive sensory analysis

Thirty-four descriptors/attributes describing appearance, aroma/odor, taste, and texture were generated to characterize the sensory properties of the fermented pastes (Table 1). There was high agreement among panelists in rating the intensities of the attributes as observed from the profile plots (data not shown) as most assessor lines followed the consensus lines closely (Tomic et al. 2010). Out of the 34 attributes, 27 were significantly different ($P < 0.05$) on product effect. The attributes not significantly different were roasted maize, *kondoole*, *chambiko*, and fermented beans aromas, *mafuta a chiwisi* odor, and readiness to be broken (Table 1 presents meanings of descriptors). Only attributes that were significantly different on product effect were used in further analyses.

Differences among samples in the following attributes: burnt roasted soybean, *chitumbuwa* and *mandazi* aromas, rancid odor, brown and yellow colors, *chitumbuwa*-like, *mandazi*-like, and fried egg-like appearances, umami, and sourness tastes and soggy texture were clearly discriminated by panelists as observed from the high F ratios (Table 3). Overall, the panel's ability to discriminate between samples was good, although Tucker plots (data not shown) showed that some assessors had low discrimination ability in a few attributes, namely graininess, roasted soybean, soaked roasted maize and *thobwa* aromas. These attributes had relatively low F ratios as well (Table 3).

Significant correlations were observed among sensory descriptors. Attributes strong in intensities in NFP had significant ($P < 0.001$) positive correlations with each other and this trend were similar in LFP (Table 4). Conversely, attributes strong in intensities in NFP

Table 3. Mean intensity scores, *F* ratios and *P*-values of descriptors/attributes based on product effect.

Attribute	100S	100SBS	90S	90SBS	75S	75SBS	*F* ratio	*P*-value
Raw soybean odor	7.17 ± 3.52^{a}	4.37 ± 1.65^{bc}	5.07 ± 2.35^{ab}	3.43 ± 1.65 ^{cd}	6.00 ± 2.84^{ab}	2.80 ± 1.73^{d}	7.51	<0.000
Roasted soybean aroma	4.21 ± 1.65^{a}	5.1 ± 1.90^{b}	4.67 ± 2.31^{ab}	4.53 ± 2.85^{ab}	4.4 ± 2.90	4.40 ± 2.55a	2.67	0.03
Burnt roasted soybean odor	2.55 ± 1.15^{a}	4.9 ± 2.23^{b}	3.67 ± 2.22^{c}	4.93 ± 1.99^{b}	3 ± 1.51^{ac}	5.10 ± 2.34^{b}	10.15	<0.000
Roasted maize aroma	3.2 ± 1.63^{a}	4.6 ± 1.96^{b}	4.21 ± 2.04^{ab}	3.33 ± 1.45^{a}	4.33 ± 3.03^{b}	3.87 ± 1.78^{ab}	2.12	0.07
Burnt roasted maize odor	2.7 ± 1.68^{a}	4.57 ± 2.37^{b}	3.43 ± 2.25^{ac}	4.03 ± 1.87^{bc}	3.4 ± 2.65^{ac}	4.43 ± 2.16^{b}	3.80	0.00
Soaked burnt roasted maize odor	3.07 ± 1.99^{ab}	3.53 ± 2.92^{a}	3.55 ± 2.72^{a}	3.13 ± 2.33^{ac}	2.37 ± 1.47^{bc}	4.00 ± 3.04^{a}	2.41	0.04
Chigumuyoyo aroma	2.13 ± 1.36^{a}	4.17 ± 2.33^{b}	3.33 ± 1.99^{c}	4.47 ± 2.42^{b}	2.83 ± 1.84^{ac}	5.67 ± 2.42^{d}	8.62	<0.000
Chitumbuwa aroma	1.9 ± 1.03^{a}	4.87 ± 2.74^{b}	3.53 ± 2.37^{c}	9.07 ± 2.75^{d}	2.83 ± 1.86^{ac}	9.44 ± 3.19^{e}	48.82	<0.000
Mandazi aroma	3.4 ± 1.48^{ac}	3.57 ± 2.03^{ab}	3 ± 1.58^{a}	4.55 ± 2.38^{b}	3.9 ± 2.07^{bc}	6.73 ± 3.05^{d}	12.54	<0.000
Fermented aroma	6.93 ± 3.66^{a}	4.07 ± 2.08^{b}	4.2 ± 2.12^{b}	5.63 ± 2.76^{c}	4.87 ± 3.09^{bc}	4.53 ± 2.37^{bc}	5.37	0.00
Matsukwa odor	3.2 ± 1.92^{a}	3.97 ± 1.94^{a}	3.37 ± 1.94^{a}	3.4 ± 2.21^{a}	3.13 ± 2.01^{a}	3.20 ± 2.37^{a}	0.44	0.83
Kondoole aroma	3.8 ± 2.66^{a}	3.43 ± 1.89^{ab}	2.87 ± 1.63^{bc}	2.8 ± 1.81^{c}	2.9 ± 2.01^{ac}	2.66 ± 1.80^{bc}	1.26	0.29
Thobwa aroma	4.10 ± 2.55^{a}	3.71 ± 2.53^{ab}	2.71 ± 1.65^{c}	3.33 ± 2.18^{ac}	2.91 ± 1.81^{bc}	3.35 ± 2.35^{bc}	2.55	0.03
Chambiko aroma	2.73 ± 1.57^{a}	2.97 ± 1.87^{a}	2.7 ± 1.91^{a}	2.73 ± 2.24^{a}	2.63 ± 1.94^{a}	2.77 ± 2.15^{a}	0.37	0.87
Fermented beans aroma	2.57 ± 1.55^{ab}	2.73 ± 2.02^{b}	2.27 ± 1.51^{a}	2.6 ± 2.04^{ab}	1.8 ± 1.03^{a}	2.3 ± 1.78^{ab}	1.18	0.33
Fried egg aroma	3.43 ± 1.91^{a}	3.2 ± 1.83^{a}	3.13 ± 1.53^{a}	2.5 ± 1.59^{b}	3.67 ± 2.43^{a}	2.2 ± 1.38^{b}	4.12	0.00
Mafuta a chiwisi odor	2.57 ± 2.27^{a}	2.5 ± 2.10^{a}	2.5 ± 1.94^{a}	2.43 ± 1.92^{a}	2.33 ± 2.26^{a}	2.67 ± 2.37^{a}	0.10	0.99
Rancid odor	9.07 ± 4.02^{a}	4.87 ± 2.36^{bcd}	5.07 ± 2.42^{c}	3.4 ± 2.16^{d}	5.8 ± 2.46^{c}	3.57 ± 2.22^{bd}	13.37	<0.000
Brown	2.83 ± 2.10^{a}	7.2 ± 3.03^{b}	3.07 ± 2.09^{a}	11.03 ± 2.40^{c}	2.8 ± 1.86^{a}	12.97 ± 1.40^{d}	101.65	<0.000
Yellow	10.07 ± 2.82^{a}	5.5 ± 2.95^{b}	9.8 ± 2.59^{a}	2.5 ± 1.31^{c}	10.6 ± 2.40^{d}	1.33 ± 0.55^{c}	74.90	<0.000
Chitumbuwa-like	3.37 ± 2.95^{a}	6.43 ± 2.25^{b}	3.7 ± 2.55^{a}	8.35 ± 2.98^{c}	2.7 ± 1.56^{d}	8 ± 3.62^{c}	23.78	<0.000
Mandazi-like	2.67 ± 2.16^{ac}	5.23 ± 2.43^{b}	3.47 ± 2.36^{c}	6.97 ± 3.30^{bd}	2.57 ± 1.72^{a}	7.63 ± 2.79^{d}	19.36	<0.000
Fried egg-like	7.73 ± 2.74^{a}	4.37 ± 2.55^{b}	7.5 ± 2.70^{a}	1.69 ± 0.81^{c}	7.73 ± 2.84^{a}	1.3 ± 0.60^{c}	42.25	<0.000
Sweetness	2.07 ± 1.36^{ac}	1.67 ± 0.71^{b}	1.83 ± 0.83^{bc}	1.4 ± 0.50^{b}	2.63 ± 1.97^{a}	1.7 ± 0.65^{b}	4.77	0.001
Saltiness	1.93 ± 0.91^{acd}	2.53 ± 1.91^{ab}	1.57 ± 0.77^{c}	2.63 ± 2.55^{a}	1.83 ± 0.95 ^{cd}	2.7 ± 1.97^{ad}	3.39	0.007
Umami	3.5 ± 1.72^{a}	5.23 ± 2.58^{b}	3.5 ± 1.81^{a}	6.07 ± 2.86^{b}	3.47 ± 1.80^{a}	7 ± 3.09^{c}	17.29	<0.000
Sourness	3 ± 1.49^{a}	7.08 ± 3.05^{b}	5.47 ± 2.85^{c}	10.07 ± 3.29^{d}	4.4 ± 2.28^{c}	10.9 ± 3.24^{d}	32.17	<0.000
Bitterness	1.8 ± 1.49^{ad}	2.48 ± 2.38^{ab}	1.6 ± 1.16^{ac}	2.9 ± 2.86^{b}	1.47 ± 0.78 ^{cd}	3.0 ± 3.49^{b}	3.11	0.01
Aftertaste	3.13 ± 2.16^{ac}	4.27 ± 2.42^{ab}	2.6 ± 1.59^{c}	4.6 ± 2.96^{ab}	2.53 ± 1.80^{c}	5.1 ± 2.68^{b}	5.09	0.000
Surface roughness	5.2 ± 2.55^{a}	6.07 ± 2.83^{a}	5.7 ± 2.44^{a}	7.5 ± 3.15^{b}	7.73 ± 2.52^{b}	8.4 ± 2.94^{b}	6.01	<0.000
Softness	7.63 ± 3.46^{a}	6.6 ± 3.29^{ab}	5.93 ± 3.26^{a}	5.03 ± 3.40^{c}	5.17 ± 2.89^{bc}	4.03 ± 2.76^{c}	7.08	<0.0001
Easiness to break	6.47 ± 2.97^{a}	6.97 ± 2.91^{a}	6.83 ± 2.65^{a}	7.5 ± 4.02^{a}	6.37 ± 3.05^{a}	6.77 ± 3.38^{a}	0.58	0.72
Graininess	3.9 ± 1.90^{a}	4.35 ± 2.35^{a}	4.5 ± 2.42^{a}	4.53 ± 2.30^{a}	6.17 ± 2.96^{b}	4.97 ± 3.09^{a}	2.89	0.02
Sogginess	8.5 ± 2.55^{a}	7.11 ± 3.05^{a}	6.31 ± 2.52^{b}	8.6 ± 2.81^{a}	5.31 ± 2.42^{b}	11.12 ± 2.85^{c}	20.79	<0.000

Intensity based on a scale of 1–15 (1 = none or least or very weak, 15 = very strong intensity). Means not sharing a superscript within a row are significantly different ($P < 0.05$). Samples coded 100S, 90S, and 75S represent naturally fermented pastes, while samples coded 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize. Full names of terms and their meanings are given in Table 1.

negatively correlated with those strong in intensities in LFP. Strong correlations were observed amongst appearance attributes ($r = -0.439$ to -0.844). Brown color strongly positively correlated with *chitumbuwa-like* and *mandazi-like* appearances and also negatively correlated with yellow color and fried egg-like appearance.

Table 4. Pearson correlations between descriptors/attributes characterizing the fermented pastes.

	RawS	BRoastdS	Chigumu	ChituA	MandA	Rancid	Brown	Yellow	ChituL	MandL	EggL	Umami	Thobwa
Brown	−0.468	0.477	0.358	0.665	0.462	−0.437	1						
Yellow	0.470	−0.391	−0.394	−0.670	−0.350	0.367	−0.844	1					
ChituL	−0.356	0.496		0.503	0.339	−0.256	0.736	−0.617	1				0.371
MandL	−0.344	0.410	0.271	0.526	0.448	−0.375	0.733	−0.563	0.672	1			0.327
EggL	0.334	−0.290	−0.299	−0.612	−0.315	0.363	−0.717	0.757	−0.444	−0.439	1		
Umami	−0.280	0.303		0.409	0.268	−0.271	0.496	−0.441	0.555	0.497	−0.374	1	
Sour	−0.366		0.527	0.719	0.219	−0.292	0.577	−0.643	0.355	0.411	−0.603	0.365	
AfterT		0.347			0.326		0.446	−0.351	0.405	0.376	−0.247	0.361	0.369
Soggy			0.353	0.491			0.362	−0.383	0.226	0.292	−0.360	0.251	
MandA	−0.231			0.297									0.404
Rancid	0.412			−0.277									
Sweet				−0.296			−0.224				0.283		
Bitter		0.248			0.224		0.336	−0.262	0.298				0.294
Rough					0.283	−0.274	0.262	−0.238	0.261			0.296	
Soft	0.221			−0.287		0.259		0.256					0.284
Salty			0.372	0.261			0.267	−0.252			−0.237		
Grainy		0.269			0.268								0.360
ChituA	−0.362	0.381	0.573										
SBRoastM		0.291			0.266				0.292	0.335			0.553
BRoastM		0.509		0.263	0.381		0.415		0.353	0.393	−0.230		0.337
	RoastS		BRoastM		Sour		EggA		Grainy	Bitter	SBRoastM		FermA
BRoastS	0.476	SBRoastM	0.335	Soft	−0.261	EggL	0.327	AfterT	0.258	0.375	0.229	RawS	0.347
BRoastM	0.346	Salty	0.244	Soggy	0.472	MandA	0.266	Rough	0.245			Rancid	0.574
ChituL	0.280	Grainy	0.295	Sweet	−0.277	Grainy	0.282	SBRoastM	0.219	0.259	1		
Thobwa	0.357			Rough	0.235	SBRoastM	0.251	Salty		0.238			
Grainy	0.270					AfetrT	0.282						
	Rough		Soggy			Sweet	0.270						
Soft	−0.228	Sweet	−0.280										

Only attributes showing significant correlations at $P = 0.001$ are presented. Full names of terms and their meanings are given in Table 1.

Appearance attributes also strongly correlated with aroma attributes and some tastes. For instance, brown color, *chitumbuwa-like*, and *mandazi-like* appearances showed significant ($P < 0.001$) positive correlations with *chitumbuwa* aroma, umami, bitterness, aftertaste, and sourness. *Mandazi* aroma positively correlated with *thobwa* aroma, fried egg aroma, umami, and aftertaste. Egg-like appearance positively correlated with sweetness. The intensities of attributes with significant positive correlations with brown color were high in LFP, while the intensities of attributes with significant positive correlations with yellow color were high in NFP. Therefore, the type of fermentation greatly influenced the appearance of the fermented pastes. Among aromas that strongly correlated with each other were fermented aroma and rancidity. Fermented aroma intensity and rancidity were highest in 100S; in addition, rancidity was high in all NFP (Table 3). High fermented aroma intensity in 100S could be due to uneven fermentation in NFP due to spontaneous fermentation by natural microflora over a long period (Kim et al. 2010). Significant positive correlations were also observed between textural properties, including roughness and graininess, which were attributes influenced by composition.

Sensory properties of the fermented pastes

Sensory properties characterizing the products are shown in PCA map in Figure 1. The first two principal components (PC1 and PC2) explained 74% of the variation. This highly explained variance in PC1 and PC2 shows that there was high systematic variation within the data, indicating that the panel discriminated well between the products. The score plot (Fig. 1A) shows product distribution in multivariate space and PC1 explains differences in the products according to type of fermentation, distinguishing NFP on the left from LFP on the right. Attributes responsible for this categorization were appearance,

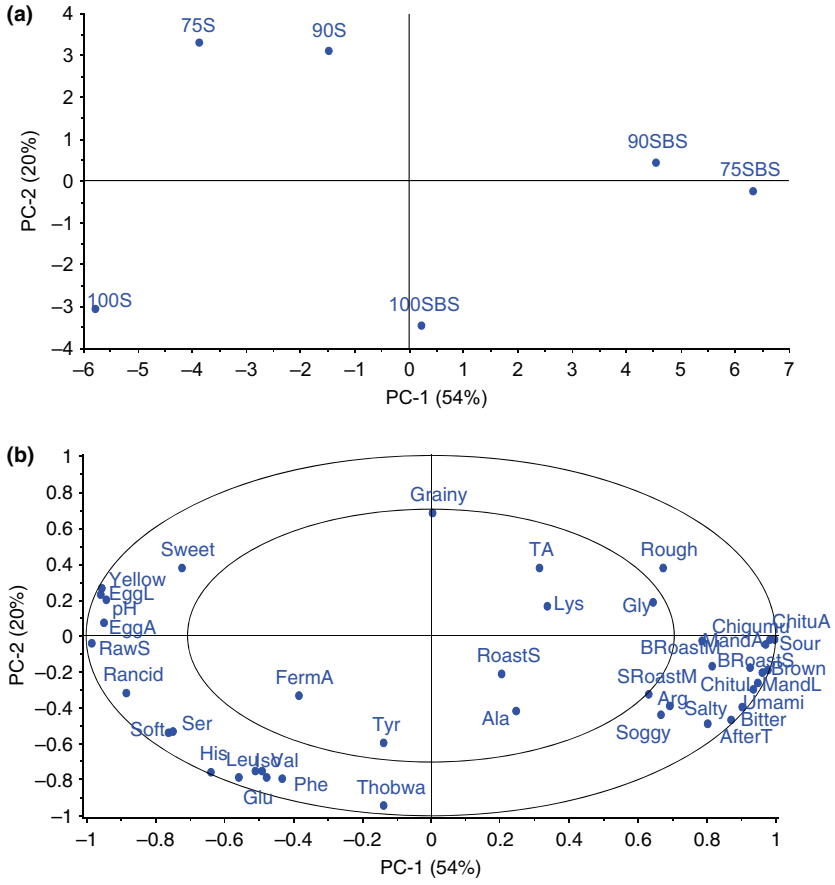

Figure 1. Principal component analysis of fermented pastes and sensory attributes. (A) Score plot showing relatedness of samples in terms of sensory, chemical, and physical properties of the pastes. (B) Correlations loading plot showing sensory properties of the pastes. On the map, 100S, 90S, and 75S represent naturally fermented pastes (NFP) while 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes (LFP). Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

some odors/aromas, taste, and pH. The mean intensities of yellow color, fried egg-like appearance, raw soybean odor, fried egg aroma, and pH were high in NFP. These attributes also loaded highly on the negative side of PC1 (Fig. 1B) and were sensory properties characterizing NFP. On the positive side of PC1, brown color, *chitumbuwa*- and *mandazi*-like appearances, burnt roasted soybean odor, *chigumuyoyo* and *chitumbuwa* aromas, umami, bitterness, aftertaste, and sourness loaded highly (Fig. 1B). These attributes had high intensities in LFP, hence characterized LFP. Most of the amino acids responsible for bitterness (histidine, arginine, tyrosine, valine, isoleucine, phenylalanine, leucine) and glutamate responsible for umami loaded highly on the negative side of PC1. Due to the comparatively higher content of bitter amino acids, it would be expected that NFP would have higher bitterness intensity compared to LFP. On the contrary, LFP were perceived to be more bitter than NFP, probably because of taste interactions in which glutamate could have suppressed bitterness in NFP and sourness could have enhanced bitterness in LFP.

The proximity of 90S and 75S; and 90SBS and 75SBS on the PCA map (Fig. 1A) indicates close similarity in terms of sensory properties, unlike 100S and 100SBS, which are clearly separated from the other NFP and LFP, respectively. This delineation is along PC2 on which graininess and sweetness loaded highly on the positive dimension, while *thobwa* aroma, fermented aroma, and softness loaded highly on the negative dimension (Fig. 1B). The intensities of fermented aroma and softness were high in 100S, while the intensities of sweetness and graininess were high in 90S and 75S.

Yellow color of NFP originated from the color of soybeans, while brown color in LFP could be due to caramelization and Maillard reactions. Color of fermented soybean pastes like *doenjang/miso* is due to the raw materials used, amino carbonyl reaction of Maillard browning, oxidative browning, enzymatic browning, and browning enhancers (Chung and Chung 2007). In this study, 100S, 90S, and 75S underwent natural fermentation, while 100SBS, 90SBS, and 75SBS were BS with a LAB-fermented product; hence, type of fermentation had a major influence on color, giving the strong browning intensity in LFP. LAB fermentations increase the amount of reducing sugars in products (Sripriya et al. 1997) and the sugars could be responsible for the browning intensity due to caramelization and participation in Maillard reactions with amino carbonyls during frying. As increasing pH values enhance both caremelization and Maillard reactions (Ajandouz and Puigserver 1999; Ajandouz et al. 2001), it would be expected that NFP would have stronger browning intensities than LFP. However, all the samples had pH values below 6.0, thus were slightly acidic and offered some stability of the amino acids that were heated in the presence of reducing sugars (Ajandouz and Puigserver 1999). The difference in browning intensity was probably due to the difference in the amount of reducing sugars, which were more in LFP than in NFP (data not shown).

Tastes of the pastes were significantly different ($P < 0.05$). NFP had low sourness intensity and their pH values were only slightly reduced from the initial. However, sweetness intensities were higher in NFP and particularly in 75S, probably because of its high maize content; hence high content of sugars resulted in higher sweet intensity. On the other hand, LFP were positively associated with sourness, umami, bitterness, saltiness, and aftertaste. The intensities of these tastes were highest in 75SBS. Chung and Chung (2007) found that fermented soybean products with high saltiness also had high umami (monosodium glutamate, MSG) and sour tastes. Although salt was not added to all the samples, its perception could be due to the presence of NaCl and KCl, which were attributed to saltiness perception in Korean-fermented soybean pastes, *doenjang* (Kim and Lee 2003). Besides, salt content alone does not sufficiently predict perceived saltiness intensity as synergistic interactions of salt and other flavor compounds also affect saltiness perception (Kim et al. 2010). Sourness in fermented products is due to organic acids, which increase during LAB fermentations. Because 75SBS had the highest maize content, it provided more fermentable sugars as substrate for organic acid production by LAB. In soybean-fermented pastes, malic, citric, succinic, and lactic acids are responsible for the sour taste (Kim et al. 2010). In this study, sour taste could have been due to succinic, lactic, and acetic acids, which were detected (data not shown). Umami taste is related to glutamic and aspartic acids (Kim and Lee 2003), which are present in soybeans and tend to increase with fermentation (Dajanta et al. 2011). Another study on similar products (Kim et al. 2010) reported high bitterness intensities, which were attributed to bitter amino acids produced during fermentation. Amino acids responsible for bitter taste include leucine and isoleucine (Namgung et al. 2010). Salts, sugars, organic acids, umami compounds, amino acids, Maillard peptides, types of base ingredients, microorganisms, and various aroma compounds contribute to flavors of fermented soybean products (Chung and Chung 2007).

A range of aromas and odors were described. NFP had high intensities of raw soybean odor, rancid odor, fermented aroma, and fried egg aroma. Raw soybean and rancid odors are among the flavors that reduce consumer acceptance of soy products (Torres-Penaranda et al. 1998). The two odors were highest in 100S. On the other hand, LFP were characterized by aromas associated with roasted soybeans and maize. In this case, LAB

fermentation was able to mask the characteristic beany and rancid odors of soybeans that are due to oxidation of polyunsaturated lipids catalyzed by lipoxygenase (Ediriweera et al. 1987). Rancid odor is associated with volatile compounds such as 3-methylbutanoic acid, 2-methylpropanoic acid, and butanoic acid, a major compound in different fermented soybean foods (Jo et al. 2011).

Textural differences were also described. 100S was rated softer than the rest of the samples while graininess and roughness intensities were high in products containing maize, particularly 75S, 75SBS, and 90SBS. Differences in composition of the products accounted for the differences in textural properties, resulting in rough appearance and large particle sizes in the products containing maize. Additionally, LFP absorbed more oil during frying than NFP; this tendency could have been due to their slightly higher moisture content (data not shown), which led to more oil uptake as the water evaporated during frying (Krokida et al. 2000).

Consumer acceptance

A total of 150 consumers participated in the consumer acceptance study but demographic information was collected on 148. A consumer was defined as a person who occasionally consumed soybeans and soybean-based products. At the time of the study, 32.4% of the participants had consumed soybean-based products within the past 2 months from the date of data collection. Soybeans were mostly consumed in porridge (69%), although some of the consumers used texturized soy products locally known as *soya pieces* as relish (side dish), roasted beans as snack, soy flour as a condiment in vegetables and other foods, in addition to using soybeans in porridge (Table 5). Soybean flour is used together with maize flour in a weaning food prepared as porridge and locally known as *Likuni Phala*. Most consumers (88%) were aware that soybeans are nutritious as they associated the promotion of its use in growth-monitoring centers and in nutrition rehabilitation programs for under-5 children.

Table 5. Ways of household soybean consumption by the consumers.

Ways of consumption	Consumers (%) $n = 129$
Porridge only	89 (69)
Porridge and soya pieces	8 (6.2)
Soya pieces only	11 (8.5)
Porridge and roasted soybeans as snack	7 (5.4)
Porridge and soy flour vegetable condiment	8 (6.2)
Porridge, roasted soybeans, and soya pieces	4 (3.1)
Used in maize flour-based snacks	2 (1.6)

External PREFMAP

To understand the attributes driving consumer liking, sensory, chemical, and physical data were regressed with consumer acceptance data using PLSR. The sensory, chemical, and physical data were used as predictor variables, while overall consumer acceptance data were used as response variables. In Figure 2, PC1 and PC2 together explain 73% of the variation in the pastes in terms of their properties and 47% of the variation in consumer preference for the pastes. The location of the samples on the map is based mainly on sensory attributes and the preference pattern shows that consumers also used the same underlying sensory properties to make their acceptability. The high density of consumers in the two left quadrants (Fig. 2B) indicates that the direction of preference was toward NFP. These samples were characterized by strong yellow color, higher pH, fried egg-like appearance, and aroma, sweetness, softness, rancid odor, and raw soybean odor. It appears that the positive impact of higher pH (low sourness intensity), sweetness, and fried egg aroma exceeded the negative impact of rancid and raw soybean odors. These two odors have been documented as deterring consumer acceptance of soybeans (Gupta 1997; Torres-Penaranda et al. 1998). Therefore, higher pH (low sourness intensity), fried egg aroma, and sweet taste seem to be the drivers of liking of the fermented soybean pastes, especially for NFP for most consumers. Nevertheless, other consumers preferred LAB-fermented pastes, which had strong brown color, sourness, bitterness, saltiness, umami, burnt roasted soybeans, and maize aromas. These attributes seem to be drivers of liking of 90SBS and 75SBS and as they load directly opposite drivers of liking of NFP, they can be considered as drivers of disliking for consumers preferring 75S, 90S, and 100S.

Taste and pleasure are among the most important predictors of food choice (Brunsø et al. 2002). Bitterness and strong sourness could have been the key attributes leading to little acceptance of LFP. Bitter taste is a major problem in the food and pharmaceutical industries due to its negative hedonic impact on ingestion (Drewnowski and Gomez-Carneros 2000; Ley 2008). In most cases, the bitter taste is not desirable and has to be eliminated from or masked in the product to increase a product's acceptance. Umami is the savory delicious taste in meat, poultry, sea foods, and fermented beans (Yamaguchi and Ninomiya 2000). Although umami is among the drivers of dislike in this study, it could not have been the reason for dislike of LFP. Its inclusion among drivers of disliking in this study is because of its significant ($P < 0.001$) correlation with sourness and aftertaste ($r = 0.365$ and 0.361, respectively) intensities. Consumption of foods that are strong in sourness is typically avoided (Breslin and Spector 2008).

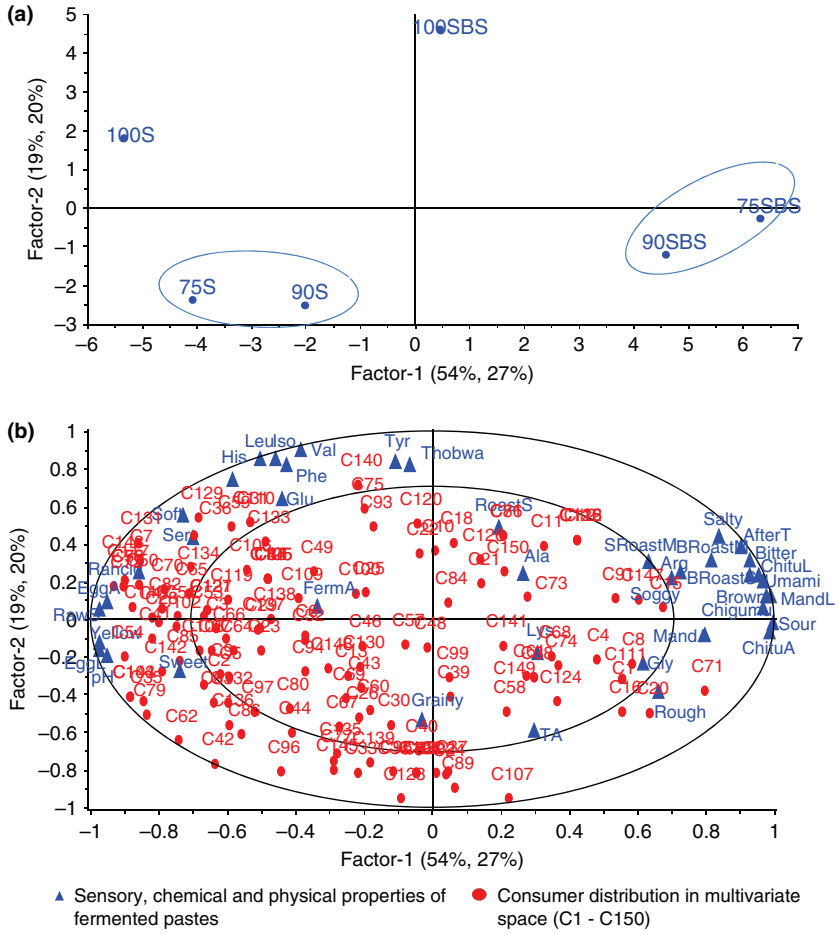

Figure 2. External preference mapping showing sensory attributes driving consumer preference of the pastes. (A) Grouping of pastes according to sensory, chemical, and physical properties and consumer preference. (B) Consumer preference pattern as influenced by sensory, chemical, and physical properties. On the map, 100S, 90S, and 75S represent naturally fermented pastes, while 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

In this study, mixed strains of LAB from *thobwa* were used in the fermentation of LFP. To improve acceptance of LAB-fermented soybeans, selection of strains that results in desirable sensory properties would be recommended. This strain selection can be achieved through identification and characterization of LAB involved in soybean fermentation.

Consumer preferences according to clusters

A visual inspection of the PREFMAP (Fig. 2) reveals heterogeneity in consumers' acceptability, although more consumers liked the NFP. Figure 2A shows four clusters as follows: 100S; 90S and 75S; 75SBS and 90SBS; and 100SBS. These clusters are mainly based on sensory properties as the PREFMAP is based on the PCA representation of sensory attributes (Resano et al. 2010). To understand this heterogeneity in consumer preference pattern more, a cluster analysis using a 6 × 150 matrix of pastes and overall consumer acceptance scores was performed. Cluster analysis assigned consumers with similar preference patterns to one group resulting in four clusters as well. A PCA of mean overall acceptance of the clusters and the pastes was then obtained (Fig. 3). The clustering pattern was slightly different from the pattern in Figure 2. Clusters 1 and 3 were composed of consumers who liked 100S and 90S, cluster 2 was composed of consumers who preferred LFP with a bias of 100SBS and 75SBS, while cluster 4 was composed of consumers that liked 75S. As seen in Figure 2, the direction of preference is biased toward NFP. The composition of consumers in each cluster is shown in Table 6. Consumers in cluster 2 disliked NFP and preferred LFP, particularly 100SBS. Consumers in cluster 3 liked 90S and 100S and disliked all the sour

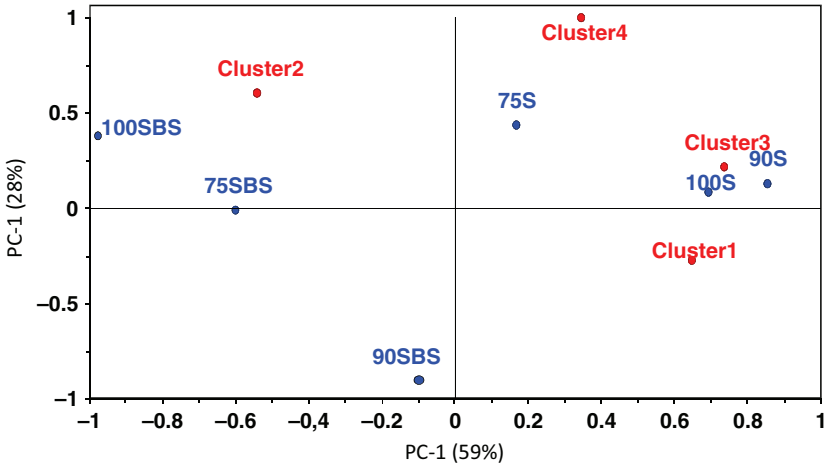

Figure 3. Principal component analysis of fermented pastes and consumer clusters. Naturally fermented pastes (100S, 90S, 75S) and lactic acid bacteria-fermented pastes (100SBS, 90SBS, and 75SBS). Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

Table 6. Demographic information of the clusters (numbers in parentheses are percentages).

Demography	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Total
Sex					
Male	22 (14.9)	3 (2.0)	2 (1.4)	4 (2.7)	31 (20.9)
Female	79 (53.4)	9 (6.1)	11 (7.4)	18 (12.2)	117 (79.1)
Total	101 (68.2)	12 (6.1)	13 (8.8)	22 (14.9)	148 (100)
Age					
14–29	53 (35.8)	5 (3.4)	5 (3.4)	16 (10.8)	79 (53.4)
30–49	28 (18.9)	5 (3.4)	4 (2.7)	4 (2.7)	41 (27.7)
50–80	20 (13.5)	2 (1.4)	4 (2.7)	2 (1.4)	28 (18.9)

Table 7. Mean overall acceptance scores for consumer clusters.

Cluster	100S	100SBS	90S	90SBS	75S	75SBS
1	6.25 ± 1.35^{a}	5.20 ± 2.1^{a}	6.23 ± 1.46^{a}	6.23 ± 0.90^{a}	6.39 ± 1.10^{a}	5.51 ± 1.84^{a}
2	2.67 ± 2.01^{b}	6.25 ± 0.87^{a}	2.75 ± 2.18^{b}	3.17 ± 2.86^{b}	6.50 ± 0.8^{a}	5.50 ± 2.02^{ac}
3	5.00 ± 2.35^{c}	1.31 ± 0.75^{b}	6.15 ± 1.68^{a}	2.69 ± 2.39^{bc}	4.38 ± 2.96^{b}	2.77 ± 2.49^{b}
4	6.46 ± 0.67^{a}	5.36 ± 1.18^{a}	6.32 ± 1.09^{a}	2.00 ± 1.31^{c}	6.00 ± 1.35^{a}	4.18 ± 2.04^{c}

Means not sharing a superscript within a column are significantly different ($P < 0.05$). 100S, 90S, and 75S represent naturally fermented pastes, while 100SBS, 90SBS, and 75SBS represent lactic acid-fermented pastes. Pastes are designated according to 100%, 90%, and 75% soybean composition, the remaining proportions being maize.

LFP products and 75S, while consumers in cluster 4 liked 75S and were slightly tolerant of the sour products except for 90SBS (Table 7).

Although 100SBS was rated lowest with an overall acceptance score of 1.31 by consumers in cluster 3 (Table 7), the same sample was rated 6.25 by consumers in cluster 2. Differences in overall acceptances of the same sample by different clusters underscore consumer heterogeneity. Attributes characterizing 100SBS also characterized LFP. These attributes were also considered as drivers of disliking by many consumers. Although the sensory attributes characterizing LFP were not necessarily highest in 100SBS, this paste stood out in terms of *thobwa* and roasted soybean aromas. These aromas loaded highly on PC2 (Fig. 2) and distinguished 100SBS from the other LFP samples, which were mainly characterized by attributes loading highly on PC1. These findings agree with the concept that consumer perception is complex and multidimensional. Consumers respond not only to a certain sensory input but also to other inputs perceived

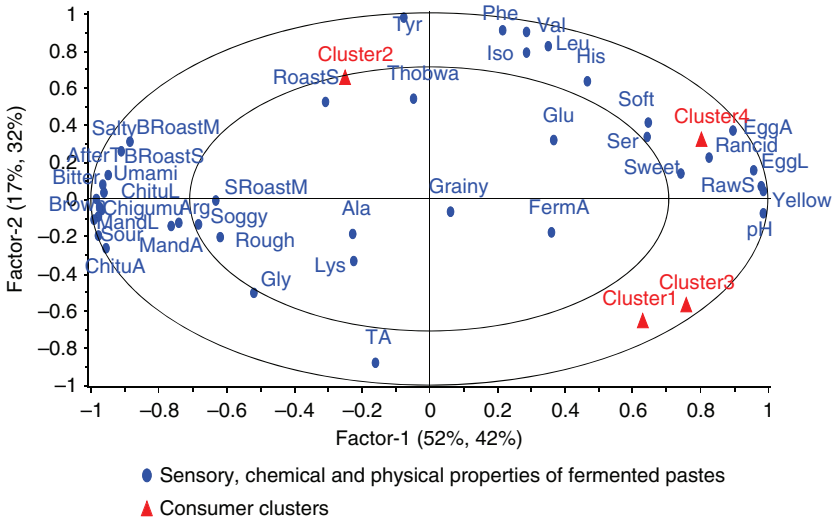

Figure 4. External preference mapping showing sensory attributes driving liking of the pastes by consumers in the clusters.

simultaneously and also to physical perceptual interactions among inputs (Costell et al. 2010).

To understand attributes driving preference of consumers in these clusters, PLSR was performed with sensory, chemical, and physical data as X matrix and means for overall consumer acceptance of the clusters as Y matrix. Figure 4 shows sensory properties driving consumer liking in the clusters. Consumers in clusters 1, 3, and 4 had similar drivers of liking, with some drivers having a greater influence in some clusters than in others. For instance, clusters 1 (n = 101) and 3 (n = 13) were characterized by consumers who liked 100S and 90S. Drivers of liking of these products were yellow color, higher pH, raw soybean odor, and fermented aroma. In this case fermented aroma was the main driver. In addition to attributes driving liking in clusters 1 and 3, sweet taste, fried egg aroma, fried egg-like appearance, rancid odor, and soft texture were drivers of consumer liking in cluster 4 (n = 22). In cluster 2, the main driver of liking of consumers (n = 12) was roasted soybean aroma and *thobwa* aroma. Attributes that loaded highly on the opposite direction of attributes driving liking of the majority of the consumers can be considered as drivers of dislike of these products. Therefore, burnt roasted soybean odor, *chigumuyoyo* aroma, soaked burnt roasted maize aroma, *mandazi* aroma, *chitumbuwa* aroma, *mandazi*- and *chitumbuwa*-like appearances, sourness, bitterness, saltiness, aftertaste, and brown color were drivers of dislike for most consumers.

Cluster 1 was the largest in terms of consumer composition followed by cluster 4 (Table 6). There were no significant differences in overall acceptance of the products by consumers of cluster 1 (Table 7), even though liking was biased toward NFP. This indicates that both naturally fermented and LAB-fermented pastes have the potential of being used by the consumers. However, to increase utilization and acceptance of the fermented pastes, it would be necessary to optimize drivers of liking influencing acceptance of NFP. Thus, optimizing pH, softness, raw soybean odor, rancid odor, fermented aroma, sweet taste, fried egg aroma, and appearance, and yellow color by increasing the desirable properties while decreasing intensities of undesirable properties would increase acceptability and utilization of fermented pastes.

Conclusions

The study concluded that the trained panel discriminated the products based on their type of fermentation; and consumers used similar discrimination in determining their preference patterns. Most consumers preferred NFP to LAB-fermented pastes. Strong intensities of yellow color, pH, sweet taste, raw soybean odor, rancid odor, fermented aroma, and soft texture in NFP were considered as positive. On the contrary, strong intensities of burnt roasted soybean odor, *chigumuyoyo* aroma, soaked burnt roasted maize odor, *mandazi* aroma, *chitumbuwa* aroma, *mandazi* and *chitumbuwa*-like appearances, sourness, bitterness, saltiness, aftertaste, and brown color, which characterized LFP were considered negative.

Consumer segmentation in liking of the products was identified, with direction of preference toward NFP. Consumers were assigned to four clusters, with the largest cluster composed of consumers who accepted all products almost similarly. This indicates that there is potential of utilization of both naturally and LAB-fermented soybean

pastes. However, optimization either by increasing or reducing intensities of drivers of liking or disliking would be recommended to increase utilization of the fermented pastes. Because of heterogeneity, optimization of attributes which were the main drivers of liking in different clusters such as pH, raw soybean odor, rancid odor, soft texture, sweet taste, egg aroma, yellow color, egg-like appearance, fermented aroma, and roasted soybean aroma would be recommended. However, as pH values of NFP were relatively high, a food safety challenge is recognized for NFP.

Being the first study on fermented soybean and soybean/maize blend pastes in Malawi, the information provided could be used in future developments of similar products for wide acceptance and utilization of soybeans.

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Conflict of Interest

None declared.

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CORRIGENDUM

Sensory evaluation and consumer acceptance of naturally and lactic acid bacteria-fermented pastes of soybeans and soybean–maize blends

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PAPER IV

Effect of natural and lactic acid bacteria fermentations on growth and survival of *Bacillus cereus* and *Escherichia coli* in pastes of soybeans and soybean-maize blends

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Abstract

Growth of *Escherichia coli* and *Bacillus cereus* in pastes of soybeans and soybean-maize blends during natural and lactic acid bacteria fermentations was investigated. Pastes containing 100% or 90% soybeans were either fermented naturally (100S, 90S) or fermented with *Lactobacillus fermentum* as starter culture (100SC, 90SC) or fermented through back-slopping using a tradition fermented cereal gruel, *thobwa* as an inoculum (100SBS, 90SBS). Each sample was singly inoculated with each pathogen. The pH of back-slopped samples decreased faster and the lowest pH was 4.8 at 24 hrs in pastes inoculated with *E. coli*. The pH values in *B. cereus* inoculated pastes were higher than in *E. coli* inoculated pastes at each sampling time from 24 hrs to 72 hrs. In NFP, the pH increased to 6.86 in 100S and 7.2 in 90S by 72 hrs. Back-slopping inhibited growth of the two pathogens more than the other two types of fermentation. At the end of the fermentations, highest *E. coli* counts were 3.1, 7.3 and 9.2 log cfu/g in 90SBS, 100SC and 100S, respectively. In pastes inoculated with *B. cereus*, highest counts by 72 hrs were 3.0, 5.0 and 8.72 cfu/g in 100SBS, 100SC and 90S, respectively. In 90SBS, *B. cereus* cells were less than 10 cfu/g at 24 and during further fermentation. There was preferential inhibition of *B. cereus* compared to *E. coli* in all fermentations. However, failure to reach critical pH value of 5.0 for *B. cereus* in *Lb. fermentum* fermentation and in natural fermentation; and pH value of 4.4 for *E. coli* at the end of all fermentations suggested food safety challenges. Therefore, a thermal processing step prior to consumption was recommended and in order to ascertain the safety of the pastes, studies on toxin production during fermentation were suggested.

Keywords: Fermentation, back-slopping, lactic acid bacteria, *Escherichia coli, Bacillus cereus,* soybean

Introduction

Fermentation is one of the oldest food processing techniques that improves food safety and leads to food preservation. Various fermentations that have been used traditionally are still practiced to produce lactic acid fermented cereal gruels, lactic acid fermented sourdoughs and alkaline fermented legumes among other foods (Kingamkono et al., 1998; De Vuyst et al., 2002; Chukeatirote et al., 2010). Fermented soybeans (*Glycine max*) have been traditionally produced and widely consumed in Asia (Chukeatirote et al., 2010). Soybeans contain 35-40% protein, 18% oil and physiologically beneficial phytochemicals (Dixit et al., 2011). Fermentation of soybeans improves nutritional quality and functional properties of the beans due to increased digestibility (Visessanguan et al., 2005) degradation of anti-nutritional factors (Reddy and Pierson, 1994; Chitra et al., 1996; Ari et al., 2012) and increased content of small bioactive compounds (Yang et al., 2011). However, high proteolytic activities in alkaline fermentations result in high ammonia production leading to a strong odor which some people find objectionable (Allagheny et al., 1996; Parkouda et al., 2009). In Malawi, utilization of soybeans is limited to a flour blend containing 80% maize and 20% soybean, used as a complimentary food (Kalimbira et al., 2004). Utilization is minimal due to the characteristic beany flavor and due to limited knowledge in processing (Coulibaly et al. 2009).

Low cost processing techniques that would eliminate or minimize objectionable flavors would increase utilization of soybeans in Malawi. With the aim of increasing soybean's utilization and acceptance in Malawi, fermented soybean pastes to be used as side dishes or meat alternatives were developed (Ng'ong'ola-Manani et al., 2014a). These pastes were naturally fermented and lactic acid bacteria (LAB) fermented. Both fermentations improved nutritional quality through increased solubility of amino acids and proteins and degradation of phytic acid (Ng'ong'ola-Manani et al., 2014b). Although consumer acceptance was biased in favor of naturally fermented pastes (NFP), relatively higher pH and lower organic acid production in NFP than in LAB fermented pastes (LFP), suggested food safety could be a challenge in NFP.

Diarrheal diseases are a major health problem in developing countries due to challenges in observing basic principles of food hygiene. Lack of cold storage facilities or fuel for hot holding in most rural households limit the implementation of principles of hygiene and

increase the risk of foodborne diseases (Nout and Motarjemi, 1997; Bonkoungou et al., 2013). On the other hand, LAB fermentation of cereal products such as *togwa* (Tanzania) were suggested as an alternative technology to safeguard food when cold and hot storage was not feasible (Nout and Motarjemi, 1997).

Moreover, Kingamkono et al. (1994, 1995, and 1998) and Svanberg et al. (1992) showed that during LAB fermentations of maize gruels inoculated with power flour (germinated sorghum flours) as starter culture to produce *togwa*, growths of different species of enteropathogens were inhibited. In the production of LAB fermented soybean pastes, Ng'ong'ola-Manani et al. (2014a) had used *thobwa* as a back-slopping material to facilitate the LAB fermentations. *Thobwa* is produced from maize gruels normally fermented using flour from germinated grains as power flour. Since LFP produced more lactic acid than NFP (Ng'ong'ola-Manani et al., 2014b) it was anticipated that LFP could be safer than NFP. Moreover, *Bacillus cereus* (6.3 to 8.3 log cfu/g) and *Escherichia coli* have been isolated from soybean alkaline fermented foods (Nout et al., 1998; Sarkar et al., 2002; Dakwa et al., 2005; Chukeatirote et al., 2010; Lee et al., 2010; Thorsen et al., 2010; Nam et al., 2012a; Nam et al., 2012b). In view of this, the fate of two enteropathogens, *E. coli* and *B. cereus* during natural and LAB fermentations were investigated. Therefore, the aim of this study was to investigate the effect of back-slopping and natural fermentations on growth of *E. coli* and *B. cereus* during fermentation.

Materials and Methods

Three types of fermentations namely natural (spontaneous) fermentation; LAB accelerated fermentation by back – slopping using a traditional fermented cereal gruel, *thobwa*; and controlled fermentation using a starter culture of a *Lactobacillus fermentum* strain previously isolated from the fermented pastes of soybeans and soybean-maize blends were studied.

Bacteria strains used

Nine *Lb. fermentum* strains previously isolated from fermented pastes of soybeans and soybean-maize blends were screened for rapid acidification in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany). The strains had been stored in MRS broth containing 15% glycerol at -80 ^{o}C and were resuscitated by sub culturing three times in MRS broth at 30 ^{o}C. Following resuscitation, the ability of the strains to acidify MRS broth at 30 ^{o}C

was determined by measuring pH. The pH was measured at 6 hrs intervals until 24 hrs and thereafter a strain that reduced pH the most was selected. The strain, *Lb. fermentum* 324c reduced pH to 4.06 by 24 hrs was used for inoculation of pastes in controlled fermentations. The *Lb. fermentum* was isolated from soybean paste at 48 hrs, was able to grow at pH 3.9, at 15 ^{o}C and 45 ^{o}C. Two enteropathoges, *E. coli* and *B. cereus* were also used. *Escherichia coli* HMG 021002 strain was isolated from traditional spontaneously fermented milk from Zimbabwe (Gran et al., 2002). *Bacillus cereus* NVH 45 isolated from whipping cream, was a gift from Professor P.E. Granum, Norwegian University of Life Sciences. The *B.cereus* strain contained the non-haemolytic enterotoxin gene (*nhe*), the haemolytic enterotoxin gene (*hbl*) and the enterotoxin T gene (Borge et al., 2001; Røssland et al., 2005). The minimum growth temperature of this strain in milk was 7 ^{o}C, and its minimum pH for growth was pH 4.9–5.0 (Borge et al., 2001; Røssland et al., 2005).

Preparation of stock cultures

Brain heart infusion broth (BHI, Merck) was used for *B. cereus* and *E. coli* culturing while MRS broth was used for *Lb. fermentum* culturing during preparation of stock cultures. *Bacillus cereus, E. coli* and *Lb. fermentum* strains were sub-cultured three times in their respective broths before finally inoculating in 9 ml of the broths and incubating at 30 ^{o}C for 18 hrs. After incubation, the cells were harvested by centrifugation at 3, 500 x *g* for 10 min at 4 ^{o}C (Eppendorf Centrifuge 5804, Hamburg, Germany). Thereafter, the cells were resuspended in 1.8 ml of BHI and MRS broths containing 15% (v/v) glycerol before transferring into sterile Eppendorf tubes and storage at -80 ^{o}C. Cell concentration of the stock cultures was predetermined before inoculating into the pastes.

To determine cell densities, stock cultures frozen at -80 ^{o}C for one week were thawed at 0 ^{o}C and serially diluted before culturing on selective media according to strain. *Bacillus cereus* was spread plated on *Cereus* selective agar (CSA; Fluka Analytical, Buchs, Switzerland) containing polymyxin B sulphate (50,000 units; P9602 Polymyxin B selective supplement, Fluka Analytical) and egg yolk emulsion (Merck) added according to manufacturer's instructions. *Escherichia coli* and *Lb. fermentum* were pour plated on Violet red bile agar (VRBA; Oxoid), and MRS agar (Merck), respectively. *Cereus* selective agar plates and VRBA plates were incubated at 37 ^{o}C for 24 hrs while MRS agar was incubated at 37 ^{o}C for 48 hrs before enumeration. One hour prior to inoculation into the pastes, tubes containing stock cultures were thawed at 0 ^{o}C. Suspensions containing cell densities of 10^2 – 10^3 cfu/g

for *B.cereus* and *E.coli*, and 10^7 - 10^8 cfu/g for *Lb. fermentum* (starter culture) were prepared in sterile water for inoculation into the pastes.

Sample preparation

Samples were prepared according to Ng'ong'ola-Manani et al. (2014a) with slight modifications (Fig 1). Samples were composed of 100% soybeans or 90% soybeans plus 10% maize. Naturally fermented pastes were designated as 100S and 90S; back-slopped samples were designated as 100SBS and 90SBS while samples containing *Lb. fermentum* as starter culture were designated 100SC and 90SC. Samples to be inoculated with *Lb fermentum* were ground after dehulling and were autoclaved at 121 ^{o}C for 15 min instead of boiling for 1 hr (Fig 1). When all the samples were prepared, a single pathogen was inoculated into the pastes to be fermented naturally. In the other fermentations, a single pathogen was simultaneously inoculated with the back-slopping material or with the *Lb. fermentum* starter culture. Sample compositions and pathogen combinations are shown in Table 1. Portions of 50 g of each sample treatment were packed in four sterile glass jars and were incubated at 30 ^{o}C. Duplicate analyses were done on all samples at 0 hrs, 24 hrs, 48 hrs, and 72 hrs. Three independent experiments were run.

Microbiological and pH Analyses

From each sample, 10 g was transferred aseptically into a sterile stomacher bag before adding 90 mL sterile diluent containing quarter strength ringer's solution (VWR International, Leuven, Belgium) and homogenising in a Colworth stomacher blender (STOMACHER 400, London, Britain) for 120 s. From the homogenates, appropriate ten-fold dilutions were prepared for enumeration of aerobic mesophiles on Plate Count Agar (PCA, Merck) and LAB on MRS Agar (Merck). *Bacillus cereus* was enumerated on CSA (Fluka Analytical) containing polymyxin B (Fluka Analytical) and egg yolk emulsions (Merk) added according to manufacturer's instructions. *Escherichia coli* was enumerated on VRBA (Oxoid). All incubations for CSA and VRBA were done at 37 ^{o}C for 24 hrs and for MRS and PCA were done at 37 ^{o}C for 48 hrs. Pour plate technique was used for *E. coli, Lb. fermentum,* LAB and PCA enumerations while spread plate technique was used for *B. cereus* enumerations.

The remaining homogenate from each sample was used for pH analysis. A pH meter (PHM210 65R048 N022, Radiometer, Copenhagen, Denmark) fitted with glass electrodes (135R 012, Radiometer, Denmark) was used.

Statistical Analysis

Analysis of variance (ANOVA) at p = 0.05 was performed in SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA) and least squares difference test was used to separate means.

Results and Discussion

Effect of fermentation on pH and growth of E. coli

Fig 2 and Fig 3 show changes in pH and viable cells of *E. coli*, respectively, in pastes fermented naturally (100S, 90S), fermented with *Lb. fermentum* as starter culture (100SC, 90SC), and in pastes fermented through back-slopping using *thobwa* as inoculum (100SBS, 90SBS). In all these fermentation types, *E. coli* was added as an enteropathogen test strain. There were significant differences ($p < 0.05$) in pH according to fermentation type at 24 hrs. The pH of the back-slopped pastes (BSP) decreased significantly ($p<0.05$) faster than in the other fermentations, and BSP had the lowest pH of 4.84 and 5.0 at 24 hrs in 90SBS and 100SBS, respectively. Naturally fermented pastes (NFP) had pH of 6.21 and 6.58 in 90S and 100S, while pastes fermented with *Lb. fermentum* as starter culture (SCP) had pH of 5.69 and 5.74 in 90SC and 100SC, respectively. At 48 hrs, only NFP had significantly higher pH and at 72 hrs only 100S had significantly higher pH. After 72 hrs, BSP and SCP had a pH of 5.2 while NFP had pH of 5.74 and 6.14 in 90S and 100S, respectively (Fig 2). Although the pH of BSP decreased faster than the rest, failure to reach critical values of $pH \leq 4.4$ which is the minimum pH for *E. coli* growth (Bell, 2002) raises a food safety concern. In our earlier studies, pH dropped to 4.36 at 24 hrs and by 72 hrs, the pH had reached 4.01 in 90SBS. In 100SBS, pH dropped to 4.26 by 72 hrs (Ng'ong'ola-Manani, 2014b). Thus although back-slopping accelerates the initial phase of the fermentation process and minimizes failure risks to fermentation (Svanberg et al., 1992; Holzapfel, 2002), it is still subject to variation in product quality (Holzapfel, 2002).

Nevertheless, back-slopping significantly inhibited *E. coli* proliferation in the fermented pastes (Fig 3). Significant differences in *E. coli* populations due to fermentation types were observed from 24 hrs and during further fermentation. Viable cells of *E. coli* in BSP increased 1.3 fold of the initial level throughout fermentation, while in NFP and SCP, *E. coli* cells increased 4.0 and 3.0-3.5 fold, respectively. In BSP, *E. coli* increased from 2.4 log cfu/g at 0 hrs to 3.2 - 3.5 log cfu/g at 24 hrs and remained at a constant level of 3.0 log cfu/g during further fermentation. In SCP, *E. coli* increased from 2.0 – 2.3 log cfu/g at 0 hrs to 6.8-7.6 log

cfu/g at 24 hrs and by 72 hrs, the cell count was 7.2 log cfu/g. In NFP, *E. coli* increased from 2.3 log cfu/g at 0 hrs to 8.8-9.2 log cfu/g and by 72 hrs, viable cells of *E. coli* were about 9.1 log cfu/g.

Failure of all the fermentations to reach pH of ≤ 4.4 explains the survival and growth of *E. coli* during fermentation. The relatively faster acidification in BSP compared to SCP and NFP, partly explains the higher inhibition of *E. coli* growth. In BSP, it can be suggested that other inhibitory compounds in addition to increasing acidity were responsible for the higher *E.coli* growth inhibition. In a study by Drago et al. (1997), the effect of *Lactobacillus* strains on growth of *E. coli* and *Salmonella enteritidis* was investigated in culture media that were periodically replaced by freshly prepared medium in order to keep the pH value constant. Growth of the two enteropathogens was inhibited; therefore they concluded that the inhibition of the pathogens could not be ascribed simply to acidification of the medium. Even though in their experiment, Drago et al. (1997) could not fully explain the mechanism of inhibition, but they could also not rule out the possibility of the production of bacteriocins and antibiotics by the lactobacillus strains under study. Besides, lactobacilli are known to exert antibacterial activity through production of lactic acid, hydrogen peroxide, short chain fatty acids, antibiotics and bacteriocins (Drago et al., 1997).

Trends in LAB counts (Fig 4) and total aerobic bacteria counts (Fig 5) in pastes inoculated with *E. coli* were almost similar throughout fermentation. An initial LAB count of 6.0 – 8 log cfu/g in BSP and SCP and the counts reached ca. 9.0 log cfu/g at 24 hrs and remained unchanged during further fermentation (Fig 4). On the contrary, a low initial LAB count of 2.0 to 2.5 log cfu/g was observed in NFP. This increased to 5.5 – 6.3 log cfu/g after 24 hrs fermentation and after 72 hrs, the counts were 7.6 and 9.4 log cfu/g in 100S and 90S, respectively (Fig 4). Although the LAB population increased significantly ($p<0.05$) throughout fermentation in NFP, a high initial LAB population is required in order to inhibit enteropathogen growth as observed in BSP and SCP. These results agree with the report of Sahlin (1999) in which initial LAB numbers as well as their rate of growth were important in relation to rate of production of lactic acid, development of pH and *E. coli* growth inhibition.

The microbial profile of BSP and SCP was almost similar for LAB (Fig 4) and total aerobic (Fig 5) counts. However, BSP were more effective in suppressing proliferation of *E. coli* than the single strain culture in SCP (Fig 3). The mixture of LAB strains in BSP probably offered a competitive advantage over the single strain culture in SCP. Interactions of mixed strains in

BSP could have been synergistic resulting in fast acidification of the medium (as evidenced by a relatively fast pH drop in BSP) and hence pronounced suppression of *E. coli* growth. This was in accordance with Holzapfel (2002), who reported that mixed strain cultures produce favorable synergistic effects such as degradation of undesirable factors, flavor production and accelerated ripening and maturation. Drago et al. (1997) also reported that mixed lactobacilli strains exerted more inhibitory effect on the growth of *E. coli* and *Salmonella enteritidis* than single culture strains.

Effect of fermentation on pH and growth of B. cereus

In pastes inoculated with *B. cereus*, BSP and SCP showed pH trends (Fig 6) similar to those observed in pastes inoculated with *E. coli*. In BSP, the pH dropped to 5.0 after 24 hrs fermentation and after 72 hrs the pH of 90SBS was 4.8 while that of 100SBS was 5.2 (Fig 6). On the contrary, increases in pH were observed during further fermentation in NFP. For instance, in 100S, the pH increased after 48 hrs from 6.11 to 6.86 while in 90S, the pH increased from 5.87 after 24 hrs to 7.2 after 72 hrs (Fig 6). The increases in pH could be attributed to *B. cereus* metabolism. *Bacillus* spp. are highly proteolytic and may degrade proteins into amino acids, subsequently producing ammonia which leads to an increase in pH (Sarkar and Tamang, 1995; Dakwa et al., 2005; Parkouda et al., 2009).

Higher *B. cereus* population in NFP (Fig 7) compared to BSP and SCP explains the increase in pH in NFP and not in the other pastes. Viable cells of *B. cereus* in NFP increased from 2.2 log cfu/g to ca. 8.0 log cfu/g after 24 hrs and remained relatively unchanged throughout fermentation. While in SCP, the counts increased to about 5.0 log cfu/g after 24 hrs fermentation and the count remained as such during further fermentation in 100SC. On the other hand, *B. cereus* population began to decline after 48 hrs in 90SC and by 72 hrs, the count was 3.7 log cfu/g. The decline could probably be explained in terms of increasing acidity due to availability of more fermentable sugars provided by the maize in 90SC than in 100SC. This could have allowed the less competitive LAB which was already high in numbers to metabolize faster in 90SC than in 100SC and produce more acids that could have led to more inhibition of *B. cereus* growth in 90SC than in 100SC. Back-slopping resulted in most *B. cereus* growth inhibition than SCP and NFP (Fig 7). Viable cell counts fluctuated between 2.0 and 3.0 log cfu/g in 100SBS while in 90SBS, the highest counts were 2.4 log cfu/g and after 24 hrs and during further fermentation, viable cells were less than 10 cfu/g (indicated as 1 log cfu/g) hence they could not be detected. This could also be due to high

LAB metabolic activities in 90SBS due to presence of more fermentable sugars resulting in more production of metabolites with inhibitory effects.

Since BSP and SCP were inoculated, their LAB populations were between 7.4 to 8.0 log cfu/g at 0 hrs, the populations increased to ca. 9.0 log cfu/g and remained relatively unchanged during further fermentation (Fig 8). On the other hand, LAB population of NFP was between 2.0 and 2.5 log cfu/g at the beginning of the fermentations, and the highest LAB count was 6.0 log cfu/g in 90S while in 100S the count increased to 5.0 log cfu/g after 48 hrs and decreased to 1.4 log cfu/g by 72 hrs. Higher LAB in 90S than in 100S could be due to the presence of more fermentable sugars in the former. Low LAB population in NFP explains high proliferation of the two enteropathogens. The trends of total aerobic count of pastes inoculated with *B. cereus* (Fig 9) were similar to those of the pastes inoculated with *E. coli*.

Escherichia coli and *Bacillus cereus* growth inhibition

The results indicated that the use of back-slopping or single strain starter culture, inhibited growth of *B. cereus* more than growth of *E. coli*. However, only a single strain of each pathogen was used in this study. Kingamkono et al. (1998) also observed a preferential growth inhibition rate of LAB between *Campylobacter jejuni* and *E. coli*. In their study, *C. jejuni* was inhibited to a higher level. In another study, Kingamkono et al. (1994; 1995) used fermenting cereal gruel *togwa* to determine growth inhibition of seven species of enteropathogens. They observed that *B. cereus* was more sensitive than enterotoxigenic *E. coli* (ETEC) for LAB. They attributed this to the inducible acid-tolerance response system in some *E. coli* strains that protects them against severe acid stress for longer periods and to the presence a double cell membrane in ETEC that make them less sensitive to growth-inhibiting factors such as bacteriocins. Acid tolerance has also been reported in *E. coli* O157:H7 (Leyer et al., 1995). The above reasons could also explain the relatively higher survival of *E. coli* in the fermented pastes, especially in BSP and SCP.

Although it is generally agreed that the critical pH for *B. cereus* inhibition is 5.0 (Røssland et al., 2005; Senesi and Ghelardi, 2010; Valero et al., 2003), the growth of this organism has also been inhibited at higher pH values. Wong and Chen (1988) observed that when *B. cereus* and LAB were inoculated at the same time into nonfat milk medium, *B. cereus* growth was not inhibited until after 16 hrs when the pH values of the mixed cultures were about 5.3 to 5.7. Therefore, it was concluded that the inhibition of vegetative cells of *B. cereus* in mixed cultures may be attributed to higher lactic acid levels production by LAB cultures, or to other

factors such as hydrogen peroxide production, nutrient depletion, or a decrease in the oxidation-reduction potential. From Wong and Chen (1988) studies, *B. cereus* seemed to be more sensitive to low pH, such that even at pH 5.7 its growth could still be inhibited. On the other hand *E. coli* can tolerate lower pH values down to 3.42 for some hrs (Leyer et al., 1995).

The failure of NFP to inhibit both enteropathogens was because of the low initial LAB flora (Fig. 4 and Fig 8). The low initial LAB flora resulted in a delayed decrease in pH and eventually, delayed inhibition of the growth of the enteropathogens (Kingamkono et al., 1995). A fast lactic acid production during early hours of fermentation is required to achieve inhibition even in *B. cereus* (Røssland et al., 2005) whose minimum pH for growth is higher than for *E. coli*. The use of a previously fermented gruel, *thobwa* in back-slopped samples also had an added advantage. Cereal gruels prepared from previously germinated grains contain amylolytic enzymes (Mosha & Svanberg 1983) that hydrolyse starch into fermentable sugars which are the main substrates for LAB. Availability of fermentable sugars facilitates rapid metabolism and growth of LAB (Kingamkono et al., 1994).

Food safety implications

Bacillus food-poisoning usually occurs because spores survive cooking or pasteurization and then germinate and multiply when food is inadequately refrigerated or reheated (Ehling-Schulz et al., 2004). Two types of foodborne illnesses namely emetic and diarrhea syndromes are due to *B. cereus.* The emetic syndrome is an intoxication caused by a preformed heat stable emetic toxin called cereulide. The infectious dose is estimated to be around 10^5–10^8 cells/g of ingested food, however some studies reported illnesses from foods containing 10^3 cells/g food (Ehling-Schulz et al., 2004; Ceuppens et al., 2011). Heat stability in cereulide for 90 min at 121°C and for 2 hrs at 121 ^oC and pH 7 has been reported, withstanding frying, roasting and microwave cooking. In addition, the toxin withstands exposure to a pH range of 2.0 – 11.0 and is resistant to pepsin and trypsin digestion (Ceuppens et al., 2011; Kotiranta et al., 2000). Cereulide production can occur at a wide temperature range of 8 ^oC to 40 °C (optimum is 20 °C and 30 ^oC), and its production is impaired by reduced O_2 levels (atmospheres of 0–2% O_2 and 20% CO_2 in N_2 inhibit production) and by low pH values i.e. < 5.6 (Ceuppens et al., 2011).

The diarrhoeal syndrome on the other hand, is an infection requiring live cells or spores to be ingested together with food to cause an illness (Ceuppens et al., 2011). The vegetative cells produce enterotoxins in the small intestines (Ceuppens et al., 2011). Various infectious dose

ranges of $10^4 – 10^8$ cells/g (Logan, 2012), $10^5 – 10^7$ cells/g (Granum and Lund, 1997) and $10^3 – 10^7$ cells ingested (Schraft and Griffiths, 2006) have been reported. Heat labile enterotoxins Hbl, Nhe, CytK (Ghelardi et al., 2002; Granum and Lund, 1997) and hemolysin II (HlyII) are the most important in diarrhoeal syndrome (Ceuppens at al., 2011). Optimal temperature for production is 30 ^oC and minimum is 6 ^oC. Anaerobic conditions lead to slow bacteria growth but increased toxin production, and low glucose is required for the production. The optimal pH for production is 7.0 – 9.0 and slow production has been reported between 5.0 – 6.0 (Ceuppens et al., 2011).

Since viable cells of *B. cereus* at 72 hrs ranged from 0 to 3 log cfu/g in BSP and 3.7 to 5.3 log cfu/g in SCP while it was > 5 log cfu/g in NFP and pH values at 72 hrs were 5.30 and 5.35 in 90SC and 100SC, respectively; 4.86 and 5.30 in 90SBS and 100SBS, respectively; and 7.2 and 6.86 in 90S and 100S, respectively. Therefore BSP could be considered relatively safe with regards to *B. cereus* poisonings based on the facts presented above. However, because the minimum pH for growth for *B. cereus* is 5.0 (Senesi and Ghelardi, 2010), then pH values slightly higher than 5.0 should be considered borderline. Further, there could be a risk of production of the diarrhea toxins even under slow growth due to creation of anaerobic environment. Another challenge with *B. cereus* is the possibility of sporulation which enables the organism to survive certain heat treatments like pasteurization. Therefore, to establish the safety of use of back-slopping in these pastes, further studies on enterotoxin production and sporulation during fermentation are recommended.

Although natural and *B. subtilis* fermentations of soybeans and other legumes have generally been regarded as safe (Allagheny et al., 1996; Steinkraus, 1997; Chukeatirote et al., 2010; Parkouda et al., 2009), the presence of *B. cereus* in some of these products (Sarkar et al., 2002; Leejeerajumnean, 2003; Chukeatirote et al., 2010) and the fact that the preparation and the fermentation is usually made in homes using rudimentary equipment and sometimes under poor hygienic conditions, means that the risk of food borne diseases is still existent (Parkouda et al., 2009). Therefore, this study suggested that NFP could only be safe if there were no pathogen contamination during food preparation and during fermentation. Besides, the environmental factors during fermentation in this study (limited O_2, and increase in pH) suggested the possibility of enterotoxin production in case of contamination; therefore, investigation of enterotoxin production in NFP was also recommended.

Escherichia coli are a diverse group of organisms that are normally harmless as commensals in humans and animals (Moriel et al., 2012). However, its acquisition of a combination of mobile genetic elements makes *E. coli* a highly adapted pathogen capable of causing gastroenteritis as well as extraintestinal infections of the urinary tract, bloodstream and central nervous system (Croxen and Finlay, 2010; Moriel et al., 2012). Diseases from *E. coli* affect hundreds of millions of people annually. Eight *E. coli* pathovars and their mechanisms of disease have been extensively studied. These pathovars can be broadly classified as either diarrhoeagenic *E. coli* or extraintestinal *E. coli* (ExPEC) (Croxen and Finlay, 2010; Moriel et al., 2012). Diarrhoeagenic *E. coli* strains include enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Croxen and Finlay, 2010; Nataro and Kaper,, 1998). EPEC is a major cause of infant diarrhea in developing countries (Nataro and Kaper, 1998).

Escherichia coli are facultative anaerobes that do not require oxygen for growth although they grow better in aerobic conditions (Jones et al., 2007). The temperature range for growth of pathogenic *E. coli* is 7 – 8°C to 46°C, with an optimum temperature of 35 – 40 °C (ICMSF 1996). The minimum pH for growth is 4.4 (Bell, 2002), but some EHEC strains like *E. coli* O157:H7 have a high degree of acid tolerance surviving 2 to 7 hrs exposures at pH 2.5 and 37°C (Buchanan and Doyle, 1997). *Escherichia coli* O157:H7 has an acid-adaptive response, and the expression of this system enhances its survival in the presence of lactic acid and in acidified food products including fermented foods (Leyer at al., 1995). Acid tolerance is probably an important component of virulence for *E. coli* O157:H7 and it allows a small number of cells to cause illness by their being protected in the gastric tract (Leyer et al., 1995). Leyer et al. (1995) demonstrated that acid-adapted *E. coli* O157:H7 cells survived substantially longer in cider. In their study, acid-adapted cell population decreased about 10-folds from 10^5 cfu/ml after 28 hrs and the cells were detected at 60 cfu/ml after 81 hrs in cider that had an initial pH of 3.42 and was stored at 6.8 ^oC.

The infectious dose for some Shiga toxin-producing *Escherichia coli* (STEC) strains (O111:H2 and O157:H7) is as low as 1 to 100 cells while those for ETEC and EPEC are higher (Paton and Paton, 1998). For ETEC, the infectious dose is 10^6 to 10^{10} cells/g (Qadri et al., 2005). In this study, the final pH values for all fermentations containing *E.coli* were above 4.4 with minimum population of 3 log cfu/g in BSP. Thus for the fermentations to inhibit *E.*

coli growth to safe levels, process optimization of back-slopping material through repetitive recycling of inoculum before final use in the pastes to select the best adapted strains is recommended. Further, strain selection for starter cultures with attributes like fast acidification, degradation of anti-nutritional factors, and improvement of aroma and texture as criteria would be desirable. Possibly mixed strain cultures could be suitable for improved inhibition of pathogens in the fermented pastes.

Although fermentation improves safety of foods, this study suggested that it cannot be considered as a critical control point for elimination of risks of biological hazards in these pastes. Thus prevention of contamination with pathogenic strains would be the best way to ensure safety. Nevertheless, the pastes were to be heat treated before consumption. Fortunately, the *E. coli* strain with lowest infectious dose (*E. coli* O157:H7) can be controlled readily through traditional thermal processing techniques; although the heating process ought to be sufficient to assure a low probability of the pathogen surviving (Buchanan and Dowley, 1997).

Conclusions

From this study, it was concluded that back-slopping has potential of producing fermented soybean pastes with reduced risk of foodborne illnesses from *Bacillus cereus*, although process optimization is recommended through repeated recycling of inoculum to select the best adapted strains. Soybean pastes naturally fermented with *Bacillus subtilis* have always been considered as safe, but they could pose a risk of infection if contamination by pathogens occurs during fermentation and food preparation. Because of acid tolerance of some *E. coli* strains during fermentation, then a thermal processing step should be applied before consumption to ensure safety.

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Table 1: Sample composition, fermentation type and pathogen combination

Pathogen	**Natural fermentation**	**Back-slopping**	**Controlled fermentation**
E. coli	100S + *E. coli*	100SBS + *E. coli*	100SC + *Lb. fermentum* + *E. coli*
	90S + *E. coli*	90SBS + *E. coli*	90SC + *Lb. fermentum* + *E. coli*
B. cereus	100S + *B. cereus*	100SBS + *B. cereus*	100SC + *Lb. fermentum* + *B. cereus*
	90S + *B. cereus*	90SBS + *B. cereus*	90SC + *Lb. fermentum* + *B. cereus*

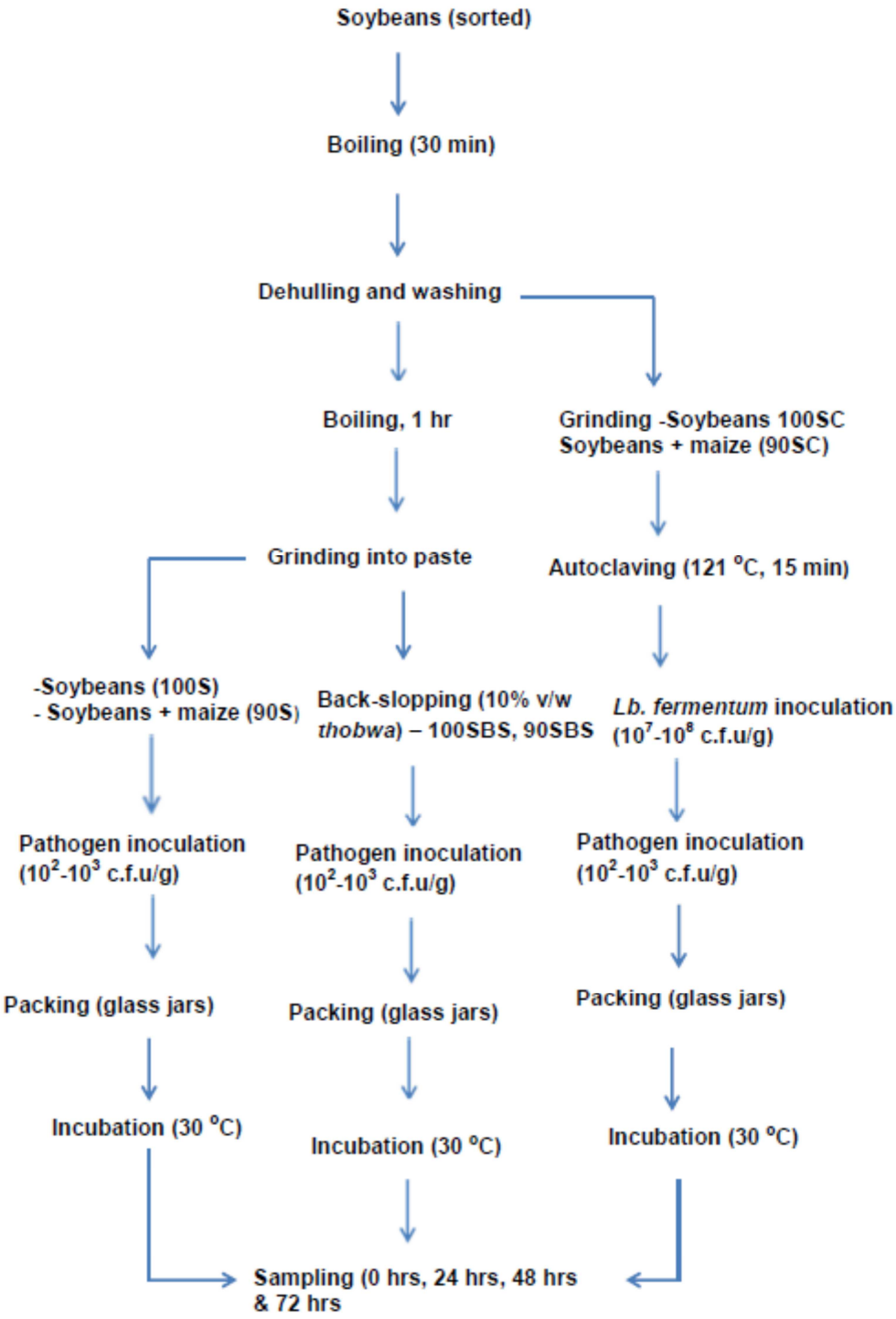

Fig 1: Flow chart for preparation of pastes of soybeans and soybean-maize blends.

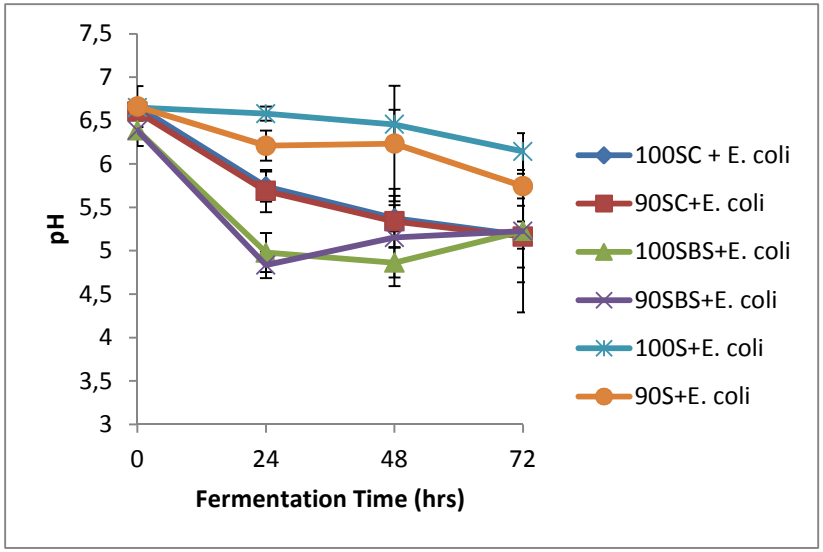

Fig 2: Changes in pH during fermentation of pastes of soybeans and soybean-maize blends inoculated with *E. coli*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

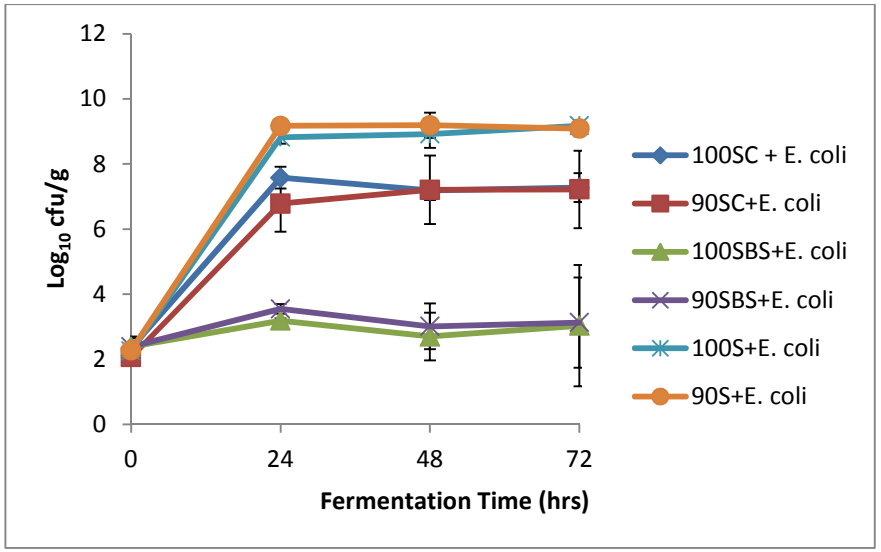

Fig 3: Changes in *E. coli* population during fermentation of pastes of soybeans and soybean-maize blends. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

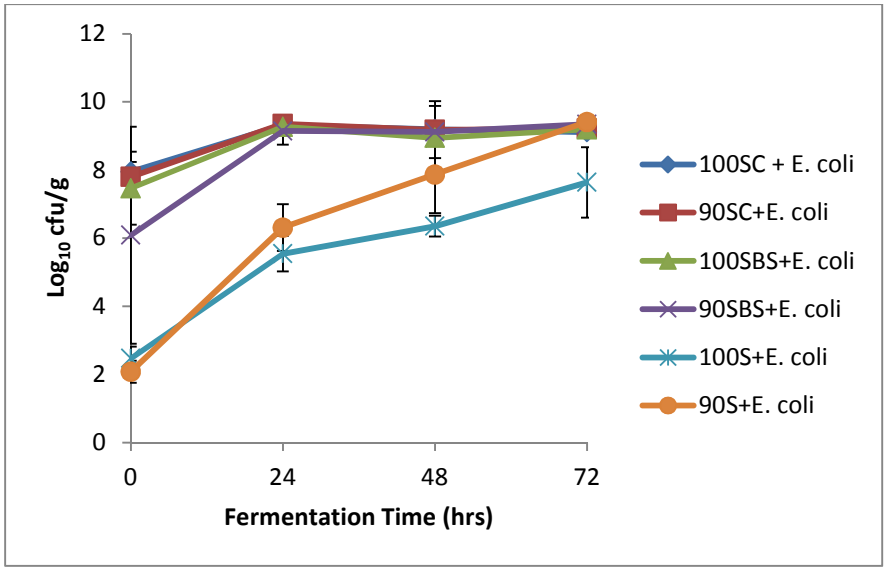

Fig 4: Changes in lactic acid bacteria population during fermentation of pastes of soybeans and soybean-maize blends inoculated with *E. coli*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

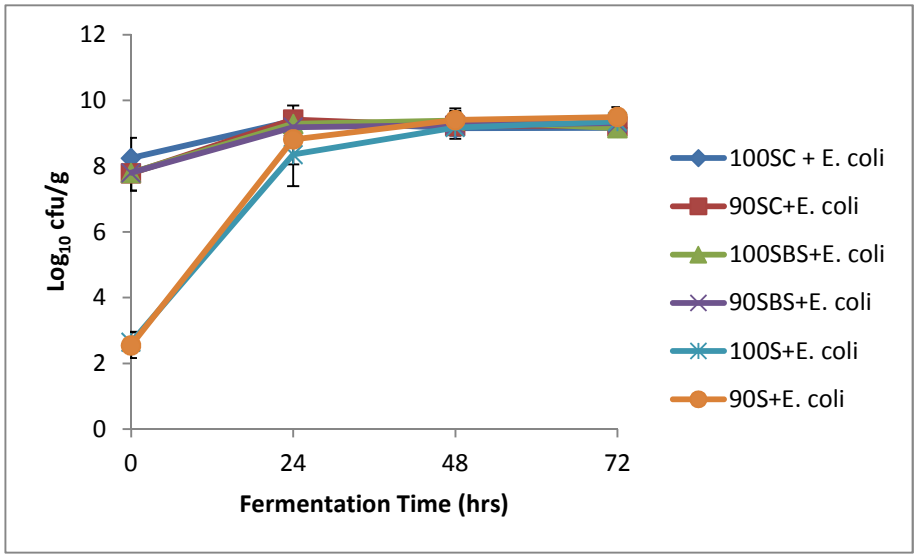

Fig 5: Changes in total aerobic bacteria population during fermentation of pastes of soybeans and soybean-maize blends inoculated with *E. coli*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

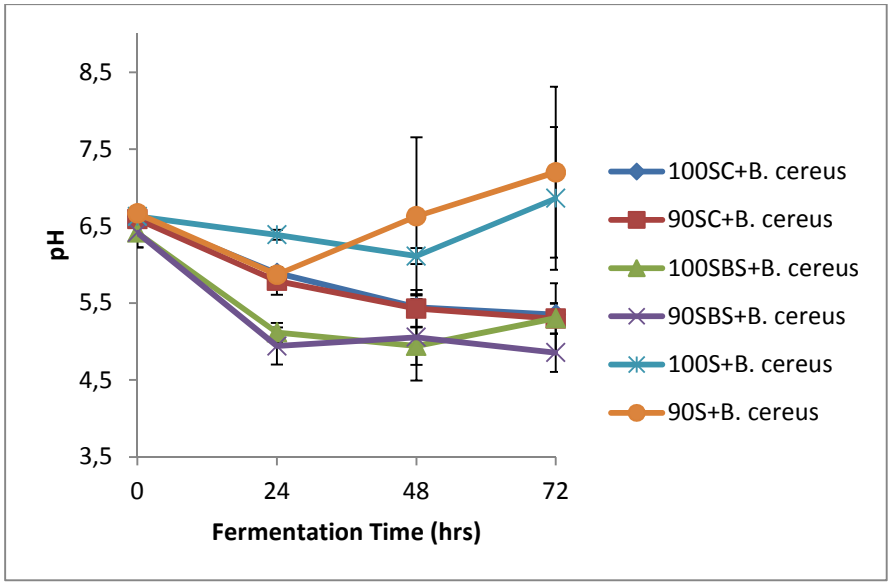

Fig 6: Changes in pH during fermentation of pastes of soybeans and soybean-maize blends inoculated with *B. cereus*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

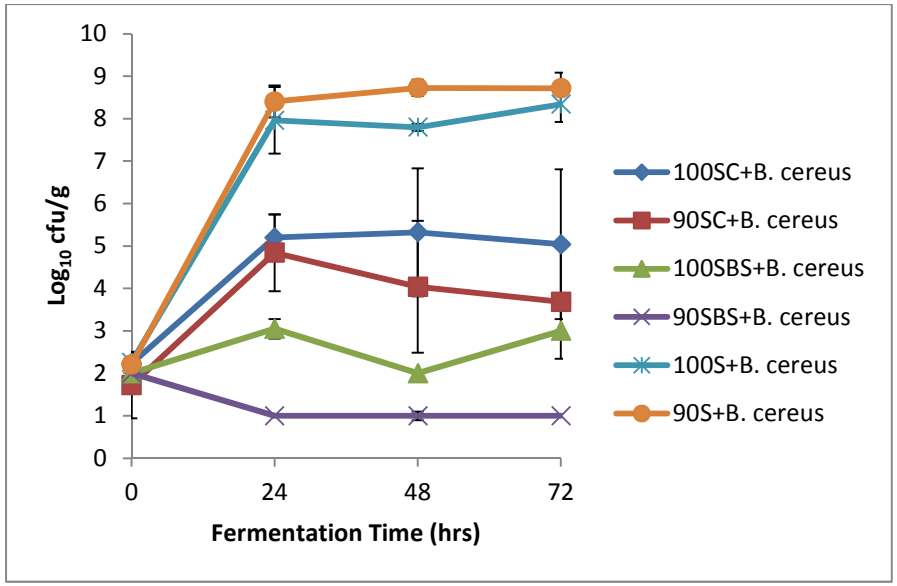

Fig 7: Changes in *B. cereus* population during fermentation of pastes of soybeans and soybean-maize blends. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

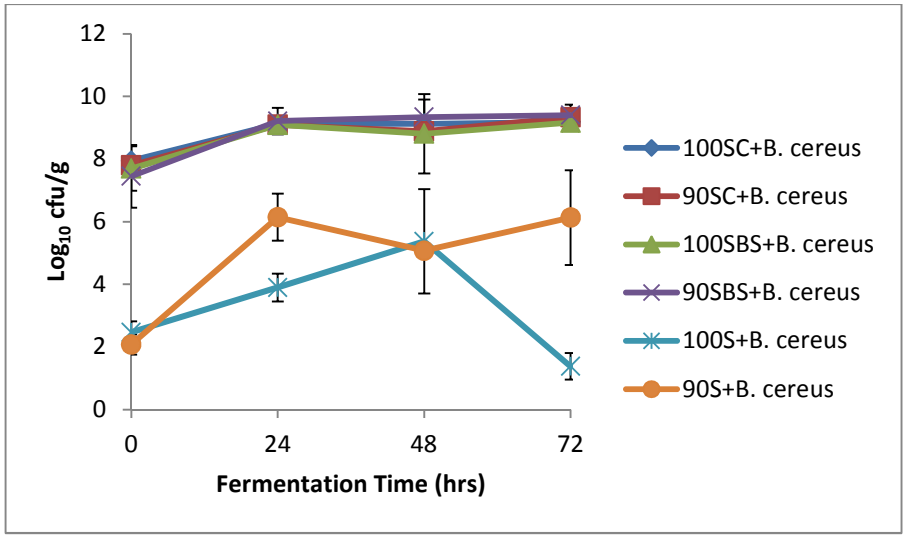

Fig 8: Changes in lactic acid bacteria population during fermentation of pastes of soybeans and soybean-maize blends inoculated with *B. cereus*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S, and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.

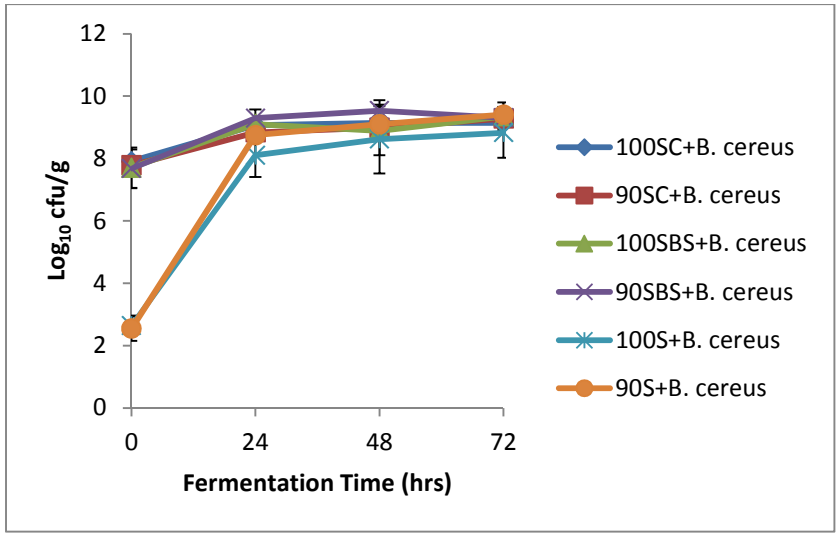

Fig 9: Changes in total aerobic bacteria population during fermentation of pastes of soybeans and soybean-maize blends inoculated with *B. cereus*. Samples coded 100SC and 90SC represent pastes fermented with *Lb. fermentum*; 100S and 90S represent naturally fermented pastes; while 100SBS and 90SBS represent back-slopped pastes. Pastes are designated according to 100% and 90% soybean composition, the remaining proportions being maize.