London Metropolitan University

Characterization of Lactic Acid Bacteria from *Nono*, a Nigerian Naturally Fermented Milk Product.

This thesis is submitted in partial fulfilment of the requirements for the degree of doctor of philosophy,

School of Human Science,

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DECLARATION

I hereby declare that this thesis:

Characterization of Lactic Acid Bacteria from *NONO*, a Nigerian Naturally Fermented Milk Product is my own work. Where other people's work has been used, was properly and clearly acknowledged in the text.

This work has not been accepted in substance for any degree or at any institution and has not been submitted for any award other than that of Doctor of Philosophy degree at School of Human Science, Microbiology Research Unit, London Metropolitan University.

Signature: .....

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ABSTRACT

The aim of this research was to enumerate, isolate, identify and characterize lactic acid bacteria (LAB) from *Nono*, a Nigerian naturally fermented cow milk product for the selection of multifunctional starter cultures to develop a controlled fermentation process for *Nono* production. This study focused on screening the diversity of the LAB associated with *Nono* and characterizing their potential probiotic and functional properties including tolerance to acidic pH and bile salt, antimicrobial activity against indicators of food borne pathogens, and resistance to antimicrobials.

The LAB were enumerated and isolated from *Nono* using MRS, M17 and MRS + L-Cysteine agars. These media were selected to harvest a diverse range of LAB associated with *Nono*. Further, they were identified using conventional phenotypic methods including Gram, catalase and oxidase and the Standard Analytical Profile Index (API 50 CHL) identification system. Genotypic methods including repetitive element sequence-based polymerase chain reaction (rep-PCR) and sequencing of the 16S rRNA, phenylalanyl-tRNA synthase α-subunit (*pheS*) and RNA polymerase, alpha subunit (*rpoA*) genes were used to identify the bacteria. The sequences were analysed using the Genbank and Eztaxon databases. Phenotyping revealed a bacterial count at a level of 10^7 CFU/ml for all samples. A total of 128 LAB were isolated and characterized as Gram positive, catalase and oxidase negative and non-spore forming bacteria. The shape and organization of the isolates were variable: rod, cocci, vibrio, V-shaped and coccobacillus bacteria organized as single, pairs or groups. The rep-PCR allowed the differentiation of multiple groups within the same species and sequencing of the 16S rRNA, *pheS* and *rpoA* genes allowed the identification of various genus and species including *Lactobacillus fermentum (40%), Lactobacillus*

senioris (2%), Lactobacillus delbrueckii (23%), Streptococcus thermophilus (22%) Streptococcus infantarius (10%), Leuconostoc pseudomesenteriodes (2 %) and Enterococcus thailandicus (1%).

Further characterisation of the isolates for probiotic and functional properties focused on seven isolates selected on the basis of differences in their rep-PCR profiles. These include *Lactobacillus fermentum*, *Lactobacillus senioris*, *Lactobacillus delbrueckii*, *Streptococcus thermophilus*, *Streptococcus infantarius*, *Leuconostoc pseudomesenteriodes,* and *Enterococcus thailandicus*. The isolates were screened for tolerance to different acidic pH and bile salt concentrations to characterise their resistance to gastric acid and bile. The survival of the isolates at different acidic pH varied according to the isolates and incubation time, *Lactobacillus fermentum* followed by *Lactobacillus senioris* survived better at pH 3 and pH 4 for 3 h incubation compared to other test isolates. All the isolates survived high concentration 1.5% and 2% of the bile salt for 3 h incubation. The isolates were further screened for antimicrobial activities against indicators of pathogenic bacteria including *Samonella enteritidis serovar Typhimurium* variant DT124, *Escherichia coli* NCTC 12900, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* CMCC 1930 and *Bacillus cereus* LMG 1356. Inhibitory activity of the test organisms was evaluated using a spot test and also spectrophotometric method by measuring and comparing the optical density (OD) of the indicator bacteria after the 24 h incubation period in both test and control experiment. The test isolates exhibited varying levels of inhibition against common Gram positive and Gram negative foodborne pathogens. Among the seven species of LAB screened for antimicrobial activity, *Lactobacillus fermentum* not only showed broad antimicrobial activities against the indicators but also exhibited antilisterial activity against

Listeria monocytogenes and this is of significant spotlight in the starter culture selection. The susceptibility of the selected LAB to 18 antimicrobials was evaluated by screening the Minimal Inhibitory Concentration (MIC) for each antimicrobial. This was followed by the detection of resistance genes by PCR. The ability of two isolates of LAB to transfer to other bacteria the *tet*(S), *tet*(M) genes coding for tetracycline resistance and *aad*(E) gene coding for streptomycin resistance was investigated by conjugation experiments.

The latter experiements revealed a variable antimicrobial susceptibility according to the LAB isolate and the antimicrobial tested. The *tet*(S), *tet*(M) were detected in the isolates of *Enterococcus thailandicus (52)* and *Streptococcus infantarius(10)*. Additionally, *aad*(E) was detected in *Enterococcus thailandicus (52)*. The conjugation experiments suggested that the *tet*(S) gene was transferable *in vitro* from isolates 52 and 10 to *E. faecalis* JH2-2 and *aad*(E) from 52 only to *E. faecalis* JH2-2. Both *tet*(S) and *aad*(E) are located at least on plasmids that have mediated the transfer of the genes to *Enterococcus faecalis* JH2-2 because positive amplicons were obtained in the donors and transconjugants by amplification of the gene from plasmid DNA samples.

This research concluded that various genus, species and sub-species of LAB are involved in the production of *Nono*. The data obtained in this research are relevant for the selection of multifunctional starter cultures for a control production of *Nono* in Nigeria.

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Chapter One: Introduction

1 Traditional African fermented foods

The production of foods by fermentation is one of the oldest food processing technologies dating back to ancient times. Traditional fermented products have been consumed for thousands of years all over the world and continue to remain popular within many communities. Fermentation, in microbiological terms, is a process in which plant and animal substrates are transformed into desirable food products by the metabolic activities of microorganisms or enzymes (Caplice and Fitzgerald, 1999).

Food fermentation is an important aspect of the traditional norms and culture among indigenes of many communities and is of great economic significance as it serves as a local preservation technology particularly in rural communities where lack of modern facilities for enhancing the shelf-life of food for both individual and commercial use remains certain. The fermentation process also improves the organoleptic characteristics as well as nutritional quality of the final fermented product (Jashbihai and Baboo, 2008; Kebede *et al.*, 2007).

Most traditional African fermented food production rely on natural fermentations or back slopping methods where leftover fermented product or previously used fermentation utensil are used as an inoculum for subsequent fermentations (Terlabie *et al.*, 2006). These methods are considered unreliable as their subsequent fermentation processes are uncontrolled (Okonkwo 2011; Yabaya *et al.*, 2012). Traditional methods relies on the knowledge of local population which is passed down from one generation to the next; very few traditional fermented products are processed on an industrial scale using starter cultures and controlled conditions

Traditional fermented milk products are consumed all over Africa, for example, *nunu* produced in Ghana (Akabanda *et al.*, 2013), *zabady*, an Egyptian traditional fermented milk and *Amasi*, produced in South Africa and Zimbabwe (Gadaga *et el.*, 1999). Spontaneous food fermentation, characterized by the non-ultilisation of starter cultures has a long history in Africa. It is usually carried out on a small scale at a household or small enterprise scale and serves as a source of income for many local communities especially women (Oyewole 1997). Milk fermentation is an important technological process, particularly for extending the shelf-life of a highly perishable food as well as improving its organoleptic and nutritional characteristics.

Fermented milk products have a characteristic sour taste and have been shown to have enhanced digestibility due to the increased availability of amino acid from protein degradation activities of fermenting organisms (Shori 2012). It has been established that the predominant organisms responsible for the fermentation of milk are lactic acid bacteria (LAB), particularly from genera *Lactobacillus*, *Streptococcus* and *Lactococcus* (Collado and Hernandez 2007). LAB are an important indigenous microflora in raw milk and their activities are responsible for acidity and viscosity characteristics associated with the fermented product (Samet-Bali and Attia, 2012)

1.1 Nono

Nono is a Nigerian fermented milk product mostly associated with the Hausa and nomadic Fulani ethnic groups residing in the Northern part of the country (Adesokan *et al.*, 2011; Obande and Azua (2013). Aernan *et al.* (2011) and Evans *et al.* (2013) noted that among the Fulanis, it is believed that it is the responsibility of men to retrieve the milk from the cow and for women to process it into *Nono* and sell in the markets (Figure 1.1). Natural fermented milks such as *Nono* can be found in other

placed in an old (used) calabash and inoculated with some previously fermented milk as a starter culture (Evans *et al.*, 2013). The inoculated milk is then allowed to stand in a calabash at ambient temperature (*ca.* 30 - 35°C) for 18-24 h for fermentation to occur. Contrastingly, it has been reported that in some tribes in Nigeria, *Nono* is prepared by keeping fresh cow milk in a plastic container or calabash at room temperature for more than 24 h or overnight to allow spontaneous microbial activity to occur and turn the milk sour without the addition of an inoculum (Evans *et al.*, 2013). However, as part of this study, it was observed that in the Eastern part of Nigeria, *Nono* is produced locally by heating cow's milk to a certain temperature (not specified due to confidential traditional method used). The heated milk is then allowed to cool down for some time and then poured into an old calabash with the belief that this contributes to the natural fermentation because of the leftover fermented milk that may remain inside the calabash. The heated milk is left in the calabash at room temperature overnight for fermentation to occur. Processing practices appear to be influenced by geographical location which may have an effect on microorganisms involved during the fermentation and subsequently on the final fermented product.

Nono processing is characterized by unhygienic practices and unreliable fermentations leading to products of poor sensory quality which can be unacceptable for customer's satisfaction (Res *et al.*, 2013; Okeke and Okwori 2011; Omotosho *et al.*, 2013 and Savadogo *et al.*, 2014). Also, due to non- standardized production conditions, the shelf-life of the product is unpredictable and hence it is usually consumed fresh. Microbiological evaluation studies have noted the presence of potential pathogenic organisms in *Nono* due to poor handling (Okonkwo, 2011).

Lactic acid and other antimicrobial compounds produced by fermentation organisms contributes to the safety of the *Nono* however, the risk of contamination as well as growth of food borne pathogens cannot be overruled. Furthermore, the temperature, pH and fermentation time associated with *Nono* production are poorly known and/or controlled. Since the production steps have not been standardized, as each tribe has their own production process, the product quality varies. Technological potential during fermentation will be an important factor for the selection of starter culture for controlled fermentations.

Climatic conditions of fermenting regions can also determine indigenous microflora and hence the quality of the final fermented product. Savadogo *et al.* (2004) noted that in cold climates, mesophilic bacteria (e.g. *Lactococcus* spp., *Leuconostoc* spp.) dominate natural milk fermentation, while hot climates allowed thermophilic bacteria such as *Streptococcus* spp., and *Lactobacillus* spp. to dominate the milk fermentation. Studies aimed at improving the fermentation technology must investigate the microbial flora of raw milk.

1.2 Lactic acid bacteria

Lactic acid bacteria (LAB) are a group of bacteria described as Gram positive, catalase negative, non-sporulating, rod and cocci which produce lactic acid as their primary metabolite from fermentative respiration (Khalid, 2011). They are heterogeneous and widely distributed in nature and are not only found in milk but in other plant materials such as cereals, numerous fermented foods as well as the animal and human gastrointestinal and urogenital tracks.

Based on their metabolic pathways, LAB can be divided into two groups; homofermentative LAB which convert sugar to lactic acid and heterofermentative

LAB which produce lactic acid and other organic compounds such as acetic acid and carbon dioxide. Heterofermentative LAB lack the enzyme aldolase and cannot ferment sugar though the glycolytic pathway. LAB can be characterized by their rate of growth at different temperatures, pH of media used for their isolation and also their tolerance to acid and bile (Halasz, 2009).

LAB in fermented milk produces lactic acid and highly valued metabolites involved in the flavor, texture development and antimicrobial activity associated with the fermented product (Agrawal and Prakash, 2013; Papagianni, 2012). LAB acidifies the milk during fermentation by producing lactic acid. The low pH increases the storage quality and palatability of the milk product (Evans *et al.*, 2013; Mohammed and Ijah 2013; Nwachukwu *et al.,* 2010). Most LAB are aero-tolerant or anaerobic and lack the ability to synthesize cytochome and porphyrins. LAB utilizes different metabolic pathways for the fermentation of carbohydrates such as hexoses, pentoses and disaccharides. During fermentation, hexose sugars are split into two triose-phosphates and though oxidation and de-phosphorylation steps in glycolysis to pyruvic acid. The final stages of the fermentation process involve the reduction of pyruvate to lactic acid. During pentose fermentation, pentose is converted to ribulose-5-phosphate via the pentose-phosphate pathway by epimerases (Khelid, 2011). LAB also possesses hydrolytic enzymes, enabling them to break disaccharides into simple sugars. This metabolic reaction is important during milk fermentation.

1.3 LAB in fermented milks

LAB, particularly those from the genera *Lactobacillus*, *Lactococcus, Streptococcus, Pediococcus* and *Leuconostoc* are widely distributed in the environment and are an important microflora of raw milk (Azadina and Khan-Nazer, 2009).

Lactobacillus spp. usually constitutes the predominant LAB microflora present in many fermented milk products (Angelis and Gobbetti 2001). *Lactobacillus* spp. including *Lactobacillus bulgaricus* and *Lb. acidophilus* are commonly reported to be the predominant *Lactobacillus* species isolated from traditional fermented milk. Other species such as *Lb. acidophilus, Lb. casei* and *Lactobacillus fermentum* have also been isolated from traditional fermented milk (Collado and Hernandez, 2007; Gonfa *et al.*, 2001; Gueimonde *et al.*, 2004; and Mathara *et al.*, 2008). *Lactobacillus* spp. have been isolated from different traditional fermented milks from around the world such as *Zabady*, an Egyptian traditional fermented milk, *Laban*, Lebanon traditional fermented milk product (Chammas *et al.,* 2006), *Kefir* Eastern Europe traditional fermented milk (Mainville *et al.*, 2006), *Amasi*, South African and Zimbabwean traditional fermented milk (Gadaga *et el.*, 1999).

Lactobacillus can be distinguished from other genera according to their carbohydrate fermentation pathway and have been noted to make a major contribution to acid production during fermentation (Angelis and Gobbetti, 2001). Some *Lactobacillus* spp. have been reported to produce bacteriocins and hydrogen peroxide which in addition to lactic acid act as antimicrobial compounds to inhibit the growth of pathogenic bacteria during fermentation (Ogueke, 2007).

Streptococcus thermophilus is one of the most widely used commercial starter cultures for yoghurt production among *Streptococcus* spp. It belongs to the group of

homofermentative LAB and produces carbonyl compounds such as acetaldehyde during pyruvate metabolism. The occurrence and growth of *Streptococcus thermophilus* in fermented milk can be influenced by processing conditions such as temperature. The ability to isolate *Streptococcus* spp. from food products relies on media used and incubation conditions (Gezginc *et al.*, 2014). *Streptococcus thermophilus* is a thermophilic LAB with good acidifying activity as inoculation of 1% (v/v) of starter culture leads to a decrease in pH from 6.6 to 5.3 within 6 h at 42°C although the maximum acidification rate of *Streptococcus thermophilus* (0.0085 $pH.min^{-1}$) is much lower than *Lb. delbrueckii subsp. bulgaricus* (0.0111 $pH.min^{-1}$) (Chammas *et al.,* 2006). Other important roles of *Streptococcus thermophilus* during milk fermentation are technological properties including urease and proteolytic activity (Iyer *et al.*, 2010). M17 is the elective medium for isolation of *Streptococcus thermophilus* at 45°C anaerobically (Tabasco *et al.*, 2007) though, Ashaf and Shah, (2011) explained that M17 can be suitable for enumeration of *Streptococcus* aerobically at 45°C because the incubation condition inhibits the growth of other LAB such as *Lb. paracasei* subsp. *Paracasei* that are found under incubation temperature of 37°C and *Lb. delbrueckii* subsp. *bulgaricus* and *Bifidobacteria. lactis* (*B. lactis)* that grow under anaerobic conditions. The optimal growth rate for *Streptococcus thermophilus* is around 45°C (Collado and Hernandez, 2007; Khedid *et al.*, 2009; Westenberg, 2008).

Lactococcus is a homofermentative coccus shaped, Gram positive, non-motile LAB. *Lactococci* were recognised as a genus after the characterization of specific species of *Lactobacillus* and *Streptococcus* spp. though chemotaxonomic analysis and 16S rRNA gene sequencing (Casalta and Montel, 2008). *Lactococcus lactis* subsp *lactis* synthesizes exopolysaccharides to improve the texture and viscosity of fermented

milk (Casalta and Montel, 2008). *Lactococcus lactis* is a species of genus *Lactococcus,* it is found naturally in nitrogen and carbon rich substrate such as milk (Sander *et al.*, 1999). The latter author further explained that *Lactococcus lactis* has a specific protective mechanism that makes it survive environmental stress. It resists proteolytic activities though; some food products (e.g. fresh milk) *Lactococcus lactis* is regarded as a spoilage organism. Their presence in food systems can be controlled by choosing specific conditions, either to promote lactococcal proliferation when desired or to prevent spoilage in products that need no lactococcal fermentation. *Lactococcus lactis* spp. is among the predominant LAB that are found in the fermented milk (Abdelgadir *et al.*, 2001; Al-Otaibi, 2012; Mathara *et al.*, 2004; Mohammed and Ijah, 2013). M17 agar is a selective medium best for enumeration of lactococci anaerobically at 37°C (Al-Otaibi, 2012).

Leuconostoc spp are Gram positive, facultative anaerobic, catalase negative cocci and oval-shaped, present in pairs and chains, and is non- motile. It has been reported to be resistant to the antibiotic vancomycin (Ogier *et al.*, 2008). According to the pre-cited author, they are often present in traditionally prepared fermented milk and the co-metabolism of lactose and citrate by *Leuconostoc* leads to the production of diacetyl. *Leuconostoc* spp have been isolated from sub-Saharan African traditional fermented milk products including *Kule noato*, *ergo* and *sethemi* (Gonfa *et al.*, 2001; Mathara *et al.*, 2004 and Kebede *et al.*, 2007).

Pediococcus spp are spherical shape Gram positive bacteria that are arranged in pairs or tetras (Stiles and Holzapfel, 1997). Their carbohydrate utilization pathway is variable, most species produce D (-) and L (+) lactic acid from glucose and some can withstand strong environmental conditions such as high pH, salt concentration and

temperature. *Pediococcus* spp. grows insufficiently in the milk product because of their irregular utilization of lactose. They have been found in ergo Ethiopian traditional fermented milk (Gonfa *et al.,* 2001).

Enterococcus spp. are Gram positive and catalase negative cocci and were considered as a member of *Streptococcus* genus in the early 90's (Murray 1990). Enterococci are facultative anaerobic cocci which grow in pairs and short chain; they can grow at 45°C and at pH 9.6 and survive at 60°C for 30 min (Nannini and Murray 2006). *Enterococcus* spp. have been used as starter cultures in the manufacturing of cheese due to their lactic acid production. Their usual habitat is in gastrointestinal tracts of warm-blooded animal and also plants (Imran *et al.*, 2010). Most of the widely used antibiotics have poor activity against enterococci which results in outbreak of enterococcal infections. However, it has been reported that enterococci has the ability to produce infection in normal host specifically intra-abdominal and urinary infection (Nannini and Murray 2006).

1.4 Methods for the identification and characterization of microbial communities

Various methods are used to characterize the microbial composition of samples of different origins such as food, human clinical materials, animals, plants and soil. These techniques include general and traditional phenotyping based on, e.g., morphological and biochemical characterization and genotypic characterization that are based on the screening of genomic elements. Both culture-dependent and culture-independent methodologies can be used. The culture-dependent techniques required the enumeration, isolation and purification of single microorganisms before their characterization and identification. For the culture-independent methods, a prior

isolation of the microorganisms is not needed. They are usually genome-based techniques where genomic elements such DNA are directly retrieved from a sample and analysed.

1.4.1 Bacterial phenotyping

Phenotypic characterization of microorganisms includes exclusively culture-dependent methods such as enumeration, isolation, and purification of the microorganisms. Further, the identification and characterization of bacteria are performed by using conventional techniques such as Gram staining, catalase and oxidase determination, growth in different pH, and salt concentration, degradation and use of various compounds such as sugars and proteins, production of toxins and resistance to antibiotics. One tool used to screen the ability of bacteria to ferment and assimilate carbohydrates is the Standard Analytical Profile Index (API system). The method allows the determination of the carbohydrates' fermentation profile of a particular isolate that can be used to differentiate it from other microorganisms. Moreover, it gives an indication of the technological properties of the isolates that can be beneficial for the selection of the microorganisms for specific tasks such fermentation of materials rich in sugars. For LAB, the particular API kit used is API 50CHL. Analysis of carbohydrate fermentation profiles using the manual sheet provided by the manufacturer or the apiweb software provides a basic presumptive identity of the microorganisms to genera and species level.

Phenotyping is an important step in the identification and characterization of microorganisms, but it does not allow a full and reliable identification, therefore, they are accompanied where possible with genotyping using molecular biology tools (Towner and Cockayne, 1993).

1.4.2 Bacterial genotyping

These include an array of methods that can be culture-dependent or independent. Genotypic identification of microorganisms presents clear advantages over phenotyping and includes fast result delivery and enhanced accuracy. Methods such internal transcribed spacer, polymerase chain reaction (ITS-PCR), random and amplification polymorphic PCR (RAPD-PCR) allow a characterization of microbial community to the species level mainly. Different species exhibiting the same DNA profile can be clustered and further characterized. Techniques including e.g. repetitive sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) can allow the differentiation of group of species at subspecies and even strain level (Liu *et al.*, 1997; Daffonchio *et al.*, 1998; Da Silva *et al.*, 1999; Yamada *et al.*, 1999; Herman and Heyndrickx, 2000; Mendo *et al.*, 2000).

Repetitive sequence-based PCR (Rep-PCR) is a typing method of microbial genome which is used to examine an isolate specific DNA pattern from PCR amplification. Rep-PCR uses DNA primer complementary to naturally occurring repetitive DNA sequences which are located along most bacteria genome. The amplification is done with a single or multiple sets of primers (Olive and Bean 1999). Rep-PCR allowed typing to subspecies and strain levels. The method was successfully used to type different bacteria including LAB. Using, the technique, Ouoba *et al.* (2010) were able to differentiate various groups within specific species of LAB from African alkaline fermented foods. For example isolates of *Weissella confusa* exhibited six different DNA profiles, *Enterococcus casseliflavus* four, *Lactobacillus plantarum* two and *Weissella cibaria* two. Using the same technique, Anyogu *et al.*

(2014) made interspecies differentiation of three groups of *Lactobacillus plantarum* and two of *Weissella confusa* isolated during submerged cassava fermentation.

Partial and full sequencing of various genes using specific primers have allowed the identification of the genus, species and even subspecies of microorganisms. The most common technique used for bacteria genotypic identification is the sequencing of the 16S rRNA gene. Reasons for sequencing the 16S rRNA gene are various including e.g. its occurrence in almost all bacteria, the stability of its function that has not changed over time and its size (1500 bp) that is long enough for informatics purposes (Janda and Abbott, 2007). The sequences obtained from sequencing the 16 S rRNA gene are analysed in databases such as GenBank (Altschul *et al.*, 1990) and EZtaxon (www.ezbiocloud.net) to provide the potential identity of the bacteria investigated. This method usually provides reliable information about mainly the genus of microorganisms and the species to some extend especially when sequences are analysed in the EZtaxon server. However, this approach does not always permit a differentiation of closely related species. Therefore, other genes such as *gyr*A, *gyr*B, and *rpo*A, *phe*S genes involved in the replication of DNA can be sequenced leading to a better delineation of species (Yamamoto, 1995; De Clerck and De Vos, 2004; Anyogu *et al.*, 2014).

For LAB, *phe*S and *rpo*A gene sequencing has been proven to be powerful in the identification and differentiation of closely related species that cannot be discriminated by16S rRNA gene sequencing (Naser *et al.*, 2005; Naser *et al.*, 2007). The latter authors also explained that both *pheS* and *rpoA* gene sequencing can differentiate also subspecies of some LAB such as those belonging to the *Lactobacillus* species. Sequencing of the two genes was used successfully to

discriminate LAB species isolated from food materials. This is the case of Anyogu *et al.* (2014) who were able to use the techniques to discriminate *Lactobacillus plantarum* from *Lactobacillus pentosus* and *Weissella confusa* from *Weissella cibaria* and *Weissella salipscis*. The pre-cited species were not differentiable by 16S RNA gene sequencing.

One culture-independent technique used to analyse microbiota is the denaturing gradient gel electrophoresis (DGGE). The technique allows the separation of sequence-specific of PCR-derived rRNA gene amplicons according to their mobility by applying linearly increasing denaturing conditions such as augmenting formamide/ urea concentrations (Strathdee and Free, 2002). Non-PCR-based molecular techniques, including microarray, fluorescence in situ hybridization, and DNA-DNA hybridization are also powerful techniques.

The current trend is the use of whole genome sequencing techniques. However, the access is quite limited due to e.g. cost especially when a large number of microorganisms need to be identified. In recent years, various new methodologies have been developed including e.g. metagenomics, metatranscriptomics, metaproteomics, and single-cell genomics. The new methodologies have modernized microbial characterization and allowed scientists to investigate directly natural microbial communities in situ, including a screening of their genes, transcripts, proteins, and metabolites.

1.5 Selection procedures for multifunctional starter cultures

1.5.1 Starter cultures

In a general sense, starter culture may be described as a preparation containing high numbers of viable microorganisms, which may be added, to bring about desirable changes in a food substrate. Being adapted to the substrate, such starters will serve to accelerate the fermentation process. This enables stricter control of a fermentation process, the outcome of which may thus be predictable. Because of the long tradition and high standards attained, starter cultures in the dairy industry served as an example or `model´ for the application of starters to other food commodities. Microorganisms used in starter cultures include bacteria, yeast and moulds. The first `pure´ starter cultures (*Lactococcus lactis*) introduced simultaneously in Denmark and Germany (Storch, Copenhagen/ Weigmann, Kiel) in 1890 were used for the fermentation of milk for cheese and sour milk processing (Holzapfel, 1997). A multifunctional starter culture is referred to bacteria that have both fermentation technological properties and probiotic attributes.

1.5.2 Probiotics

A probiotic can be defined as a living microorganism which when taken in large quantity can improve the health status of the consumer (human or animal). Live microbial food supplements include species of mainly *Lactobacillus*, *Bifidobacteria and Streptococcus* and affect beneficially the host by improving e.g. the intestinal microbial balance (Saarela *et al.*, 2000; Schrezenmeir and DE Vrese, 2001). They are mostly used in fermented dairy products (Dune *et al.*, 2001; FAO/WHO, 2002). Such products are reported to have several potential health benefits such as helping

with lactose intolerance, diarrhoea control, mucosal immune response, blood cholesterol concentrations, and cancer (Table 1.1).

Table 1. 1 Probiotics and related health benefits (Dune *et al.*, 2001; Negendra, 2007; Saarela *et al.*, 2000)

Probiotics	Health benefits
Lactobacullus acidophilus	Diarrhoea treatment, cholesterol reduction, inhibition of colon cancer
Bifidobacterium bifido	Treatment of viral diarrhoea, modulation of intestinal bacteria
Lactbacillus reuteri	Treatment of rotavirus diarrhoea, acute diarrhoea
Lactobacillus acidophilus LC1	Immune enhancing, vaccine adjuvant, adherence to human intestinal cells, balancing of intestinal microflora
Lactobacillus acidophilus NCFO1748	Lowering of fecal enzymes, prevention of radiotherapy-related diarrhea, treatment (24, 25) of constipation

Dairy products are the most common carriers of probiotics, but non-dairy foodstuffs such as fruit juices and cereal based products supplemented with probiotics are also manufactured to help with some disorders such as lactose intolerances (Prado *et al.*, 2008). In the feed sector, administration of probiotics (*Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus* and *Bifidobacterium* species) to animals including cattle, pigs, and chickens promotes e.g. the enhancement of their general health, a faster growth rate, increased production of milk and eggs (Fijan 2014; Musa *et al.*, 2009).

1.6 Selection criteria of starter cultures and probiotic bacteria

1.6.1 Requirements for the selection of starter cultures

Starter cultures are selected especially for a substrate or a raw material (milk, meat, cereals, legumes, roots, and tubers, etc.) and may have some potential attributes (Holzapfel, 1997) such as:

- To improve the nutritional value by a biological enrichment, e.g. though the biosynthesis of vitamin, essential amino acids, and proteins

- To improve the sensory quality (taste, aroma, visual appearance, texture, consistency)

- To have antimicrobial activity against foodborne pathogens - To improve the toxicological safety by degrading toxic components

- To reduce the preparation procedures by an acceleration of the fermentation

- To have probiotic properties contributing to the improvement of the general wellbeing and health.

The first most important criteria in the selection of starter cultures is the identification of the microorganisms using well-defined methods including both phenotypic and genotypic methods. Once well identified, the microorganisms can be screened for specific technological properties which are variable according to the raw material being fermented. For example, for a raw material rich in proteins such as African locust beans seeds which are fermented to produce nutritious foods such as Soumbala and dawadawa, the ability of the bacteria to degrade proteins into more digestible peptides and essential amino acids is important (Ouoba *et al.*, 2003). For cassava tubers and leaves that contain cyanogenic compounds, it is crucial that the

bacteria responsible for the fermentation possess the capacity to degrade such toxic components (Kobawila *et al*, 2005; Ahaotu *et al*., 2017). It is also important that the selection takes into account the ability of the bacteria to ensure the safety of the product by inhibiting and/or inactivating the presence of pathogenic microorganisms. It is strongly recommended that starter cultures do not produce toxins and not harbour toxin and antimicrobial resistance genes. Thus, before use, the pre-cited safety criteria should be accessed to protect the health of the consumers. After selecting potential starter cultures, the variability of the cultures should be noted and the role of each isolate determined. The identified cultures should be evaluated in fermentation trials with relevant raw material and their technological properties described (Sanni, 1993).

Various studies have been conducted to select starter cultures for controlled production of traditional fermented foods. Akabanda *et al*. (2013) used technological properties assessment to select starter cultures for *Nunu* fermentation in Ghana (West Africa). Selection criteria included acidification properties, proteolytic, lipolytic and antimicrobial activities as well as the ability to produce exopolysaccharide. Starter cultures of *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, and *Leuconostoc mesenteroides* were screened and shown to allow a rapid acidification and possess high proteolytic activity. Moreover, the isolates used in single and mixed cultures generated yoghurt with desirable organoleptic properties.

1.6.2 Requirements for the selection of probiotics

The Lactic Acid Bacteria Industrial Platform (LABIP) workshop on probiotics confirmed that "probiotics may be consumed either as a food component or as a non-

food preparation". This supported criteria listed below for the selection and assessment of probiotic lactic acid bacteria. These criteria may be summarized as follows:

- Human origin, nonpathogenic behavior, resistance to technological processes (i.e. viability and activity in delivery vehicles).
- Resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to persist within the gastrointestinal tract.
- Production of antimicrobial substances, ability to modulate immune responses, and ability to influence metabolic activities (e.g., cholesterol assimilation, lactase activity, and vitamin production) (Dune *et al.,* 2001)

For the potential probiotics to reach the gut where they can exert their properties, they need to survive the human gastric acidic juice in the stomach (pH 1.5-2 for an individual who is fasting) and the bile secretion in the small intestine. It is the reason why these parameters should be thoroughly screened during a selection process. Safety is one of the most significant criteria for the selection of probiotics. Therefore, the microorganisms should be generally recognised as safe (GRAS) i.e. non-pathogenic and non-toxic. Furthermore, they should not possess transferable antimicrobial resistance genes (Gueimonde *et al.* 2013; Saarela *et al.* 2000).

1.7 Benefits of the use of multifunctional starter cultures for controlled fermentation

Besides the fact that the preparation of most African fermented food including *Nono* is still traditional family arts, the fermentation is by uncontrolled inoculation as most of the African fermented foods. Starters are normally not used and therefore

variations in the quality and the stability of the products are often observed (Sanni, 1993). One major factor that contributes to the optimisation of fermented foods' production process is the use of attested and effective starter cultures. This guarantees product of consistent quality, taste and flavour and enhanced safety. Also, the fermentation time may be shortened (Odunfa and Adewuyi, 1985). Examples of advantages related to the use of LAB multifunctional starter cultures are depicted in (Table 1.2).

Table 1. 2 Typical examples of functional starter cultures or co-cultures and their advantages for the food industry (Leroy and De Vuyst, 2004)

Advantage	Functionality	Lactic acid bacteria
Food Preservation	Bacteriocin production	*Lactococcus lactis subsp. Lactis*
	-Dairy products	
	-Fermented meat	*Enterococcus spp.*
		Lactobacillus Curvatus Lactobacillus sakei
		Pediococcus acidilactici Enterococcus faecium
	-Fermented Olives	*Lactobacillus plantarum*
	-Fermented vegetable	*Lactococcus lactis*
Organeloptic	-Production of exopolysaccharides	Several lactobacilli streptococci
	-Production of amylase aroma generation	Several lactobacilli Several strains
	Enhanced sweetness	*Lactococcus lactis*
	-Homoalanine-fermenting starters	
	-Galactose-positive/glucose-negative starters under development malolactic fermentation	*Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus Oenococcus oeni*
Technological	Bacteriophage resistance	Several strains
	Prevention of overacidification inlactose-negative	lactose-negative
	Autolysing starters	*Lactobacillus delbrueckii subsp. bulgaricus*
	-Phage-mediated	*Lactococcus lactis subsp. lactis*
	-Bacteriocin-induced	*Lactococcus lactis*

Table 1.2 (contd) Typical examples of functional starter cultures or co-cultures and their advantages for the food industry (Leroy and De Vuyst, 2004)

Advantage	Functionality	Lactic acic bacteria
Nutritional	Production of nutraceuticals	*Lactobacillus plantarum*
	-Low-calorie sugars (e.g., sorbitol and mannitol)	*Lactococcus lactis*
		Lactococcus lactis
	-Production of oligosaccharides	*Lactobacillus delbrueckii subsp.*
	-Production of B-group vitamin (e.g., folic acid)	*bulgaricus, Lactococcus lactis, Streptococcus thermophilus*
		Several strains
		l(+)-lactic acid-producing strains
	-Release of bioactive peptides	
	Reduction of toxic and anti-nutritional compounds	*Streptococcus thermophilus*
	-Production of l(+)-lactic acid isomer	*Several strains*
	-Removal of lactose and galactose	*Lactobacillus plantarum, Lactobacillus acidophilus*
	-Removal of raffinose in soy	
	-Reduction of phytic acid content, amylase inhibitors, and polyphenolic compounds	*Enterococcus faecalis*

1.8 Acid pH and bile tolerance of LAB

Microorganisms such as LAB are found to survive the unfavourable and peristaltic conditions in the stomach (Chadwick *et al.* 2003). They increase progressively in the small intestine, from the duodenum (upper part) along to the jejunum and ileum (lower parts) though, their numbers in the duodenum are lower (10^3 per gram), and also transit time are short because of the secretion of bile, which inhibits bacterial growth. As reported by Farahmand (2015) the gastrointestinal tract (GIT) of the human body contains variable chemical and physical characteristics of which certain microbial communities could not survive in it due to pH and bile salt content of each compartment. Microorganisms that survive in the GIT are termed probiotics and lactic acid bacteria are among the most important probiotic microorganisms typically associated with the human gastrointestinal tract (Holzapfel *et al.*, 2001). According to the author, the organism might not dominate the GIT but it has beneficial effect on the human health.

The dominant bacteria in the large intestine are non-spore-forming anaerobes, including *Bifidobacterium* and *Lactobacillus* (Harzallah and Belhadj 2013; Shigwedha and Jia 2013) though, the large intestine, due to availability of nutrients and slow transit time, is convenient for microbial growth due to favourable pH conditions. Furthermore, for LAB to survive and establish within the human Gastro Intestinal Tract (GIT), it must exhibits some of the desirable properties of starter cultures/probiotics include their ability to resist the acidity (pH 2.5-pH 3.5) of the stomach and the exposure to bile in the upper part of the intestine (Holzapfel *et al.*, 1998).

There are needs for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, as the bacteria passes through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Chou and Weimer, 1999). The author further explained that the time of travelling of bacteria from entrance through the stomach to the intestine is 90 min therefore, strains selected for use as starter culture should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits

1.9 Antimicrobial compounds produced by LAB

LAB plays a role in inhibiting other microorganisms, particularly pathogenic foodborne bacteria (Gonfa *et al.*, 2001). Since the early 1900s, LAB have been the source of bio-preservatives bacteria depending on their interaction with the food product. The inhibition property of LAB has a significant role in improving the storage quality and safety of fermented foods including milk. Factors which contribute to microbial inhibition by LAB include low pH, organic acid, bacteriocins, hydrogen peroxide, ethanol, diacetyl, nutrient depletion and low redox potential. It has been widely reported that the major antimicrobial effect of LAB in traditional lactic fermented food products is acid production at low pH because of the production of lactic acid and acetic acid (Patil *et al.*, 2010; Anyogu *et al.*, 2014). LAB has a significant role in human health; they contribute to the area of osmoregulation, autolysis and produce bacteriocins and bacteriophages. The US FDA has approved LAB as Generally Recognized As Safe (GRAS) for use as medical implantation, cosmetics, food additive and pharmaceuticals (Nikita and

Hemangi, 2012). A variety of organic acid is produced by LAB as part of their normal metabolism. These organic acids have been shown to have antimicrobial activity when they disrupt the cell membrane of the bacteria and interfere with the active form (Blom and Mortvedt 1991). Notwithstanding their complexity, the whole basis of lactic acid fermentation centres on the ability of LAB to produce lactic acid, which inhibits the growth of other non-desirable organisms (Evans *et al.*, 2013).

1.9.1 Bacteriocin as an antimicrobial compound

Bacteriocins are antimicrobial peptides produced by different types of bacteria including LAB that inhibit Gram-positive bacteria of the same nutritive requirements (Sewa *et al.*, 2012). Bacteriocins are specifically known as natural compounds that are capable of influencing the quality and safety of food products. As reported by Jeevaratnam *et al.* (2005), many bacteriocins such as Nisin (a commercially exploited bacteriocin used in large scale) have been isolated, characterized and confirmed to exhibit an antimicrobial activity against pathogenic and foodborne pathogens. The author further explained the various ways in which bacteriocins could be used as food preservative. The compound could be applied by inoculating the food with LAB (starter cultures) that produce a bacteriocin or by a straight addition of a purified or semi-purified bacteriocin as a preservative or the use of a product previously fermented with bacteriocin producing strain. All these methods depend on the approach of the biological preservation required.

Bacteriocins are known to be ribosomally synthesized and extracellularly they exert a bacteriostatic and bacteriocidal activity. To identify bacteriocin- producing strain, antagonism assays can be performed as follows: 2 h incubated cell-free supernatants from colonies of the test bacteria treated with proteolytic enzymes such as protease

and proteinase K are overlaid with soft agar inoculated with indicator bacteria (Gram positive and Gram negative food borne pathogens) or sensitive strain. After an incubation time of 24-48 h at the adequate temperature, the apparition of clear zones indicative of an inhibitory activity is recorded. Daba *et al.* (1991) reported the inhibitory effects of *Leuconostoc mesenteroides* against several species of *Listeria monocytogenes* due to bacteriocin production. Also, LAB isolated from Nigerian fermented food products were able to produce bacteriocins when tested against Gram-positive and Gram-positive bacteria including *Shigella flexneri* and *Salmonella Typhimurium* (Sanni *et al.*, 1999; Ogunbanwo *et al.*, 2003).

Bacteriocins have been classified as following:

1. Lantibiotics: active peptide membrane that contains thio-ether amino acid lanthionine and β-methyl lanthionine and which are usually low molecular weight peptides. Example: nisin produced by *Lactococcus lactis subsp lactis*.

2. Small heat-stable peptides: bioactive peptides which have no amino acid residues. They are pediocin-like bacteriocin with conserved N-terminal sequence Tyr-Val and two cysteines forming S-S bridge that possess anti-listeria activity.

3. Large heat stable labile bacteriocins: they possess large molecular weight. Examples: Helviticin, Lactacin A and B.

4. Constituted by bacteriocins that form a large complex with other chemicals such as carbohydrate or lipid required for activity.

The most common bacteriocin produced by bacteria associated with food is related to class 1 and 2. Nisin is the best known bacteriocin that is considered as GRAS to use in food ingredient (Cleveland *et al.*, 2001; Jeevaratnam *et al.*, 2005),

1.9.2 Hydrogen peroxide as an antimicrobial compound

Hydrogen peroxide has antimicrobial activity because of it oxidizing effect on thiol of proteins and cell phospholipids. Hydrogen peroxide in addition to lactate, NADH, and pyruvate is generally produced by LAB in the presence of O_2 (Oxygen) and can easily activate the lactoperoxidase system in milk (Anyogu *et al.*, 2014).

Hydrogen peroxide alters gene expression in many cell types. Alterations in nuclear import of transcription factors or similar key proteins may be responsible for these changes (Michael *et al.*, 2000). It can also be as a forerunner for the production of bactericidal free radicals such as superoxide (O2-) and hydroxyl (OH.) radicals which can damage DNA. Davidson *et al.* (1983) have reported using H_2O_2 produced by *Lactobacillus* and *Lactococcus* strains to inhibit *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotropic microorganisms in foods. In raw milk, H_2O_2 activates the lactoperoxidase system, producing hypothiocyanate (OSCN-), higher oxyacids (O_2SCN- and O_3SCN-) and intermediate oxidation products that are inhibitory to a wide spectrum of Gram-positive and Gram-negative bacteria (Conner 1993).

1.9.3 Diacetyl as an antimicrobial compound

Diacetyl is an aroma compound that is mostly produced from citrate fermentation within LAB genera. It inhibits mainly the growth of Gram-negative bacteria by reacting with the arginine-binding protein, thus affecting the arginine utilization. The inhibition activity depends on the concentration of the compound; for instance, Diacetyl at 344 μg/mL inhibited strains of *Listeria*, *Salmonella*, *Yersinia*, *Escherichia coli*, and *Aeromonas* (Jay 1982). Production of diacetyl from lactic fermentation is low; therefore, its food preservative property is limited. However, it

may act synergistically with other antimicrobial factors and contribute to combined preservation systems in fermented foods (Jay 1982).

1.10 Antimicrobial resistances in bacteria

Antimicrobial resistance can be defined as a stage whereby microorganisms (e.g. bacteria, fungi, parasites) have developed the ability to become resistant to one or multiple antimicrobials (such as antibiotics, antivirals and antimalarials) to which they were originally sensitive to. Currently, many pathogenic microorganisms have developed multi-resistances to various antibiotics and this constitutes a global public health threat. Development of antimicrobial resistances is due to environmental and human factors though e.g. misuse and overuse of drugs. The World Health Organization (WHO) invites all key stakeholders such as policy makers, patients, the public, practitioners, pharmacists, the pharmaceutical and food and feed industries to act and take responsibility for combating antimicrobial resistance (Bax *et al.*, 2001; WHO 2014).

An antimicrobial is a substance that is synthetized to kill or inhibit the growth of microorganisms. Antimicrobials which include antibiotics are substances such as penicillin and gentamycin that produced by microorganisms which other microorganisms can be sensitive or resistance to. Other antimicrobials include synthesized compounds such as sulfonamides and quinolones, semi-synthesised (generated by chemical alteration of natural compounds) compounds such as methicillin, amoxicillin, amikacin and ampicillin and natural compounds from plants (quercetin and alkaloids) and animals (lysozyme) (Berger-bachi, 2002; worldatlas, 2016). It is clear then that an antibiotic is an antimicrobial but all antimicrobials are not antibiotics. However, the term "antibiotics" is commonly used to designate

antimicrobials and the two words may be used interchangeably in the current manuscript.

1.10.1 Class of antibiotics and their mode of actions

There are various classes of antibiotics. Those with similar structural classes have comparable patterns of toxicity, effectiveness and allergic potentials. They consist of a variety of drugs but each one is still unique in its own way. Antibiotics are classified based on their structures, functions and/or spectrum of activity (Table 1.3) (Forbes *et al.*, 1998).

a: Structural classification (Molecular structure)

- β-lactams: beta-lactam ring.
- Aminoglycoside: the side chains attached to the basic structures varies.

b: Function (Mode of action) Targets for antibiotics

- Inhibition of cell wall synthesis
- Inhibition of protein synthesis
- Inhibition of membrane function
- Inhibition of nucleic acid synthesis
- Anti-metabolites

c: Spectrum of activity

- Narrow spectrum
- Broad spectrum

Tetracycline compounds are broad-spectrum bacteriostatic antibiotics that have effect against a multitude of organisms. They are commonly used for the treatment of infections such as moderately severe rosacea and acne. They can also treat respiratory tract infections, intestinal infections, ear infections, sinus infections and urinary tract infections as well as Lyme disease, gonorrhea, and Rocky Mountain spotted fever (New Health Advisor 2014 http://www.newhealthadvisor.com/Classification-of-Antibiotics.html). Resistance to Tetracycline compounds have been observed frequently among *Lactobacillus* species and the Minimal Inhibitory Concentrations (MIC) is variable according to the species.

Aminoglycosides are another class of antibiotics that include compounds such as Kanamycin, Neomycin, Streptomycin and Gentamycin. Aminoglycosides and tetracycline are able to bind the 30S ribosomal subunit and act as inhibitors of protein synthesis, but aminoglycosides further bind to the 30S ribosome to freeze the 30S initiation complex and stop further reactions. Chloramphenicol, lincomycin, and macrolides, such as erythomycin bind to the 50S ribosome and inhibit transpeptidation

Table 1. 3 Classification of antibiotics and their mechanism of action (Berger-Bachi, 2002; Forbes *et al.*, 1998).

Antibiotics	**Mechanism of action**
Beta-lactams antibiotics (Penicillin, Cephalosporin)	Target and bind to penicillin-binding protein(PBPs), Inhibits bacteria cell wall production
Microlids (Erythomycin, Clarithomycin, Troleandomycin)	Target and bind to 50s ribosomal subunit to inhibit translocation and transpeptidation process, Hinders bacteria protein production
Tetracycline (Doxycycline, Minocycline)	Target and bind 30s ribosomal subunit to prevent aminoacyl-tRNA to attach to RNA-ribosome complex, Inhibits bacteria ability to produce protein
Fluoroquinolones(Ciprofloxacin, Levofloxacin, Norfloxacin and Ofloxacin)	Prevent bacteria from producing DNA
Aminoglycisides (Kanamycin, Streptomycin, Gentamycin and Neomycin)	Target and bind to the 30s ribosomal subunit to cause misreading of the genetic code which results in inhibition bacteria protein synthesis

1.10.2 Development and spread of antibiotic resistance

Resistant bacteria have long existed before human's knowledge of the therapeutic use of antibiotics. In the early 1940s-1950s, Streptomycin, Chloramphenicol, and Tetracycline were developed and Streptomycin was very effective against causative agents of Tuberculosis. Dozens of antibiotics are manufactured worldwide and use to fight various diseases caused by different microorganisms such as bacteria (Patel *et al.*, 2012). However, due mainly to human factors, resistances to many antibiotics have occurred and are increasing. This constitutes a global public health threat that many countries and international organizations are currently trying to target.

There are many factors that cause the occurrence of antibiotic resistance namely:

- Over-prescription of antibiotics
- Patients not finishing the entire antibiotic course
- Overuse of antibiotics in livestock and fish farming
- Poor infection control in health care settings
- Poor hygiene and sanitation
- Absence of new antibiotics synthesis

According to the WHO (2015), the occurrence of antimicrobial resistances may be more prevalent in countries without standard treatment guidelines and where antibiotics are often over-prescribed by health workers and veterinarians and over-used by the public.

Antimicrobial resistance in bacteria can be intrinsic i.e. naturally occurring in some types of bacteria or related to mutations or acquired though transfer from other microorganisms. The resistance can also occur when a bacterium is in contact with the antibiotic agent concerned (European Commission 2008).

Bacteria use a complex array of mechanisms to transfer and spread resistant determinants (Ouoba *et al.*, 2008). Each bacteria strain possesses many types of resistance mechanisms though, it varies among species. The resistance mechanisms can be of a biochemical and/or genetic type. Biochemical mechanisms produce resistance by e.g. antibiotic inactivation that reduces the concentration of the active drug without modifying of the compound itself. Genetic types of mechanisms involved e.g. mutations or horizontal transfer of genes among bacteria. These mechanisms involved the transfer of genetic elements such as conjugative plasmids, transposon and also insertion or deletion of fractions of the genome (Patel *et al.*, 2012; Jahan *et al.*, 2015). Mutations occur mostly on chomosomes and it can cause genetic changes in multiple regions of the genome. Conjugation is the most significant in vivo mechanism of gene transfer between different bacterium species. It is operated by a direct cell to cell contact (Courvalin, 1994; Gever *et al.*, 2003).

Lactic acid bacteria such as species belonging to the genera *Lactobacillus*, *Enterococcus*, *Leuconostoc*, and *Pediococcus* are commonly found in humans, animals and plants and constitute important components of the food chain. Many of them have been reported to harbor antimicrobial resistance genes that are located on the chomosome, plasmids, and transposons that can be transferred to other bacteria. In a study that investigated the antimicrobial susceptibility of the LAB of African and European origins, Ouoba *et al.* (2008) detected the presence of the *erm*B gene

encoding resistance to Erythomycin in an isolate of *Lactobacillus reuteri*. They further demonstrated by conjugation experiments that the *Lactobacillus* isolate was able to transfer the gene to an isolate of *Enterococcus faecalis*. The isolate of *Lactobacillus* had probiotic properties, but due to the presence of the transferable erythomycin resistance gene its intended usage as probiotic for control of diarrhea was abandoned.

The food chain is the main route of antibiotic resistance transfer between bacteria in animal and human populations (Devirgilis *et al.*, 2011). An example of a possible route of transfer is depicted in (Figure 1.2). Also, International food trade and travel are channels by which resistant microbes are spread around the world and cause the death of many people irrespective of the country of origin (European Centre for Disease Prevention and Control (ECDC) 2018).

Fermented milk such as *Nono*, have been shown to have human health benefits and a rich microbial biodiversity including probiotic organisms (Beukes *et al.*, 2001; Omotosho *et al.*, 2013). The general theme of these studies is assessing the microbial quality and LAB diversity in the fermented milk when produced under uncontrolled conditions (Figure 1.1). However, only a few studies have investigated fermenting organisms from the perspective of improving the technology (Akabanda *et al.*, 2013). With regard to *Nono*, studies focused on the identification of fermenting organisms have been conducted using mainly phenotypic techniques which are known to be unreliable as compared to molecular typing methods (Anyogu *et al.*, 2014; Ouoba *et al.*, 2008).

Overall, although information has recently started to emerge, There are still big gaps in our knowledge and understanding of *Nono* including:

- Establishing the traditional method or methods of production of *Nono* in Nigeria.
- Determination of the dominant lactic acid microflora of *Nono* produced in different locations.
- Setting up optimum conditions for the production of a safe, consistent product with enhanced shelf-life.
- From the study, strains with technological and probiotic potential will be selected to be used as starter cultures for controlled fermentation

Table 1. 4 A summary of selected research studies on Nigerian *Nono*

	Research Subject	Source
1	Screening of lactic acid bacteria strains isolated from "*Nono*" for Exopolysccharides (EPS) production	(Bukola *et al.*, 2008)
2	Production of Nigerian *Nono* using lactic starter cultures	(Adesokan *et al.*, 2011)
3	Microbial contaminants associated with "*Nono*"	(Aernan *et al.*, 2011)
4	Screening locally produced *Nono* for occurrence of pathogenic *Yersinia species* in fermented milk	(Okeke and Okwori, 2011)
5	Extent of microbial contamination of *Nono* and fresh cow milk in Makurdi, Benue state, Nigeria	(Obande and Azua, 2013)
6	Microbial quality and HACCP concept in the production of" *Nono*" in Minna Niger state, Nigeria	(Omotosho *et al.*, 2013)
7	Comparative study of microbial quality of hawked *Nono* and packaged yoghurt sold in Bida Metropolis	(Mohammed and Abdullahi, 2013)
8	Physico-chemical and microbiological analysis of "*Nono*" consumed within Kaduna town, North-Western Nigeria	(Eqwaikhida *et al.*, 2014)
9	Bacteriological quality of fermented milk sold locally in Samaru and Sabongeri market, Zaria, Nigeria	Yabaya *et al.*, 2012)

1.12 Objectives of the study

The main objective of the study is to characterize the community of LAB involved in the production of *Nono* in order to select multifunctional starter cultures for controlled fermentations leading to a product with improved nutritional quality, safety, stability and marketability.

To achieve the pre-cited objective the following specific objectives containing each various activities were or will be pursued.

1. Determination of the identity of the bacteria

Activities: Enumeration, isolation and identification of the bacteria using phenotypic and genotypic methods

2. Determination of the functional properties of the bacteria

Activities: screening the tolerance of the bacteria to acid and bile salt and antimicrobial activity against indicators of pathogenic bacteria

3. Determination of the safety of the bacteria with regards to transferable antimicrobial resistance genes

Activities: determination of the MIC for various antibiotics, detection of the presence of specific antimicrobial resistance genes, determination of the transferability of the resistant genes, determination of the genetic background of any gene transfer.

Chapter Two: Enumeration, isolation and identification of LAB from *Nono*

2 Introduction

Food substrates such as cereals, cassava and milk undergo lactic acid fermentation due to their high sugar content. During fermentation, these sugars are converted into lactic acid as the primary end product by LAB. There are various type of LAB that can be differentiated according to factors such as morphology, growth at different temperature, mode of glucose fermentation, lactic acid production, growth at different salt concentration, acid and alkaline tolerance and range of sugar utilization (Nikita and Hemangi, 2012). Moreover, they exhibit various genotypic features that can be targeted to identify and characterize them (Anyogu *et al.*, 2014; Ouoba *et al.*, 2012; Sawadogo-Lingani *et al.*, 2007).

Studies have been carried out on the enumeration and isolation of LAB present in some traditional African fermented milks including those from Zimbabwean *amasi* (Gadaga *et al.*, 2000), Ghanaian *nunu* (Akabanda *et al.*, 2010), fermented milk from the Fulani community in Burkina Faso (Savadogo *et al.*, 2004), Kenyan *suusac* (Lore *et al.*, 2005) and South African *Sethemi* (Kebede *et al.*, 2007). LAB from the Sub Sahara Africa fermented milks include species from e.g. the genera *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Enterococcus* and *Pediococcus* (Abdelgadir *et al.*, 2001; Gonfa *et al.*, 2001; Kebede *et al.*, 2007; Mathara *et al.*, 2004; Okonkwo, 2011). Assessing the diversity and dynamics of microbiota during *Nono* production has been the focus of a number of studies. However, the microorganisms implicated have been identified for the most part using phenotypic methods which have been shown not to be sufficient for an accurate identification of bacteria (Gever *et al.*, 2001; Ouoba *et al.*, 2008). A number of techniques can be used to identify bacteria to species and subspecies level and to

differentiate closely related species. These include methods such as (ITS)-PCR, (RFLP), Repetitive Element Palindromic (Rep)-PCR, (PFGE) and sequencing of specific genetic elements such 16S rRNA, *gyr*B, *gyr*A, *rpo*B, *phe*S genes (Branco *et al.*, 2006; Ouoba *et al.*, 2008; Otlewska *et al.*, 2010).

2.1 Objectives

The general objective of this study was to investigate the microbial diversity of LAB involved in the traditional fermentation of milk for *Nono* production. To achieve this main objective, specific objectives that include various activities were pursued including:

- Isolation and determination of the number of bacteria using different agars media

- Phenotypic characterization of the bacteria including the screening of macroscopic, microscopic and biochemical features using conventional methods

- Genotypic characterization of the bacteria including typing and identification at species and subspecies level by rep-PCR and sequencing of specific genes

2.2 Materials and methods

2.2.1 Media preparation

Three different types of media were used namely deMan-Rogosa Sharpe (MRS) Agar (Oxoid, CM0361 Basingstoke, UK), MRS + L-Cystein (MRSL) (C1276-10°C Minimum 98% TLC), M17 Agar (Oxoid, CM0785) and Maximum Recovery Diluent (MRD, Oxoid CM0733). All media were prepared according to the manufacturer's instructions and sterilised by autoclaving at 121°C for 15 minutes prior to usage.

2.2.2 Sample collection

Sampling was performed in different areas of Abia State, Eastern region of Nigeria (West Africa). A total of eight samples of *Nono* were collected from eight different *Nono* producers. Two from producers at Eket Islamic Mosque Umuahia and six other samples from farm settlements at Lokpa-Nta Fulani village (Table 2.1). All samples were collected in sterile containers and kept on ice before being transported for preliminary analysis to Michael Okpara University of Agriculture (MOUA), Centre for Molecular, Bioscience and Biotechnology (Nigeria). The preliminary analyses aimed at isolating the bacteria mainly. Further, the isolates were transported to London Metropolitan University (London, UK) for advanced characterization.

2.2.3 pH measurement

The pH of the samples was measured at least two times with a calibrated pH meter (Whatman PHA 2000 Portugal). This was performed at time of arrival at the laboratory and before the microbial analysis. After calibrating the pH meter with buffers pH 7 and pH 4, the round glass electrode was inserted into the *Nono* samples and the pH values recorded.

Table 2. 1 *Nono* sampling locations and ages

Samples	Locations	Age of samples	Type of samples
1	Lokpa Fulani Village	Overnight	Heated cow milk
2	Lokpa Fulani Village	Overnight	Heated cow milk
3	Lokpa Fulani Village	Overnight	Heated cow milk
4	Lokpa Fulani Village	Overnight	Heated cow milk
5	Lokpa Fulani Village	Overnight	Heated cow milk
6	Lokpa Fulani Village	Overnight	Heated cow milk
7	Eket Islamic Musque	One day	Heated cow milk
8	Eket Islamic Musque	One day	Heated cow milk

2.2.4 Enumeration and isolation of LAB from *Nono*

Isolation of the LAB was carried out using three different media including MRS agar, MRSL agar and M17 as shown in (Table 2.2). *Nono* sample (1 ml) was homogenized with 9 ml of MRD to make an initial dilution of 10^{-1}; the suspension was used for further dilutions up to 10^{-7} by adding 100 µl of the previous suspension into 900µl of MRD in a sterile Eppendorf tube. All mixtures were homogenized using a vortex mixer. Further, 100 µl of the last four dilutions (10^{-4}-10^{-7}) were inoculated unto each type of agar using glass spreader. The plates were incubated anaerobically in an anaerobic jar (Oxoid AG0025) and an anaerogen gas kit (Oxoid BR0038) added to create an anaerobic condition. MRSL and MRS agar plates were incubated at 37°C and M17 plates at 45°C for 48 h. After incubation, colonies were enumerated and recorded as CFU/ml.

Table 2. 2 Media and incubation conditions used for the enumeration of LAB from *Nono*

Media	T°C	Duration (h)	Incubation
M17	45	48	Anaerobic
MRS Agar	37	48	Anaerobic
MRS+L-cysteine	37	48	Anaerobic

After 48 h of incubation, the morphology of the colonies on the plates was visually examined and colonies with different colours, shapes and sizes were randomly selected and aseptically streaked on the corresponding medium (e.g. MRSL/MRS/M17) to purify the culture. Further, a single pure colony was picked

aseptically with a sterile loop and stored in a Microbank cryovial (Pro-Lab Diagnostics, Birkenhead, UK) at -20°C until required for further analysis.

2.2.5 Determination of the macroscopic and microscopic characteristics of LAB from *Nono*

Each isolate from the stored cryovial was cultured for 48 h and further subcultured for another 48 h. Colony morphology characteristics such as size, shape and colour were recorded for all pure isolates. Cell morphology was examined by microscopy using a phase contrast microscope (0.90 Dry Japan Nikon Eclipse E400) according to the manufacturer's instructions. A smear was made by mixing a drop of sterile water with a single colony on a glass slide. After covering the culture with a slide cover, a drop of immersion oil was added followed by observation of the cell morphology with the phase contrast microscope (magnification X100).

2.2.5.1 KOH (3%w/v) string test

All the colonies were initially tested for Gram reaction by wet preparation using KOH (3%w/v) as described by Lanyi (1987) and Brown (2011). A drop of 3% KOH was added onto a microscope slide and emulsified with a single bacterium colony to observe the formation or not of a string. A slimy string was indicative of a Gram-negative bacterium while the absence of slimy material was indicative of a positive Gram reaction.

2.2.5.2 Oxidase test

The bacteria were tested for oxidase reaction using a few drops of oxidase reagent (Biomerieux® REF 55635), and a strip of filter paper (Whatman No. 4, Whatman Plc., Kent, UK) according to the method described by Anyogu *et al.* (2014). A

colony was spread on the filter and the appearance of a purple colour within 20 seconds that is indicative of a positive reaction was screened.

2.2.5.3 Catalase test

To screen the isolates for catalase reaction according to Anyogu *et al.* (2014), a drop of 30 % (v/v) hydrogen peroxide (H3410, Sigma) was placed on a microscope slide, and a single colony was added. The presence of bubble indicated a positive reaction

2.2.6 Identification of the LAB using genotypic methods

2.2.6.1 Extraction of DNA

Each isolate was cultured for 48 h on the same medium from which they were enumerated. A colony of each isolate was sub-cultured on agar and incubated for 24 h anaerobically at 37°C. The DNA of a pure colony was extracted using the Instagene matrix (Bio-Red 732-6030, Hercules, CA, USA) according to the manufacturer's instructions. The extracted DNA was stored in an Eppendorf tube at -20°C until required for further analysis.

2.2.6.2 Differentiation of isolates at species and sub-species level using Rep-PCR

This was carried out according to the protocol used by Ouoba *et al.* (2008). The reaction mixture of 25 µl contained 2 µl of DNA template, 2.5 µl of PCR buffer (10x Applied Bio system N808-0161), 0.2 µl $MgCl_2$ ($25mmol1^{-1}$) (AM9530G, Applied Bio system), 4 µl of primer GTG5 (5' - GTG GTG GTG GTG GTG-3') (5 pmol ml^{-1}), 4 µl DNTP (1:25 mmol 1^{-1})

(U1511, Promega, Southampton, UK), 0.2 µl of Taq polymerase (5U; N808-0161, Applied Bio system) and 10.30 µl of autoclaved high purity water (Sigma, Gilligham

UK). Amplification was carried out in a thermocycler (GeneAmp PCR 2700 system, Applied Bio system Singapore) under the following conditions: 4 min at 94°C for initial denaturation, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 1 min and elongation at 65°C for 8 min . The amplification ended with a final extension at 65°C for 16 min and the amplified product cooled at 4°C.

2.2.6.3 Agarose gel electrophoresis

The PCR products were separated by electrophoresis on 1.5 % agarose gel (Bio-Rad, Hemel Hempstead UK) for 2 h in 1x Tris Borate-EDTA buffer (TBE, Sigma UK) at 130 V. The gel was stained with aqueous 0.5 µl/ml ethidium bromide (Sigma, UK) for 20 min and rinsed with distilled water for 10-15 min. The DNA profiles of the samples were visualized and recorded using a UV transilluminator gel documentation system (M-26X, UVP, Cambridge UK). The DNA profiles were analyzed visually and the bacteria group according to their pattern similarities.

2.2.6.4 Sequencing of the 16S rRNA gene

The method described by Ouoba *et al.* (2008) was used to sequence partially the 16S rRNA gene of the bacteria. Representative of isolates from each rep-PCR group were further identified by sequencing first their 16S rRNA gene. The process was carried out with a reaction mixture containing 1 µl of each extracted DNA, 5 µl of 10 X PCR buffer, 5 µl of DNTP (1:25 mmo1 1^{-1}) (U1511, Promega), 0.5 µl of primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') (100 mml1^{-1}), 0.5 µl of primer pE (5'-CCGTCAATTCCTTTGAGTTT-3') (100 mml1^{-1}), 0.25 µl of AmpliTaq polymerase (Applied Bio system N808-0161, 5U) and 37.75 µl of high purity autoclaved water. The amplification was carried out under the following conditions: 5 min at 95°C for initial denaturation followed by 35 cycles of denaturation at 94°C for 1min,

annealing at 55°C for 1 min, and elongation at 72°C for 1 min. The final extension was performed for 5 min at 72°C and the products cooled at 4°C. The PCR products were further electrophoresed on 0.4% agarose gel that was run in TBE buffer for 1h at 130 V. The gel was stained with ethidium bromide for 20 min and rinsed with distilled water. The DNA bands were visualized and recorded as described above.

The PCR products were purified using QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany 28104) and following the manufacturer's instructions. Sequencing was done to generate 550 bp of nucleotides with the primer pD (5'-GTATTACCGCGGCTGCTG-3') and using the ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems 4337455) to stop the reaction. The reaction mixture consisted of 4 µl of PCR product (30–90 ng), 2 µl of primer pD (20 ng/µl) and 4 µl of ABI Big Dye Terminator. The reaction was achieved using the following program: 95°C for 2 min, then 35 cycles at 96°C for 15 s, 40°C for 1 s and 60°C for 4 min.

The amplified PCR product was precipitated with 1µl of sterile 3M sodium acetate (pH 4.6) and ethanol. PCR product (10 µl) was mixed with 1µl of sterile sodium acetate and 50 µl of absolute, followed by centrifugation at 13000 rpm for 20 min. The pellets were rinsed with 250 µl 70% (v/v) ethanol and centrifuged for 10 min at 13000rpm. The supernatants were discarded and the pellets air dried and sent for sequencing (Source Bioscience, Cambridge, UK). The bacteria were identified by comparing their sequences with those contained in the GenBank database of National Centre for Biotechnology Information, (NCBI), Maryland USA, using the online Basic Local Alignment Search Tool (BLAST) program. They were further

analysed using the Eztaxon database (www.ezbiocloud.net) that contains 16S rRNA gene sequences of type strains only.

2.2.6.5 Identification of the bacteria by sequencing of the phenylalanyl-tRNA synthase (pheS) α-subunit and RNA polymerase, alpha subunit (rpoA) genes

For species that could not clearly be differentiated by 16S rRNA gene sequencing, a further identification was carried out by sequencing of the *pheS* and *rpoA* genes according to the method described by Anyogu *et al.* (2014). The reaction mixture of 50 µl for the amplification of the *pheS* gene was composed of of 36.8 µl of sterile high purity water, 5 µl of 10 X PCR buffer (Applied Biosystems), 5 µl of dNTP (1.25 mmol l^{-1}; Promega), 0.5 µl (21 mmol l^{-1}) of each forward primer, pheS-21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and reverse primer, pheS-23-R (5'-GGRTGR-ACC-ATV-CCN-GCH-CC-3'), 0.2 µl AmpliTaq DNA polymerase (5U; Applied Biosystems) and 2µl of DNA template. The thermal programme consisted of (i) 5 min at 95°C, (ii) 3 cycles of 1 min at 95°C + 2 min 15 s at 46°C + 1 min 15 s at 72°C, (iii) 30 cycles of 35 s at 95°C + 1 min 15 s at 46°C + 1 min 15 s at 72°C and (iv) a final 7 min at 72°C. The same reaction mixture were used for the *rpoA* gene sequencing but with forward primer *rpoA*-21-F (5'-CAY-CCNGCH-CGY-GAY-ATG-C-3') and reverse primer *rpoA*-23-R (5'-GGRTGR-ACC-ATV-CCN-GCH-CC-3'). The amplification was confirmed using gel electrophoresis as described above. Positive amplicons were purified using the QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) and following the manufacturer's instructions. The forward primer (3.2 mmol l^{-1}) was used for sequencing the purified products to identify the bacteria to genus and species level. The sequences were analysed in the

GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) database using the BLAST tool.

2.2.7 Determination of the carbohydrate assimilation profiles of selected LAB using API 50 CHL

From the genotypic identification results, seven different bacteria were selected for further analyses. API 50 CHL comprising 49 carbohydrates was used to test the carbohydrate assimilation profile of the selected isolates and also compare their identity obtained by this method to that obtained using genotypic methods.

Colonies of overnight culture of the isolates were harvested to make a dense suspension in 2 ml sterile distilled water. Eight drops of each dense suspension was inoculated into the medium and mixed thoroughly. The inoculated medium was used to fill the 49 different tubules in a strip containing different carbohydrates. The top of the tubules was overlaid with sterile paraffin oil to create an anaerobic condition. The strips were covered and incubated aerobically at 37°C and the reading was taking after 24 and 48 h. After inoculation and during incubation, the production of acid due to low pH leads to a colour change from purple to yellow for some carbohydrates depending on the isolate. This change of colour that shows the ability of the bacteria to metabolize specific carbohydrates was recorded in a results' sheet provided by the manufacturer. Tentative identification of the isolates was performed by entering and analyzing their metabolic profile into the APIWeb software

2.3 Results

2.3.1 Isolation, enumeration and preliminary phenotypic characterisation of LAB from *Nono*

Before isolating the bacteria, the pH of the samples was recorded. Unfermented milks exhibited a pH of about 6.8. The fermented milks exhibited various acidic pH between 4.3-5 (Table 2.3).Three different media MRS agar, MRSL and M17 agar were used for the enumeration of LAB to allow cultivation of a diversity of microbes. The colonies were enumerated and calculated as CFU/ml. After 48 h of incubation, all samples showed a bacterial count at a level of 10^7 CFU/ml. The bacteria showed similar growth pattern at different acidic pH values (Table 2.3). At total of 128 bacteria were isolated from the eight samples of *Nono* investigated. Variable colony morphologies were observed with LAB recovered from MRS agar showing more variable types of colony than those observed on M17 and MRSL agars (Table 2.4-2.6). All isolates exhibited the primary features of LAB i.e. Gram positive; catalase negative and oxidase negative. Microscopic observations revealed that a majority of the cells were rods arranged as chains, single and diplobacillus. There were also some cocci arranged in chains, single, and diplococci while some were V-shape and coccobacillus (Table 2.4-2.6).

Table 2. 3 Total microbial counts of *Nono* samples on different agar media

Samples	pH	Total counts (cfu/ml)		
		MRS	MRS + L-CYSTEINE	M17
1	4.91	3.3×10^7	3.70×10^7	3.60×10^7
2	4.9	6.44×10^7	5.88×10^7	8.76×10^7
3	4.67	6.20×10^7	7.36×10^7	1.34×10^7
4	4.99	3.60×10^7	3.18×10^7	7.02×10^7
5	4.38	5.18×10^7	5.94×10^7	7.76×10^7
6	4.55	3.64×10^7	4.02×10^7	3.08×10^7
7	4.59	4.14×10^7	6.44×10^7	4.52×10^7
8	4.43	4.12×10^7	5.32×10^7	3.62×10^7

Data represent the mean of number of colonies in two experiments expressed as mean of $\log_{10}$ cfu/ml

Table 2. 4 phenotypic characteristics of lactic acid bacteria from *Nono* on M17 agar

Samples	Isolate code	Colony Morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
1	1A1	Irregular, flat	Small single rods	+	-	-
	1C1	Irregular, flat,	Small rod in chain	+	-	-
	1E	Irregular, flat,	Small rod in chain	+	-	-
	1FA	Smooth, flat, round	Cocci in chain	+	-	-
	1FB	Irregular, flat,	Small rods in chain	+	-	-
	1FC	Irregular, flat,	diplobacillus	+	-	-
2	2C2	Smooth, shiny, round	Small rods in chain	+	-	-
	2FA	Smooth, shiny, round	Small rods in chain	+	-	-
3	Nil	Nil	nil	nil	nil	nil
4	4A1	Smooth, shiny, white spot	Cocci in chain	+	-	-
	4A2	Smooth, shiny, white spot	Cocci in chain	+	-	-
	4A3	Smooth, shiny, round	Cocci in clusters	+	-	-
	4CA	Smooth, shiny, white spot	diplococci	+	-	-
	4CB	Smooth, shiny, white spot	Cocci in chain	+	-	-
5	5A1	Tiny, smooth, round	Single rods	+	-	-
	5A3	Irregular, flat,	Single rods	+	-	-
	5B1	Smooth, white spot	diplococci	+	-	-
	5B2	Smooth, white spot	diplococci	+	-	-

Table 2.4 (Contd) phenotypic characteristics of lactic acid bacteria from *Nono* on M17 agar

Samples	Isolate code	Colony morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
	5B3	Irregular, flat,	diplococci	+	-	-
	5B4	Smooth, white spot	diplococci	+	-	-
	5B5	Smooth, round	coccobacillus	+	-	-
	5CB	Smooth, round	Small rods in chain	+	-	-
	5CC	Smooth, round	Small rods in chain	+	-	-
6	6A1	Irregular, flat,	Small rods in chain	+	-	-
	6A2	Smooth, white spot	Small rods in chain	+	-	-
	6CA	Smooth, round	Small rods in chain	+	-	-
	6CB	Smooth, round	Small rods in chain	+	-	-
7	7A1	Smooth, cream, round	long rods	+	-	-
	7A2	Smooth, cream, round	long rods	+	-	-
	7CB	Smooth, round	diplococci	+	-	-
8	8A1	Smooth, cream, round	Small rods in chain	+	-	-
	8A2	Smooth, round	Small rods in chain	+	-	-
	8A3	Smooth, cream, round	Small rods in chain	+	-	-
	8B1	Smooth, shiny round	Small rods in chain	+	-	-
	8B2	Smooth, shiny round	Small rods in chain	+	-	-
	8CA	Smooth, round	Small rods in chain	+	-	-
	8CB	Smooth, round	Small rods in chain	+	-	-

Table 2. 5 phenotypic characteristics of lactic acid bacteria from *Nono* on MRS agar

Samples	Isolate code	Colony Morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
1	1A1	Smooth, creamy, round	Single rods	+	-	-
	1A2	Smooth creamy, round	Single rods	+	-	-
	1B1	Smooth, creamy, round	Single rods	+	-	-
	1CB	Smooth, creamy, rounnd	Diplococci	+	-	-
	1CC	Smooth, creamy, round	Single rods	+	-	-
2	2A1	Shiny, smooth, round	Single rods	+	-	-
	2A2	Smooth, round	Single rods	+	-	-
	2B1	Small white,round	V-shape	+	-	-
	2B2	Small white,round	Rod in pairs	+	-	-
	2CB	Smooth, round	V-shape	+	-	-
	2CC	Smooth, round ,white spot	V-shape	+	-	-
	2CD	Smooth, cream, round	Diplobacillus	+	-	-
3	3A1	Smooth round , white	Single rods	+	-	-
	3A2	Smooth round , white	Single rods	+	-	-
	3B	Smooth, round round	Single rods	+	-	-
	3CA	Smooth, round, white	Single rods	+	-	-
	3CB	Smooth, round, white	Rod in pairs	+	-	-
	3CC	Smooth, creamy, round	Single rods	+	-	-
4	4A	Smooth, shiny, white	Single rods	+	-	-
	4B1	Tiny, creamy, round	Rods in chain	+	-	-
	4B2	Smooth, creamy, round	Rods in chain	+	-	-
	4CA	Tiny shiny round,	yeast	+	+	-
	4CB	Smooth, creamy, round	Rod in pairs	+	-	-
	4CC	Smooth, shiny, round	Cocci bacillus	+	-	-

Table 2.5 (Contd) phenotypic characteristics of lactic acid bacteria from *Nono* on MRS agar

Samples	Isolate code	Colony Morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
5	5A1	Smooth, round, white	Rod in pairs	+	-	-
	5A2	Smooth round white	Rod in pairs	+	-	-
	5B1	Smooth, round, white spot	Rod in pairs	+	-	-
	5B2	Smooth, round, white spot	Rod in pairs	+	-	-
	5CA	Smooth, creamy, round	Rod in pairs	+	-	-
	5CB	Smooth, shiny round	Single rods	+	-	-
	5CD	Smooth, creamy, round	Single rods	+	-	-
6	6A1	Smooth, round,	Single rods	+	-	-
	6A2	Smooth, creamy, round	Single rods	+	-	-
	6B1	Smooth, creamy, round	Single rods	+	-	-
	6B2	Smooth, creamy, round	Single rods	+	-	-
	6CC	Shiny, smooth, round	Single rods	+	-	-
7	7A1	Smooth, white, round	Single rods	+	-	-
	7A2	Smooth, white, round	Rod in pairs	+	-	-
	7B1	Smooth, creamy, round	Rod in pairs	+	-	-
	7B2	Smooth, creamy, round	Rod in pairs	+	-	-
	7CA	Smooth, creamy, round	Single rods	+	-	-
	7CB	Smooth, round	Diplobacillus	+	-	-
	7CC	Smooth, round	V-shape	+	-	-
8	8A1	Smooth, cream, round	Rod in pairs	+	-	-
	8A2	Smooth, creamy,	Rod in pairs	+	-	-
	8BA	Smooth, round,	Single rods	+	-	-
	8BB	Smooth, round,	Single rods	+	-	-

Table 2. 6 phenotypic characteristics of lactic acid bacteria from *Nono* on MRSL agar

Samples	Isolate code	Colony Morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
1	1A1	Smooth, round	Single long rods	+	-	-
	1B1	Smooth, round	Single long rods	+	-	-
	1D1	Smooth, round	Rod in pairs	+	-	-
	1D2	Smooth, round	Single long rods	+	-	-
	1E1	Smooth, round	Single long rods	+	-	-
	1FA	Smooth, round	Single long rods	+	-	-
	1FB	Smooth, round	Single long rods	+	-	-
	1FC	Smooth, round	Single long rods	+	-	-
2	2A1	Smooth, round	Single rods	+	-	-
	2B1	Smooth, round	Single rods	+	-	-
	2B2	Smooth, round	V-shape	+	-	-
	2CA	Smooth, shiny, round	Single rod in pairs	+	-	-
	2CB	Smooth, shiny, round	Single rod in pairs	+	-	-
3	3A	Smooth, shiny, round	Single rods	+	-	-
	3B	Smooth, shiny round	Single rods	+	-	-
	3CA	Irregular, flat,	Single rods	+	-	-
	3CB	Irregular, flat,	Single rods	+	-	-
	3CC	Smooth, round	Single rods	+	-	-
4	4A1	Tiny, smooth	Cocci in chains	+	-	-
	4A2	Tiny, smooth	Rod in chains	+	-	-
	4A3	Smooth, round	Single long rods	+	-	-
	4B2	Smooth, round	Cocci in chains	+	-	-
	4CA	Smooth, round	Single rods	+	-	-
	4CB	Smooth, round	Single rods	+	-	-
	4CC	Smooth, round	Single long rods	+	-	-

Table 2.6 (Contd) phenotypic characteristics of lactic acid bacteria from *Nono* on MRSL agar

Samples	Isolate code	Colony morphology	Cell Morphology	Gram Reaction	Catalase	Oxidase
5	5A1	Smooth, round white	diplococci	+	-	-
	5A2	Smooth, round white	diplococci	+	-	-
	5B1	Smooth, shiny, round	coccibacillus	+	-	-
	5B2	Smooth, shiny, round	coccibacillus	+	-	-
	5C	Shiny, round	Single, long, rod	+	-	-
6	6A1	Smooth, big, round	Single small rods	+	-	-
	6A2	Smooth, big round	Single small rods	+	-	-
	6B1	Smooth, cream, round	Single rods	+	-	-
	6B2	Smooth, cream round	V-shape	+	-	-
	6CA	Big round spot white	Single small rods	+	-	-
	6CC	Big round spot white	Single small rods	+	-	-
7	7A1	Smooth, round creamy	Single rods	+	-	-
	7A2	Smooth, round	Single rods	+	-	-
	7B1	Smooth, round	Rod in pairs	+	-	-
	7B2	Smooth, round	Rod in pairs	+	-	-
	7C	Smooth, round shiny	Long rods	+	-	-
8	8A1	Smooth, round	Single rods	+	-	-
	8A2	Tiny, round	Cocci in chain	+	-	-
	8B1	Tiny, round	Single rods	+	-	-
	8B2	Tiny, round	Single rods	+	-	-
	8C	Smooth, round	Single rods	+	-	

2.3.2 Bacteria identification

Out of 128 isolates, 100 were selected for molecular identification on the basis of their phenotypic characteristics. Rep-PCR allowed differentiation of the isolates at interspecies and intraspecies levels. Using the latter method, the isolates after were clustered according to their DNA profiles into 11 different groups (Table 2.7, Figure 2.1-2.6).

The combination of the 16S rRNA, *phe*S and *rpo*A gene sequencing allowed the identification of four genera of LAB including *Lactobacillus*, *Streptococcus, Leuconostoc*, and *Enterococcus* and seven species including *Lactobacillus fermentum* (40%), *Lactobacillus senioris* (2%), *Lactobacillus delbrueckii* (23%), *Streptococcus thermophilus* (22%) *Streptococcus infantarius* (10%), *Leuconostoc pseudomesenteriodes* (2 %) and *Enterococcus thailandicus* (1%) (Table 2.7). *Lactobacillus fermentum* and *Lactobacillus delbruckii* were observed in all samples irrespective of the location and production site. In terms of production site within the same location, it was seen that from Lokpa, in addition to the two common species, *Streptococcus thermophilus* was recovered from LO1, LO2, LO5 and LO6, *Streptococcus infantarius* from LO4 and LO5 and *Leuconostoc pseudomesenteroides* and *Lactobacillus senioris* from LO4 only. In Eket, additional species recovered were *Streptococcus infantarius* from E01 whereas *Enterococcus thailandicus*, *Lactobacillus senioris* and *Streptococcus thermophilus* were noticed in the sample from E02. The main difference between the two locations was the presence of *Leuconostoc pseudomesenteroides* in LO4 (Lokpa) and *Enterococcus thailandicus* in EO1 (Eket).

Using the 16S rRNA gene sequencing, some of the bacteria were not clearly separated from closely related species. This was the case of isolates belonging to species such as *Enterococcus thailandicus* which could not be separated from *Enterococcus seguinicola, Leuconostoc pseudomesonteriodes* which was not differentiable from *Leuconostoc mesonteriodes*, and *Streptococcus infantarius*, which was not differentiable from *Streptococcus lutetiensis*. Such bacteria were all clearly identified with the further *rpo*A and *phe*S genes sequencing as seen in (Table 2.7). The bacteria were identified with a percentage similarity of 98-100%. Some of the examples of sequenced data were shown in appendix (1-5).

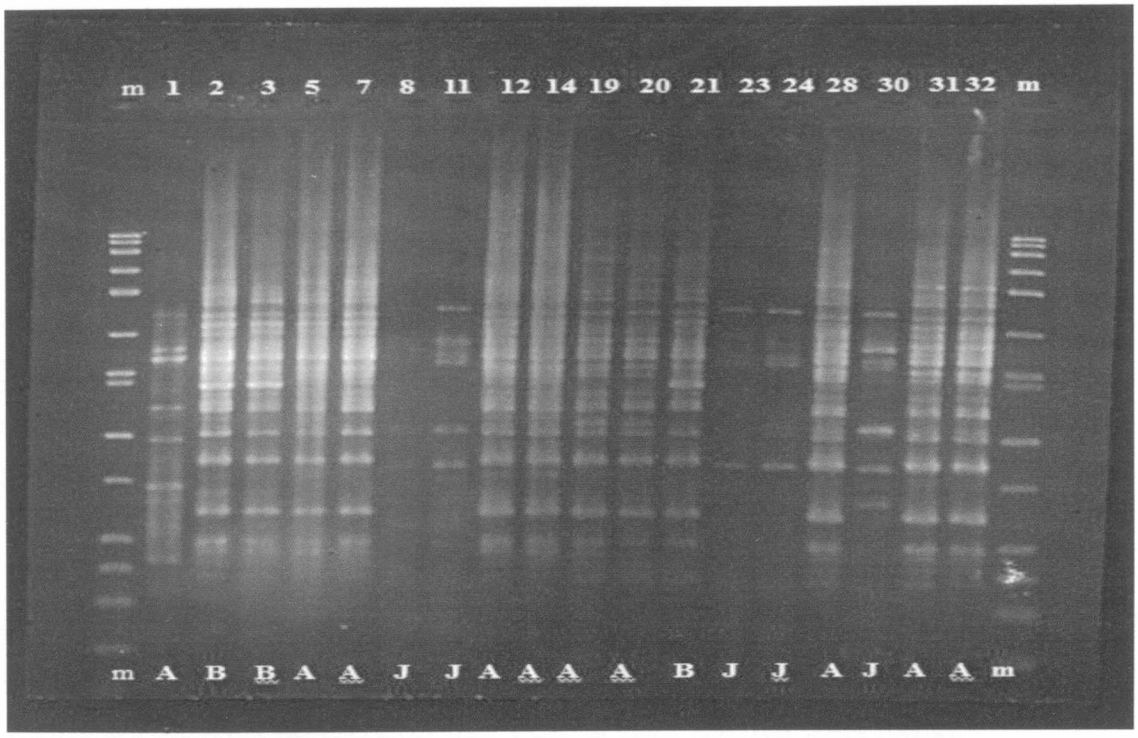

Figure 2. 1 Agarose gel image of Rep-PCR of LAB isolated from *Nono*

m: DNA marker
Isolates A, B and J were identified as A: *Lactobacillus fermentum*, B: *Lactobacillus fermentum* and J: *Lactobacillus delbrueckii* subsp. *indicus*.

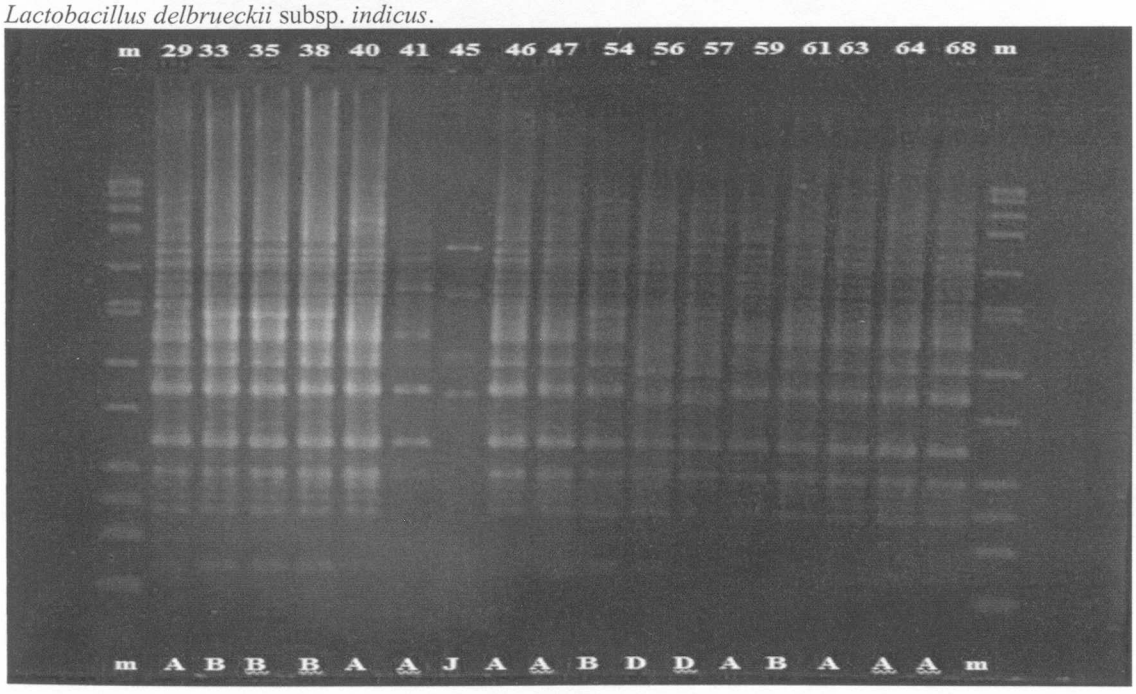

Figure 2. 2 Agarose gel image of Rep-PCR of LAB isolated from *Nono*

m: DNA marker
Isolates A, B, D and J were identified as A: *Lactobacillus fermentum*, B: *Lactobacillus fermentum*, D: *Lactobacillus fermentum* and J: *Lactobacillus delbrueckii* subsp. *indicus*.

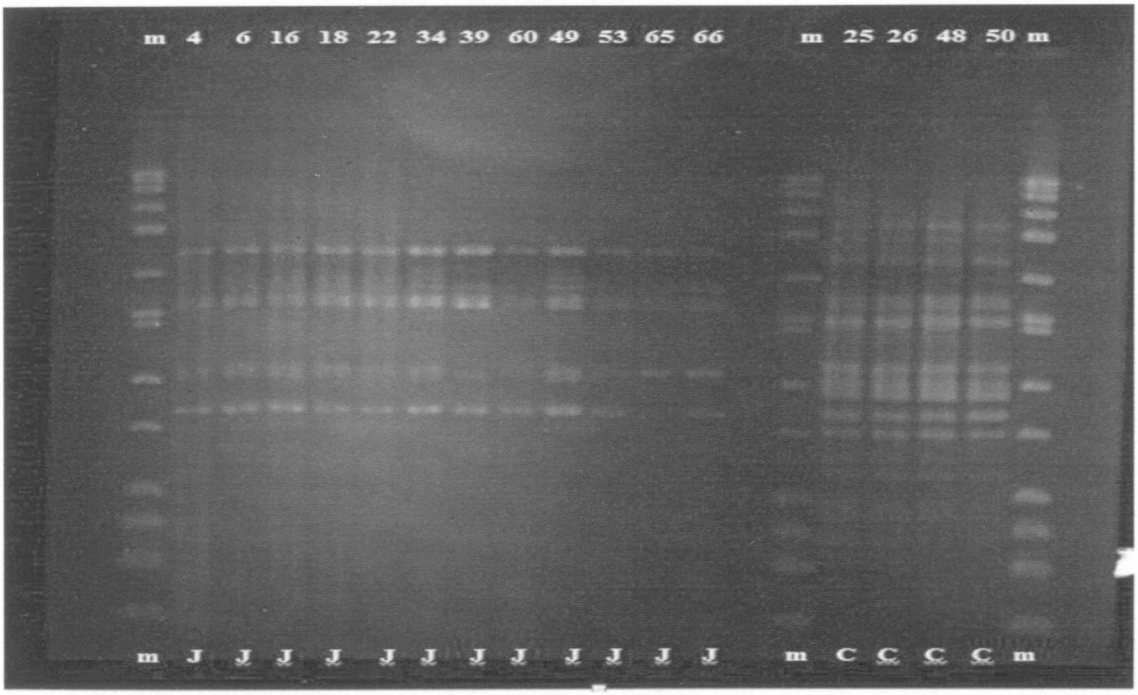

Figure 2. 3 Agarose gel image of Rep-PCR of LAB isolated from *Nono*.

m: DNA marker
Isolates C and J were identified as C: *Lactobacillus fermentum*, J: *Lactobacillus delbrueckii* subsp.*indicus*

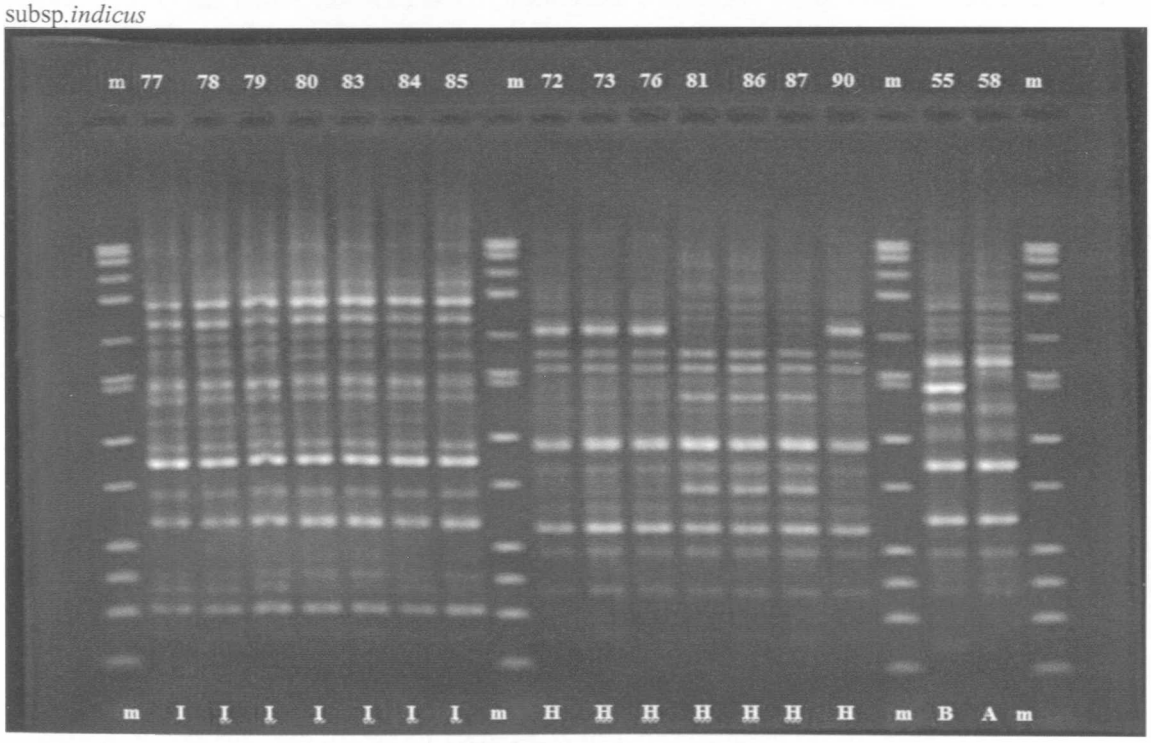

Figure 2. 4 Agarose gel image of Rep-PCR of LAB isolated from *Nono*.

m: DNA marker
Isolates A, B, I and H were identified as A: *Lactobacillus fermentum*, B: *Lactobacillus fermentum*, I: *Streptococcus infantarius subsp.infantarius* and H: *Streptococcus thermophilus*

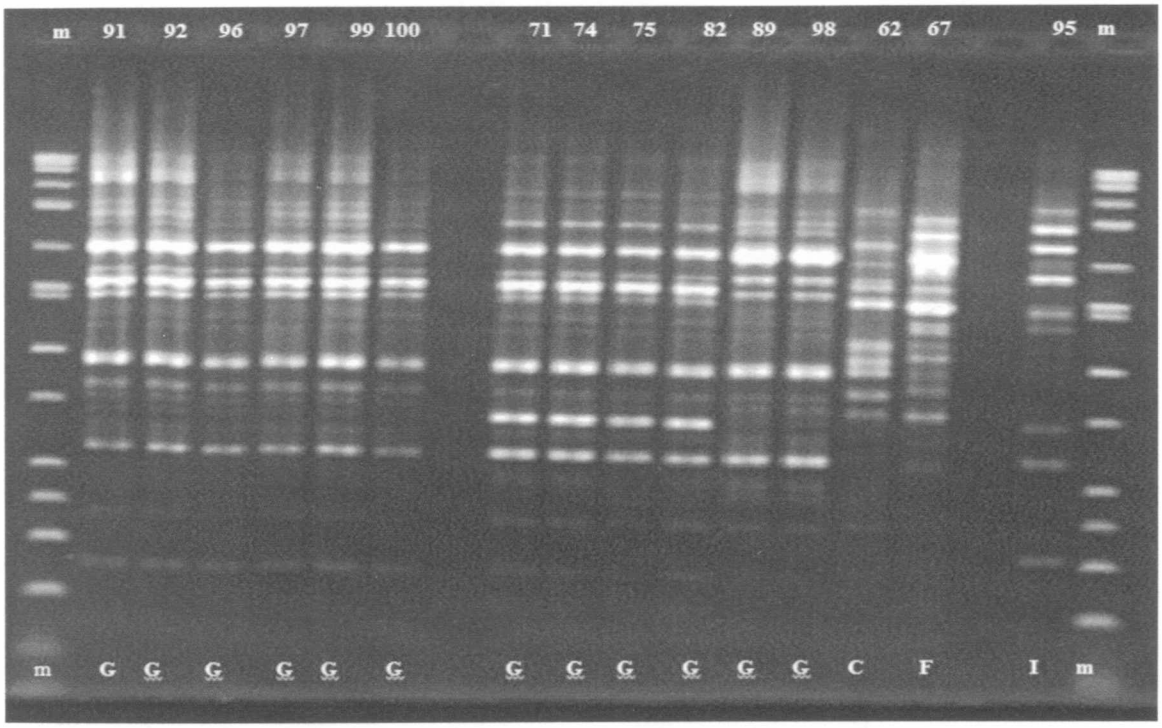

Figure 2. 5 Agarose gel image of Rep-PCR of LAB isolated from *Nono*.

m: DNA marker
Isolates C, F, G and I were identified as C: *Lactobacillus fermentum*, F: *Lactobacillus senioris*, G: *Streptococcus thermophilus*, I: *Streptococcus infantarius subsp.infantarius*.

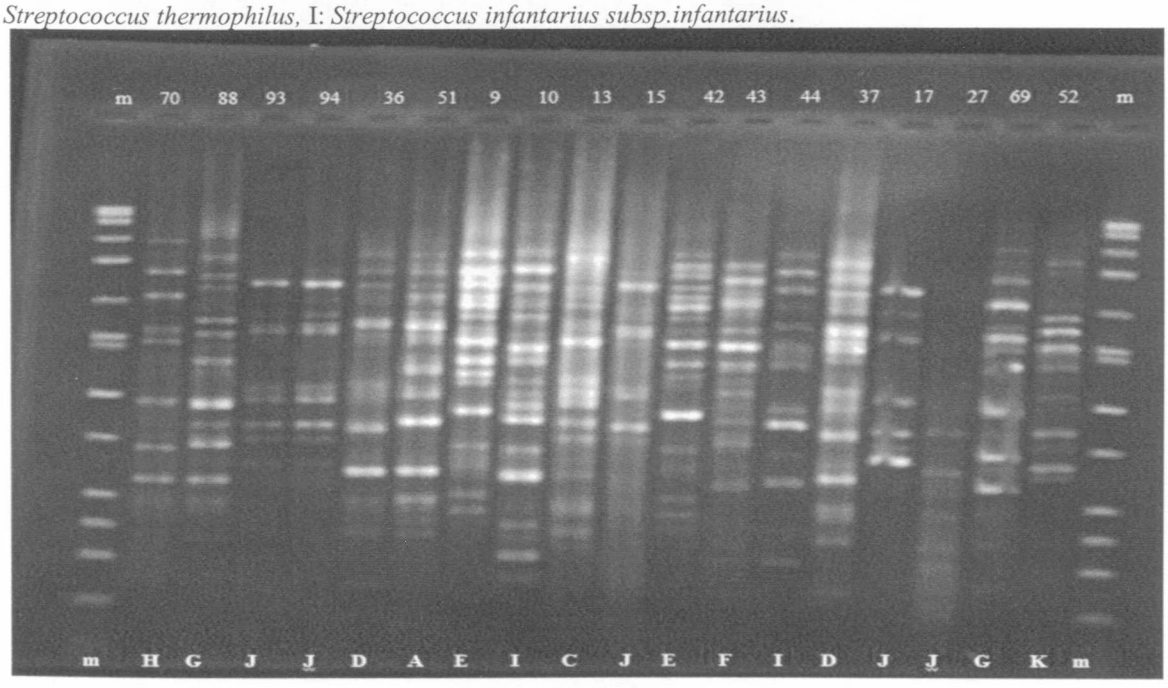

Figure 2. 6 Agarose gel image of Rep-PCR of LAB isolated from *Nono*.

m: DNA marker
Isolates A, C, D, E, F, G, H, I, J and K were identified as A: *Lactobacillus fermentum*, C: *Lactobacillus fermentum*, D: Lactobacillus fermentum, E: *Leuconostoc pseudomesenteroides*, F: *Lactobacillus senioris*, G: *Streptococcus thermophilus*, H: *Streptococcus thermophilus*, I: *Streptococcus infantarius subsp.infantarius*, J: *Lactobacillus delbrueckii* subsp. *indicus* and k: *Enterococcus thailandicus*

Table 2. 7 Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
1	LO1	A	*Lactobacillus fermentum*	-
2	LO1	B	*Lactobacillus fermentum*	-
33	LO1	B	*Lactobacillus fermentum*	-
35	LO1	B	*Lactobacillus fermentum*	-
54	LO1	B	*Lactobacillus fermentum*	-
34	LO1	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
53	LO1	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
73	LO1	H	*Stretococcus salivarius* subsp.*thermophilus* *Streptococcus vestibularis*	-
72	LO1	H	*Stretococcus salivarius subsp.thermophilus* *Streptococcus vestibularis*	-
71	LO1	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
74	LO1	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
70	LO1	H	*Streptococcus salivarius subsp.thermophilus* *Streptococcus salivarius subsp.salivarius*	*Streptococcus salivarius subsp.thermophilus*
36	LO1	D	*Lactobacillus fermentum*	*Lactobacillus fermentum*
37	LO1	D	*Lactobacillus fermentum*	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
17	LO1	J	*Lactobacillus delbrueckii* subsp.*indicus* *Lactobacillus delbrueckii* subsp.*bulgaricus*	-
69	LO1	G	*Streptococcus salivarius subsp.thermophilus* *Streptococcus salivarius subsp.salivarius*	*Streptococcus salivarius subsp.thermophilus*
3	LO2	B	*Lactobacillus fermentum*	-
38	LO2	B	*Lactobacillus fermentum*	-
5	LO2	A	*Lactobacillus fermentum*	-
19	LO2	A	*Lactobacillus fermentum*	-
56	LO2	D	*Lactobacillus fermentum*	-
57	LO2	D	*Lactobacillus fermentum*	-
4	LO2	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
18	LO2	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
39	LO2	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
55	LO2	B	*Lactobacillus fermentum*	-
76	LO2	H	*Stretococcus salivarius* subsp.*thermophillus* *Streptococcus vestibularis*	-
75	LO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	*Stretococcus salivarius subsp.thermophilus*
58	LO3	A	*Lactobacillus fermentum*	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
7	LO3	A	*Lactobacillus fermentum*	-
20	LO3	A	*Lactobacillus fermentum*	-
40	LO3	A	*Lactobacillus fermentum*	-
41	LO3	A	*Lactobacillus fermentum*	-
59	LO3	A	*Lactobacillus fermentum*	-
6	LO3	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
8	LO3	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
21	LO4	B	*Lactobacillus fermentum*	-
61	LO4	B	*Lactobacillus fermentum*	-
22	LO4	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
60	LO4	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
79	LO4	I	*Streptococcus infantarius subsp.infantarius* *Streptococcus.lutetiensis*	-
80	LO4	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	*Streptococcus infantarius subsp.infantarius*
77	LO4	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
78	LO4	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	-
9	LO4	E	*Leuconostoc mesenteroides subsp.mesenteroides* *Leuconostoc pseudomesenteroides*	*Leuconostoc pseudomesenteroides*
10	LO4	I	*Streptococcus.lutetiensis* *Streptococcus infantarius* subsp.*infantarius*	*Streptococcus infantarius subsp.infantarius*
43	LO4	F	*Lactobacillus senioris*	-
44	LO4	I	*Streptococcus infantarius* subsp. *coli* *Streptococcus infantarius subsp.infantarius*	*Streptococcus infantarius subsp.infantarius*
42	LO4	E	*Leuconostoc mesenteroides subsp.mesenteroides* *Leuconostoc pseudomesenteroides*	*Leuconostoc pseudomesenteroides*
46	LO5	A	*Lactobacillus fermentum*	-
47	LO5	A	*Lactobacillus fermentum*	-
11	LO5	J	*Lactobacillus delbrueckii* subsp.*sunkii* *Lactobacillus delbrueckii* subsp. *Indicus*	*Lactobacillus delbrueckii* subsp *indicus*
12	LO5	A	*Lactobacillus fermentum*	-
23	LO5	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
24	LO5	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
45	LO5	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
83	LO5	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	-
84	LO5	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	-
85	LO5	I	*Streptococcus.lutetiensis* *Streptococcus infantarius subsp.infantarius*	-
86	LO5	H	*Streptococcus salivarius subsp.thermophilus* *Streptococcus salivarius subsp.salivarius*	*Streptococcus salivarius subsp.thermophilus*
81	LO5	H	*Streptococcus salivarius subsp.thermophilus* *Streptococcus salivarius subsp.salivarius*	-
87	LO5	H	*Streptococcus salivarius subsp.thermophilus* *Streptococcus salivarius subsp.salivarius*	-
82	LO5	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
88	LO5	G	*Streptococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	*Streptococcus salivarius subsp.thermophilus*

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
14	LO6	A	*Lactobacillus fermentum*	-
63	LO6	A	*Lactobacillus fermentum*	-
49	LO6	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
50	LO6	C	*Lactobacillus fermentum*	-
48	LO6	C	*Lactobacillus fermentum*	-
25	LO6	C	*Lactobacillus fermentum*	
26	LO6	C	*Lactobacillus fermentum*	
90	LO6	H	*Stretococcus salivarius subsp.thermophilus* *Streptococcus vestibularis*	-
91	LO6	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
62	LO6	C	*Lactobacillus fermentum*	-
13	LO6	C	*Lactobacillus fermentum*	-
92	LO6	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
89	LO6	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
28	EO1	A	*Lactobacillus fermentum*	-
29	EO1	A	*Lactobacillus fermentum*	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
64	EO1	A	*Lactobacillus fermentum*	-
30	EO1	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. Indicus*	-
65	EO1	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
95	EO1	I	*Streptococcus infantarius* subsp. *coli* *Streptococcus infantarius* subsp. *infantarius*	*Streptococcus infantarius subsp.infantarius*
27	EO1	J	*Lactobacillus delbrueckii* subsp *indicus*, *Lactobacillus delbrueckii* subsp *bulgaricus*,	*Lactobacillus delbruckii* subsp *indicus*
51	EO1	A	*Lactobacillus fermentum*	*Lactobacillus fermentum*
94	EO1	J	*Lactobacillus delbrueckii* subsp *indicus*, *Lactobacillus delbrueckii* subsp *bulgaricus*,	*Lactobacillus delbrueckii* subsp *indicus*
93	EO1	J	*Lactobacillus delbrueckii subsp indicus*, *Lactobacillus delbrueckii subsp bulgaricus*,	-
15	EO1	J	*Lactobacillus delbrueckii subsp indicus*, *Lactobacillus delbrueckii subsp bulgaricus*,	-

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

Table 2.7 (contd) Identity of the LAB isolated from *Nono*

Isolates	Sample Location	Rep-PCR Group	Identification 16S rDNA sequencing	Identification pheS/rpoA sequencing
31	EO2	A	*Lactobacillus fermentum*	-
32	EO2	A	*Lactobacillus fermentum*	-
68	EO2	A	*Lactobacillus fermentum*	-
16	EO2	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
66	EO2	J	*Lactobacillus delbrueckii subsp.sunkii* *Lactobacillus delbrueckii subsp. indicus*	-
97	EO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	*Stretococcus salivarius subsp.thermophilus*
96	EO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
99	EO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
100	EO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
98	EO2	G	*Stretococcus salivarius subsp.thermophilus* *Stretococcus salivarius subsp.salivarius*	-
67	EO2	F	*Lactobacillus senioris*	*Lactobacillus senioris*
52	EO2	K	*Enterococcus sanguinicola,* *Enterococcus thailandicus*	*Enterococcus thailandicus*

Lokpa (Lokpa Fulani Village), Eket (Eket Islamic Musque) = Sample locations

LO1, LO2, LO3, LO4, LO5 and LO6 = Production sites from Lokpa

EO1 and EO2 = Production sites from Eket

2.3.3 Carbohydrate assimilation and phenotypic identification of selected LAB isolates

As seen in Table 2.8, the ability of the bacteria to degrade carbohydrates was variable according to the isolate and the carbohydrate. Using these profiles, the bacteria were tentatively identified (Table 2.9) generating in some cases, different identities from those provided by the genotyping. *Lactobacillus fermentum*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* showed similar identification using both methods. However, by phenotyping, *Streptococcus infantarius* was misidentified as *Leuconostoc lactis*, *Lactobacillus senioris* as *Lactobacillus brevis*, and *Enterococcus thailandicus as Carnobacterium maltaromaticum*

Table 2. 8 Fermentation profile of selected LAB from *Nono*

Carbohydrates	Lactic acid bacteria*						
	52	10	43	13	11	9	73
Glycerol	+	-	-	-	-	-	-
Erythitol	-	-	-	-	-	-	-
D-arabinose	-	-	-	-	-	-	-
L-arabinose	-	-	-	+	-	-	-
Ribose	+	-	+	+	-	+	-
D-xylose	-	-	+	+	-	+	-
L-xylose	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-
β-Methyl xylose	-	-	-	-	-	-	-
Galactose	+	+	-	+	-	+	-
D-glucose	+	+	-	+	+	+	+
D-fructose	+	+	-	+	+	+	-
D-mannose	+	+	-	+	+	+	-
L-sorbose	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-
Mannitol	+	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-
α-methyl-D- mannoside	+	-	-	-	-	-	-
α-methyl-D- glucoside	-	-	-	-	-	+	-
N-acetyl glucosamine	+	+	+	-	+	+	-
Amygdaline	+	-	-	-	-	-	-
Arbutin	+	-	-	-	-	-	-
Esculine	+	+	-	+	+	-	-
Saline	+	-	-	-	-	-	-

*Isolate: 52:- *Enterococcus thailandicus*, 10:- *Streptococcus infantarius*, 43:- *Lactobacillus senioris*, 13:- *Lactobacillus fermentum*, 11:- *Lactobacillus delbrueckii* subsp *indicus*, 9:- *Leuconostoc pseudomesenteroides* and 73:- *Streptococcus thermophiles*

Table 2.8 (contd) Fermentation profile of selected LAB from *Nono*

Carbohydrates	Lactic acid bacteria*						
	52	10	43	13	11	9	73
Cellodiose	+	-	-	+	-	+	-
Maltose	+	+	-	+	+	+	-
Lactose	+	+	-	+	+	+	+
Melibiose	-	+	-	+	-	+	-
Saccharose	+	+	-	+	-	+	+
Trehalose	+	-	-	+	+	+	-
Inuline	-	-	-	-	-	-	-
Melezitose	-	-	-	-	-	-	-
D-Raffinose	-	+	-	+	-	+	-
Amicon	-	+	-	-	-	+	-
Glycogen	-	+	-	-	-	+	-
Xylitol	-	-	-	-	-	-	-
β-gentobiose	+	-	-	-	-	+	-
D-turanose	-	-	-	-	-	+	-
D-lyxose	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	-	-
D-fucose	-	-	-	-	-	-	-
L-fucose	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-
L-arabitol	-	-	-	-	-	-	-
Gluconate	+	-	+	+	-	+	-
2 Keto-gluconate	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	+	+	+	-

*Isolate code: 52:- *Enterococcus thailandicus,* 10:- *Streptococcus infantarius*, 43:- *Lactobacillus senioris,* 13:- *Lactobacillus fermentum*, 11:- *Lactobacillus delbrueckii* subsp *indicus*, 9:- *Leuconostoc pseudomesenteroides* and 73:- *Streptococcus thermophilus*

Table 2. 9 Comparison of the LAB identification by phenotyping and genotyping

Isolates	Rep-Group	API 50CHL identification	Similarity	Sequence identification	Similarity
9	E	*Leuconostoc mesenteroides* subsp *mesenteroides/dextranicum*	100 %	*Leuconostoc pseudomesenteroides*	98%
10	I	*Leuconostoc lactis*	93 %	*Streptococcus infantarius*	99%
11	J	*Lactobacillus delbrueckii*	93 %	*Lactobacillus delbrueckii*	99%
13	C	*Lactobacillus fermentum*	100 %	*Lactobacillus fermentum*	99%
43	F	*Lactobacillus brevis*	97 %	*Lactobacillus senioris*	99%
52	K	*Carnobacterium maltaromaticum*	98 %	*Enterococcus thailandicus*	99%
73	H	*Streptococcus thermophilus*	99 %	*Streptococcus thermophilus*	98%

2.4 Discussion

In this study, the isolation and identification of LAB from *Nono*, a Nigerian traditional fermented milk product was evaluated. A decrease of the pH from 6.8 in the unfermented milk to 4.4-5.0 in the fermented milk samples was observed and confirmed that an acidic fermentation took place during the production of *Nono*. This is probably related to the production of lactic acid and other types of acids by the microorganisms responsible for the fermentation. The current results are similar to those reported; a study on *kule naoto*, the Maasai traditional fermented milk reported a pH range of 4.17-5.16 (Mathara *et al.*, 2004). For *Nunu*, the fermented milk from Ghana, a much lower pH value of 3.1 was reported (Akabanda *et al.*, 2013). This might be related to factors such as the types of the microorganisms involved in the fermentation and their particular technological properties especially related to the production of lactic acid (Wu 2001).

For the enumeration and isolation of LAB from *Nono*, the three media used were useful in the recovery of a variety of microorganisms. Some media were favourable for the isolation of particular bacteria as reported by previous studies on fermented milk products. For example, MRS agar was shown to be an adequate medium for the enumeration and recovery of *Lactobacillus* spp (Garcia-Cayuela *et al.*, 2009; Gonfer *et al.*, 2001; Gueimonde *et al.*, 2004; Tabasco *et al.*, 2007; Mathara *et al.*, 2008 and Sule *et al.*, 2014) while M17 agar is more selective for *Streptococcus* species such as *Streptococcus thermophilus* and lactococci (Westenberg, 2008; Khedid *et al.*, 2009).

The microbial count that ranged between 1.34×10^7 and 8.76×10^7 was quite similar to those reported for other African fermented milk products where a count of 10^6-10^8 was seen (Beukes *et al.*, 2001; Akabanda *et al.*, 2013; Mohammed and Ijah,

2013). With regards to the phenotypic characteristics, the isolates exhibited the basic features of LAB i.e. Gram positive, catalase and oxidase negative. Selected isolates were screened for their ability to assimilate various sugars and shown to be able to ferment some sugars such as glucose which can lead to the release of various acids including lactic acid. Thus, the acidification process observed in the fermented milk products can be attributed to the LAB present in the products.

The current study demonstrated that various genus, species, and subspecies of LAB including *Lactobacillus fermentum*, *Lactobacillus senioris*, *Lactobacillus delbrueckii* subsp *indicus*, *Streptococcus thermophilus*, *Streptococcus infantarius*, *Leuconostoc pseudomesenteriodes* and *Enterococcus thailandicus* are involved in the fermentation of milk for *Nono* production. Overall, although *Leuconostoc pseudomesenteroides* and *Enterococcus thailandicus* were observed only in Lokpa and Eket respectively, the microbial profile of the samples according to the location was not significantly different. However, within a location, some production sites exhibited quite diverse patterns even though common bacteria were seen. The similarities can be attributed to the use of the same raw material and differences to factors such as the environment, the production process and the equipment used.

Similarly to the current study, the genera *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus* also occurred in the Maasai fermented milk and the Ghanaian *Nunu* (Mathara *et al.*, 2004; Akabanda *et al.*, 2010). Also, as observed in the current research, *Lactobacillus fermentum* has been reported to be the predominant LAB in *Nunu*, followed by *Lactobacillus plantarum* (Akabanda *et al.*, 2013). The latter authors noticed that *Lactobacillus fermentum* was present from the onset of the fermentation till the end whereas other bacteria such as *Lactobacillus plantarum* and

Leuconoctoc mesenteroides were seen only either at the beginning or from the middle or at the end of the fermentation time. They suggested that this was due to the ability of the species to withstand the acid conditions appropriately. In the Maasai yogurt, *Lactobacillus plantarum* accounted for about 60% for the isolates with a reduced occurrence of *Lactobacillus fermentum*. In the traditional fermented yak milk from Qinghai, China, Sun *et al.* (2010) reported a dominance of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The variation observed are related to different factors such the production process, the sampling procedure, the storage material, the location and climate, the media and methodologies applied for the isolation of the bacteria.

A combination of phenotypic and genotypic methods was required for the adequate characterization and identification of the bacteria. Rep-PCR was shown to be appropriate for the differentiation of the isolates at interspecies and intraspecies levels and allowed to lay out the diversity of the microorganisms responsible for the fermentation. The sequencing of the *phe*S and *rpo*A genes was confirmed to be powerful and more discriminatory than the 16S rRNA gene sequencing in the identification of the bacteria. The later method is very useful for identifying many bacteria such the *Lactobacillus fermentum* isolates in the current study, especially when the sequences were analyzed using the EZtaxon server. However, for closely related species, a combination with other methods such as the sequencing of *gyrB*, *phe*S, *rpo*A and *rpo*B genes is necessary to provide the adequate bacterial identity. This was demonstrated in other studies for the identification of LAB from food materials such as cassava where *pheS* and *rpoA* gene sequencing were used to discriminate *Lactobacillus plantarum* from *Lactobacillus pentosus*, *Enterococcus faecium* from *Enterococcus durans* and *Weissella confusa* from *Weissella salipiscis*

(Anyogu *et al.*, 2014). The tentative identification of selected bacteria by phenotyping using their carbohydrates assimilation profile revealed serious discrepancy with the genotypic identification in many cases. The insufficiency of the sole phenotyping in identifying microorganisms has been widely reported (Gever *et al.*, 2001; Ouoba *et al.*, 2008; Anyogu *et al.*, 2014).

2.5 Conclusion

Various genera, species, and subspecies were identified indicating that a diverse microbial population is involved in the production of *Nono*. It was revealed that the predominant LAB species associated with *Nono* production was *Lactobacillus fermentum* and *Lactobacillus delbrueckii* subsp *indicus* that occurred in all samples. A combination of various phenotypic and genotypic methods was required to provide the appropriate identity to the bacteria. However, it appeared clearly that further sequencing of the housekeeping *rpo*A and *phe*S genes was more discriminatory in the identification of the bacteria in comparison with the sequencing of the 16S rRNA gene. The results obtained constitute an important step for the selection of multifunction starter cultures for a controlled fermentation of milk to produce *Nono* with improved nutritional quality and safety.

Chapter Three: Screening of lactic acid bacteria isolated from *Nono* for tolerance to acid and bile salt concentration.

3 Introduction

For probiotics, the ability to adapt to numerous host factors under the harsh conditions of the gastrointestinal tract is critical for colonisation. Microorganisms to be considered as probiotics face many challenges including being exposed to high acid concentration in the stomach. It is a functional characteristic that any probiotic candidate including LAB should survive the passage through the tough condition of the stomach.

Another such host element readily faced by probiotic bacteria is bile, an inherently antimicrobial detergent-like compound crucial for digestion and nutrient absorption. Not all LAB evolved to resist the bactericidal conditions of bile.

In addition to acid, the survival of probiotics, when exposed to the secreted bile salts in the small intestine is another pivotal functional property of probiotics. Such resistance depends on the bile concentration and the bacteria characteristics (Deshpande *et al.*, 2014). Therefore, it is necessary to characterise the selected LAB and examine whether they can survive in presence of the acid and bile salts in the human gastrointestinal tract.

Survival and viability of LAB are the one of the probiotic criterial in selection of probiotic cultures. Any probiotic culture must meet these probiotic potentials to be able to provide therapeutic functions during manufacturing and also survive the upper gastrointestinal part of human body. A number of factors have been stated to affect the functional properties of these bacteria including low pH, processing condition and fermentation temperature (Shah, 2000 and Makinen *et al.* 2012). Considering the selection criteria, probiotic candidate organisms need to have the following characteristics including resistance to acid/gastric acid, resistance to bile,

adhesion to the intestinal epithelium and ability to transiently colonise the gut. Since the fermented milk will be consumed orally, it is important the bacteria used as a starter culture survive the acidic conditions of the stomach and the bile secreted into the small intestine (Chou and Weimer 1999 and Nikos.kelainen *et al.*, 2001).

3.1 Effect of acid pH and % bile salt concentration on viability of LAB

The most significant role of LAB is the production of lactic acid as end-product of lactose fermentation and other metabolic activities (Widyastuti *et al.*, 2014). This acid production is the most important factor that affects their viability because their growth is reduced considerably below pH 4.3 (Lankaputra and Shah 1995). Though some bacteria survive below pH 2 for instance Balcazar *et al.* (2008) stated that *Lactobacillus plantarum, Lactococcus lactis, Lactobacillus acidophilus, Bacillus subtilis* and *Lactobacillus casei* from intestinal microbiota of fish showed viability at low pH values. Acid tolerance of bacteria is important not only for withstanding gastric stresses, but also for their use as dietary supplement.

Bile tolerance is considered as one of the functional criteria for the selection of probiotic cultures. It plays a fundamental role in specific and nonspecific defence mechanisms in the gut; the magnitude of its inhibitory effects is determined primarily by the concentrations of bile salts (Succi *et al.*, 2005). Bile secreted in the small intestine reduces the survival of bacteria by destroying their cell membranes, whose major components are lipids and fatty acids and these modifications may affect not only the cell permeability and viability, but also the interactions between the membrane and the environment (Gilliland *et al.*, 1984; Gilliland 1987). Resistance to bile salts is considered an important parameter for selecting probiotic strains. Wu *et al.* (2010) reported that the average physiological concentration of bile

in humans ranges between 0.3 to 0.5% (w/v) while, a concentration of 0.15-0.3 % of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use (Goldin and Gorbach 1992). Generally, growth may occur at lower or higher concentration depending on the strains or species that are more sensitive to the bile salts.

Considering the selection of starter culture based on their probiotic properties, the bacteria must have the ability to tolerate acid and bile salts as well as growing in the lower intestinal tract. The strain must have the ability to survive passage through the upper gastro-intestinal tract and arrive alive at its site of action (Corbo *et al.*, 2001). In this research, LAB from *Nono* will be investigated for the ability to survive the upper and lower gastrointestinal tract and function as potential probiotics.

3.1.1 Aims and objectives

However, the aim of this study was to determine the survival of LAB isolated from *Nono*. To achieve this aim, specific objectives that include various activities were pursued including.

- *In vitro* assessment of the resistance of the isolates to various pH levels of 2.0, 3.0 and 4.0.

- *In vitro* assessment of the resistance of the isolates to the different percentage of bile salts of 0 % to 2 %.

3.2 Materials and Methods

3.2.1 Microorganisms

Seven out of 11 isolates were selected for this study. The selection of these isolates was based on differences in their rep-PCR profiles and they are listed in Table 3.1.

Table 3. 1 Lactic acid bacteria used

Isolate Codes	Species name
52	*Enterococcus thailandicus*
10	*Streptococcus infantarius*
43	*Lactobacillus senioris*
13	*Lactobacillus fermentum*
11	*Lactobacillus delbrueckii subsp indicus*
9	*Leuconostoc pseudomesenteroides*
73	*Streptococcus thermophilus*

3.2.2 Inoculum preparation

After two consecutive sub cultures of isolates on MRS agar and M17 agar, a single colony was suspended in 1 ml of sterile Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK). The suspension was used to prepare an inoculum (in 5 ml MRD) with a final cell concentration of 10^7-10^8 cfu/ml (equivalent to 0.5 McFarland standard) using a sensitre nephelometer (TREK Diagnostic systems, West Sussex, UK). The microbial suspension (10^7cfu/mlml) was used as an inoculum for further experiments.

3.2.3 *In vitro* assessment of the resistance of the test isolates to various pH levels of 2.0, 3.0, 4.0 and 7.0

Acid resistance test was performed according to the method of Prasad *et al.* (1998). 1000 µl of each microbial suspension (10^7 cfu/ml) was inoculated in each 9 ml of phosphate buffer solution (PBS) adjusted to pH 2 to 7 by adding 1 M HCl. The inoculums of different pH were incubated at 37 °C for 3 h under anaerobic conditions. Viable cell counts were determined using a plate counting method involving MRS agar and M17 by taking each sample for enumeration from different pH for every half an hour. The enumerated samples were incubated for 48 h anaerobically at 37 °C. The viable colonies were counted and the data were expressed as $\log_{10}$ cfu/ml.

3.2.4 *In vitro* assessment of the resistance of the isolates to the different percentage of bile salts

Bile salt tolerance of the test isolates were ascertained in sterile PBS containing 1.0 to 2.0 % (w/v) bile salts according to Prasad *et al.* (1998). The process was carried out as described in section 5.2.3 but the control was used as microbial suspension (10^7cfu/ml) in PBS without bile salt (0 % bile salt). Viable cells were enumerated on MRS and M17 agar plate and incubated for 48 h anaerobically at 37°C. The viable colonies were counted and data were expressed as $\log_{10}$ cfu/ml.

3.2.5 Data analysis

Each experiment was conducted at least two times and data were analysed using Microsoft excel to determine the mean and standard deviation of the number of the viable colonies also, statistical significant differences set at $P \leq 0.05$ to compare the

means using 1 way ANOVA. The results were expressed as mean ± standard deviation in $\log_{10}$ cfu/ml.

3.3 Results

3.3.1 Acid and Bile salt

The effect of pH on the viability of the test isolates were presented in (Table 3.2 to 3.4) while that of % bile tolerance were summarised in (Table 3.5 to 3.7). Considering the survival of the test isolates in pH conditions, it showed that, the viabilities of all tested isolates were completely lost at pH 2 after 0 h incubation (Table 3.2). At pH 3, the survival rate of the test isolates were greater than at pH 2 at all tested sampling intervals. *Lactobacillus fermentum* and *Lactobacillus senioris* among all the test isolates exhibited longer surviving hours of up to 3 h incubation but *Lactobacillus fermentum* exhibited the highest viability count up 10^7 cfu/ml for 3 h incubation. There were significant differences $P<0.001$ among the tested strains compared to the 10^7 cfu/ml of control (pH 7). All the test isolates were viable at pH 4 after 3 h of incubation. Survival rate of all strains varies dependent on the isolate and the time of exposures. However, no significant differences were observed at pH 4 conditions in comparison to the cfu/ml of control (pH 7) $P>0.05$.

All tested bacteria exhibited tolerance to higher % bile salt concentrations (1 – 2 %, w/v). At 1 % bile concentration, there were no differences in the viability of the test isolates in comparison to the $\log_{10}$ cfu/ml of control (0 %) for 3 h incubation except *Streptococcus thermophilus* which showed reduced viability count after 30 min of incubation. Also, at 1.5 % bile salt concentration after 1h 30 min (Table 3.6), *Streptococcus thermophilus* lost its survival. However, there were no differences in the viability of the test isolates at 2 % bile concentration in comparison to the $\log_{10}$

cfu/ml of control (0 %) for 3 h incubation except *Streptococcus thermophilus* which showed reduced viable count at 2 to 3 h incubation. Generally, *Lactobacillus fermentum* among all test isolates exhibited the highest viability count $\log_{10}$ cfu/ml for longer incubation while *Streptococcus thermophilus* exhibited the least compared to $\log_{10}$ cfu/ml of control (0 %) at 1 – 2 (w/v) % bile concentrations.

Table 3. 2 Survival of LAB in PBS adjusted to pH 2 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

	Viable count (log_{10} cfu/ml)											
	pH7						pH2					
Strains/time	0 h	30 min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	8.20 ± 0.08	8.37 ± 0.04	6.29 ± 0.00	7.90 ± 0.08	8.07 ± 0.10	8.14 ± 0.20	7.90 ± 0.08	-	-	-	-	-
Strep. infantarius	7.60 ± 0.04	7.53 ± 0.08	7.31 ± 0.05	7.34 ± 0.15	7.20 ± 0.04	7.31 ± 0.11	3.94 ± 5.58	-	-	-	-	-
L. senioris	7.78 ± 0.13	7.72 ± 0.04	7.67 ± 0.06	7.60 ± 0.00	7.04 ± 0.00	7.68 ± 0.06	7.52 ± 0.06	-	-	-	-	-
L. fermentum	7.85 ± 0.00	7.60 ± 0.06	7.40 ± 0.05	7.47 ± 0.07	7.49 ± 0.04	7.48 ± 0.02	7.47 ± 0.07	-	-	-	-	-
L.delbrueckii subsp indicus	7.76 ± 0.04	7.72 ± 0.01	7.73 ± 0.12	8.15 ± 0.68	7.71 ± 0.14	7.77 ± 0.06	7.57 ± 0.02	-	-	-	-	-
Leu. pseudomesenteroides	7.43 ± 0.15	7.51 ± 0.05	7.41 ± 0.29	7.67 ± 0.29	7.74 ± 0.13	7.52 ± 0.02	7.64 ± 0.03	-	-	-	-	-
Strep. thermophilus	7.38 ± 0.03	6.95 ± 0.07	6.83 ±0.18	7.73 ± 0.02	6.78 ± 0.04	6.84 ± 0.03	2.79 ± 3.95	-	-	-	-	-

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

Table 3. 3 Survival of LAB in PBS adjusted to pH 3 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

	Viable count (log_{10} cfu/ml)											
	pH7						pH3					
Strains/times	0 h	30 min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	6.33 ± 0.08	6.32 ± 0.04	6.29 ± 0.00	6.27 ± 0.08	6.18 ± 0.10	6.18 ± 0.20	6.31 ± 0.04	6.09 ± 0.05	3.79 ± 0.10	3.48 ± 0.09	3.16 ± 0.09	2.90 ± 0.26
Strep. infantarius	6.86 ± 0.04	6.81 ± 0.08	6.52 ± 0.05	6.51 ± 0.15	6.53 ± 0.04	6.46 ± 0.11	6.38 ± 0.21	3.30 ± 0.09	1.78 ± 0.21	-	-	-
L. senioris	7.78 ± 0.13	7.72 ± 0.04	7.67 ± 0.06	7.60 ± 0.00	7.04 ± 0.00	7.68 ± 0.06	7.62 ± 0.02	7.59 ± 0.51	6.87 ± 0.04	6.59 ± 0.01	6.58 ± 0.02	5.98 ± 0.19
L. fermentum	7.85 ± 0.00	7.60 ± 0.06	7.40 ± 0.05	7.47 ± 0.07	7.49 ± 0.04	7.48 ± 0.02	7.47 ± 0.01	7.47 ± 0.18	7.48 ± 0.11	7.40 ± 0.14	7.20 ± 0.00	7.32 ± 0.00
L.delbrueckii subsp indicus	6.68 ± 0.04	5.88 ± 0.01	5.88 ± 0.12	5.94 ± 0.68	5.75 ± 0.14	5.35 ± 0.06	5.87 ± 0.07	5.88 ± 0.09	5.12 ± 0.16	5.05 ± 3.43	4.53± 0.08	4.26 ± 0.03
Leu. pseudomesenteroid	6.56 ± 0.15	6.59 ± 0.05	6.36 ± 0.29	6.51 ± 0.29	6.19 ± 0.13	6.12 ± 0.02	6.33 ± 0.16	3.60 ± 0.19	-	-	-	-
Strep. thermophilus	5.94 ± 0.03	5.97 ± 0.07	5.79 ±0.18	5.72 ± 0.02	4.91 ± 0.04	4.51 ± 0.03	4.37 ± 0.18	2.00 ± 0.43	1.70 ± 0.34	1.78 ± 0.21	-	-

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

Table 3. 4 Survival of LAB in PBS adjusted to pH 4 after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

					Viable count (log_{10} cfu/ml)							
	pH7						pH4					
Strains/time	0 h	30 min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	8.20 ± 0.08	8.37 ± 0.04	6.29 ± 0.00	7.90 ± 0.08	8.07 ± 0.10	8.14 ± 0.20	8.17 ± 0.12	8.57 ± 0.01	8.10 ± 0.14	8.02 ± 0.03	8.30 ± 0.00	8.06 ± 0.08
Strep. infantarius	7.60 ± 0.04	7.53 ± 0.08	7.31 ± 0.05	7.34 ± 0.15	7.20 ± 0.04	7.31 ± 0.11	8.04 ± 0.00	7.64 ± 0.01	7.35 ± 0.17	7.32 ± 0.09	7.21 ± 0.13	6.93 ± 0.04
L. senioris	7.78 ± 0.13	7.72 ± 0.04	7.67 ± 0.06	7.60 ± 0.00	7.04 ± 0.00	7.68 ± 0.06	7.69 ± 0.02	7.62 ± 0.12	7.67 ± 0.14	7.77 ± 0.06	6.95 ± 0.07	7.24 ± 0.67
L. fermentum	7.85 ± 0.00	7.60 ± 0.06	7.40 ± 0.05	7.47 ± 0.07	7.49 ± 0.04	7.48 ± 0.02	7.31 ± 0.08	7.57 ± 0.00	7.42 ± 0.03	7.50 ± 0.02	7.60 ± 0.06	7.41 ± 0.04
L.delbrueckii subsp indicus	7.76 ± 0.04	7.72 ± 0.01	7.73 ± 0.12	8.15 ± 0.68	7.71 ± 0.14	7.77 ± 0.06	7.82 ± 0.02	7.72 ± 0.05	7.74 ± 0.15	7.79 ± 0.04	7.72 ± 0.06	7.75 ± 0.05
Leu. pseudomesenteroid	7.43 ± 0.15	7.51 ± 0.05	7.41 ± 0.29	7.67 ± 0.29	7.74 ± 0.13	7.52 ± 0.02	7.68 ± 0.01	7.58 ± 0.12	7.50 ± 0.12	7.63 ± 0.14	7.38 ± 0.08	6.29 ± 0.08
Strep. thermophilus	7.38 ± 0.03	6.95 ± 0.07	6.83 ±0.18	7.73 ± 0.02	6.78 ± 0.04	6.84 ± 0.03	7.28 ± 0.19	6.98 ± 0.19	6.52 ± 0.05	5.19 ± 0.06	5.48 ± 0.16	4.72 ± 0.17

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

Table 3. 5 Survival of LAB in PBS containing 1% bile salt (w/v) after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

					Viable count (log_{10} cfu/ml)							
	0 % bile						1 % bile					
Strains/time	0 h	30min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	7.68 ± 0.07	7.68 ± 0.06	7.72 ± 0.03	7.28 ± 0.62	7.83 ± 0.06	7.75 ± 0.09	7.55 ± 0.10	7.67 ± 0.03	7.63 ± 0.09	7.77 ± 0.15	7.64 ± 0.07	7.61 ±0.11
Strep. Infantarius	8.18 ± 0.47	7.87 ± 0.00	7.87 ± 0.00	7.65 ± 0.00	7.87 ± 0.01	7.84 ± 0.01	7.70 ± 0.07	7.75 ± 0.11	7.41 ± 0.02	7.77 ±0.03	7.76 ± 0.01	7.47 ± 0.03
L. senioris	7.59 ± 0.12	7.68 ± 0.06	7.76 ± 0.08	7.73 ± 0.01	7.17 ± 0.66	7.65 ± 0.05	7.66 ± 0.03	7.69 ± 0.04	7.61 ± 0.01	7.57 ± 0.06	7.58 ± 0.03	7.69 ± 0.02
L. fermentum	8.29 ± 0.05	8.19 ± 0.06	8.18 ± 0.04	8.30 ± 0.09	8.22 ± 0.25	8.29 ± 0.35	8.25 ± 0.10	8.16 ± 0.06	7.44 ± 0.20	7.68 ± 0.05	7.59 ± 0.19	7.18 ± 0.81
L.delbrueckii subsp indicus	5.60 ± 0.07	5.83 ± 0.00	5.83 ± 0.14	5.90 ± 0.18	5.83 ± 0.12	5.73 ± 0.14	5.56 ± 0.14	5.16 ± 0.07	5.34 ± 0.16	5.47 ± 0.01	5.20 ± 0.14	5.23 ± 0.09
Leu. Pseudomesenteroides	7.94 ± 0.14	7.74 ± 0.07	7.72 ± 0.04	7.61 ± 0.08	7.68 ± 0.04	7.29 ± 0.16	7.96 ± 0.17	7.57 ±0.02	7.47 ± 0.08	6.85 ± 0.00	6.74 ± 0.01	6.39 ± 0.16
Strep. Thermophilus	6.32 ±0.06	5.90 ± 0.06	5.72 ± 0.04	5.39 ± 0.09	5.29 ± 0.05	5.26 ±0.07	5.40 ± 0.08	4.66 ± 0.08	3.83 ± 0.84	3.03 ± 0.07	2.78 ± 0.13	2.86 ± 0.10

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

Table 3. 6 Survival of LAB in PBS containing 1.5 % bile salt (w/v) after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

					Viable count (log_{10} cfu/ml)							
	0 % bile						1.5 % bile					
Strains/times	0 h	30min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	7.68 ± 0.07	7.68 ± 0.06	7.72 ± 0.03	7.28 ± 0.62	7.83 ± 0.06	7.75 ± 0.09	7.65 ± 0.03	7.75 ± 0.07	7.60 ± 0.03	7.59 ± 0.02	7.57 ± 0.04	7.72 ± 0.03
Strep. infantarius	8.18 ± 0.47	7.87 ± 0.00	7.87 ± 0.00	7.65 ± 0.00	7.87 ± 0.01	7.84 ± 0.01	7.87 ± 0.00	7.81 ± 0.01	7.82 ± 0.02	7.82 ± 0.08	7.79 ± 0.06	7.60 ± 0.06
L. senioris	7.59 ± 0.12	7.68 ± 0.06	7.76 ± 0.08	7.73 ± 0.01	7.17 ± 0.66	7.65 ± 0.05	7.67 ± 0.03	7.59 ± 0.01	7.64 ± 0.02	7.60 ± 0.02	7.61 ± 0.03	7.64 ± 0.03
L. fermentum	8.29 ± 0.05	8.19 ± 0.06	8.18 ± 0.04	8.30 ± 0.09	8.22 ± 0.25	8.29 ± 0.35	8.22 ± 0.20	8.18 ± 0.14	7.63 ± 0.04	7.50 ± 0.04	7.55 ± 0.13	7.44 ± 0.13
L.delbrueckii subsp indicus	6.33 ± 0.07	6.26 ± 0.00	6.35 ± 0.14	6.24 ± 0.18	6.20 ± 0.12	5.33 ± 0.14	5.50 ± 0.01	5.41 ± 0.10	5.05 ± 0.21	4.87 ± 0.04	4.94 ± 0.14	5.16 ± 0.02
Leu. pseudomesenteroid	7.94 ± 0.14	7.74 ± 0.07	7.72 ± 0.04	7.61 ± 0.08	7.68 ± 0.04	7.29 ± 0.16	4.02 ± 5.69	7.80 ± 0.01	7.82 ± 0.07	7.59 ± 0.05	6.90 ± 0.68	6.99 ± 0.12
Strep. thermophilus	8.00 ±0.06	7.83 ± 0.06	7.74 ± 0.04	7.80 ± 0.09	7.67 ± 0.05	7.71 ±0.07	8.11 ± 0.05	7.34 ± 0.00	7.12 ± 0.16	6.45 ± 0.10	-	-

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

Table 3. 7 Survival of LAB in PBS containing 2 % bile salt (w/v) after 0, 0.5, 1, 1.5, 2 and 3 h incubation at 37 °C under anaerobic condition.

					Viable count (log_{10} cfu/ml)							
	0 % bile						2 % bile					
Strains/times	0 h	30min	1 h	1.30min	2 h	3 h	0 h	30min	1 h	1.30min	2 h	3 h
E. thailandicus	7.68 ± 0.07	7.68 ± 0.06	7.72 ± 0.03	7.28 ± 0.62	7.83 ± 0.06	7.75 ± 0.09	7.70 ± 0.05	7.64 ± 0.06	7.62 ± 0.02	6.97 ± 0.70	7.62 ± 0.02	7.65 ± 0.05
Strep. infantarius	8.18 ± 0.47	7.87 ± 0.00	7.87 ± 0.00	7.65 ± 0.00	7.87 ± 0.01	7.84 ± 0.01	7.86 ± 0.01	7.87 ± .01	7.85 ± 0.03	7.88 ± 0.00	7.87 ± 0.00	7.86 ± 0.00
L. senioris	7.59 ± 0.12	7.68 ± 0.06	7.76 ± 0.08	7.73 ± 0.01	7.17 ± 0.66	7.65 ± 0.05	7.74 ± 0.07	7.60 ± 0.01	7.63 ± 0.03	7.66 ± 0.02	7.66 ± 0.05	7.60 ± 0.09
L. fermentum	8.29 ± 0.05	8.19 ± 0.06	8.18 ± 0.04	8.30 ± 0.09	8.22 ± 0.25	8.29 ± 0.35	8.24 ± 0.23	8.27 ± 0.13	7.79 ± 0.06	7.83 ± 0.05	7.87 ± 0.01	7.76 ± 0.10
L. delbrueckii subsp indicus	6.33 ± 0.07	6.26 ± 0.00	6.35 ± 0.14	6.24 ± 0.18	6.20 ± 0.12	5.33 ± 0.14	5.63 ± 0.14	5.43 ± 0.07	5.34 ± 0.08	5.37 ± 0.09	5.43 ± 0.07	5.47 ± 0.06
Leu. pseudomesenteroide	7.94 ± 0.14	7.74 ± 0.07	7.72 ± 0.04	7.61 ± 0.08	7.68 ± 0.04	7.29 ± 0.16	8.10 ± 0.21	7.86 ± 0.00	7.70 ± 0.02	7.69 ± 0.04	7.33 ± 0.41	4.91 ± 0.00
Strep. thermophilus	8.00 ±0.06	7.83 ± 0.06	7.74 ± 0.04	7.80 ± 0.09	7.67 ± 0.05	7.71 ±0.07	7.92 ± 0.11	7.32 ± 0.34	7.52 ± 0.03	6.35 ± 0.17	5.17 ± 0.02	5.40 ± 0.11

Bacterial counts were determined by plate counts on MSR and M17 agar plates.
Data represent the mean of number of viable colonies in two experiments expressed as mean ± standard deviation in log_{10} Cfu/ml.
Data were considered significantly different when $P < 0.05$.

3.4 Discussion

The pH of gastric juice is the main factor that determines the survival of bacteria that pass from the stomach to the intestine (Balcazar *et al.*, 2008). Generally, during production and consumption of fermented milk, the bacteria involved were exposed to different environmental conditions such as oxygen and oxygen-derived radicals, acids, bile, osmotic, heat and cold stress, which could negatively affect their viability and functionality (Zomer *et al.*, 2009). In this study, the strains tested could not show any survival at pH 2, this result is similar to the findings by Hassanzadazar *et al.*, (2012) where the author screened Lactobacilli isolate from Koozeh cheese for acid and bile tolerance properties. This could be due to severity of the pH to the bacteria. Studies by Hassanzadazar *et al.*, (2012) and Chan *et al.*, (2011) also confirmed that exposing gastric acid microflora to pH ≤ 2 after 3 h incubation caused a reduction in the viability count of the bacteria intensively. The viability of some of the isolates increases at pH 3. Though, it was reported that the best pH of the stomach when food (e.g. yoghurt) is ingested is probably 4 to 4.5 (Vernazza *et al.*, 2006a) but, according to Holzapfel *et al.*, (1998) the pH of the stomach generally ranges from pH 2.5 to pH 3.5 and fermented food products have a pH of about 4.5 in which probiotic microorganisms have to survive for long periods even during refrigerated storage (Jia *et al.*, 2010).

Lactobacillus senioris and *Lactobacillus fermentum* showed good survival in pH 3 though, there were significant differences with other isolates after 1.5 h of incubation $P<0.001$ but *Lactobacillus senioris* and *Lactobacillus fermentum* showed no significant differences compared to control. At pH 4 the tested strains indicated no significant differences compared to 10^7 cfu/ml of the control. These observations

were similar with other studies from (Devirgiliis *et al.*, 2009) which explained that *Lactobacillus* species was found to be more tolerant to acid environment than the other genera of lactic acid bacteria (cocci). Hence, this property makes *Lactobacillus* species abundant in the final phases of many food fermentations, *Lactobacillus* strains were viable even after being exposed to pH values of 2.5 – 4.0, but showed reduced viability at lower pH values below pH 2.5 (Wang *et al.*, 2010). The acid tolerance of the tested strains varies in this study and this might be due to the changes in the composition of the cell membrane, differences in induction of H+ - ATPase activity resulting in the removal of protons (H+), alkalization of the external environment, (Cotter and Hill, 2003).

As reported by Kumari *et al.* (2016) Human bile concentration varies from 0.5 to 2 % depending on the type of food being consumed also, bile plays a significant role in the clearly defined or undefined defend mechanisms of the gut. Bile salts are toxic for living cells because they disrupt the structure of cell membranes. This means that tolerance to bile was considered as one of the essential properties required for probiotic bacteria to survive in the small intestine (Wang *et al.*, 2010).

In this study all the tested strains survived at all % bile salt concentration of 1 to 2 % (w/v) for 3 h. Similar studies have assessed the survival of LAB at concentrations of 0.5 % (w/v) up to 2 % (w/v) (Giri *et al.*, 2012), the author observed the higher tolerance of LAB isolated from fish intestine at 2 % bile concentration. Though, Hassanzadazar *et al.* (2012) stated that some of the LAB isolated from Koozeh cheese survived 0.3 % bile salt concentration for 4 h while some could not survive also, Maragkoudakis *et al.* (2006) explained that *Lactobacillus* strains of dairy origin survived at 0.3 % bile concentration for 4 h when screened for probiotic potential.

3.5 Conclusion

Considering the standard pH condition of the stomach and the concentration of % bile salt, all the isolates met the requirement of tolerance to acid and % bile salt concentration although, some isolates had decreased viability after certain hours of incubation. Generally, *Lactobacillus fermentum* followed by *Lactobacillus senioris* exhibited the highest viability compared to other isolates in both pH conditions and % bile salt concentrations. Though, there were significant differences in the viability of the isolates at pH 3 compared to control pH 7 but at 1 – 2 % (w/v) bile concentration there were no significant differences $P>0.05$ compared to control 0 % bile concentration.

In conclusion, the strains of LAB tested were found *in vitro* to possess desirable probiotic properties. These isolates are good for further investigation *in vivo* studies to determine their health benefits and potential to be used as multifunctional starter culture in the manufacturing industry.

Chapter Four: Screening Lactic Acid Bacteria from *Nono* for antimicrobial activities against selected indicators of pathogenic bacteria

4 Introduction

Traditional processing methods used during the spontaneous fermentation of milk for *Nono* production can provide opportunities for microbial contamination with spoilage and pathogenic organisms from the environment. Poor hygiene practices and use of unsanitary utensils have been associated with small scale production of traditional fermented foods and can negatively impact on the microbial quality and stability of the fermented product (Anyogu *et al.*, 2014; Oguntoyinbo, 2014).

It has been well established that LAB dominate the fermentation of milk products (Abdelgadir *et al.*, 2008; Akabanda *et al.*, 2013). Furthermore, LAB such as those belonging to the genera *Lactobaccillus, Lactococcus*, *Enterococcus* and *Pediococcus* are known to produce a vast range of antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocins which can inhibit the growth and survival of spoilage and pathogenic bacteria during fermentation processes and in the final fermented products (Klaenhammar, 1998; Saidi *et al.*, 2011). A variety of organic acid is produced by LAB as part of their normal metabolism. These organic acids have been shown to have antimicrobial activity when they disrupt the cell membrane of the bacteria and interfere with the active form (Blom and Mortvedt 1991). Notwithstanding their complexity, the whole basis of lactic acid fermentation centres on the ability of LAB to produce lactic acid, which inhibits the growth of other non-desirable organisms (Evans *et al.*, 2013).

Bacteriocins are specifically known as natural compounds that are capable of influencing the quality and safety of food products. As reported by Jeevaratnam *et al.* (2005), many bacteriocins such as Nisin (a commercially exploited bacteriocin used in large scale) have been isolated, characterized and confirmed to exhibit an

antimicrobial activity against pathogenic and foodborne pathogens. The author further explained the various ways in which bacteriocins could be used as food preservative. There is now an increasing interest to study the ability of fermenting LAB strains to produce antimicrobial peptides (bacteriocins) in addition to their main antimicrobial effect of low pH and acid stress and potential use in the food industry as functional starters (Heradio-Castro *et al.*, 2015; Azizi *et al.*, 2017). Despite this, nisin remains the most studied and only bacteriocin approved for use in food (O'Sullivan *et al.*, 2002). Hydrogen peroxide inhibition activity may be due to the oxidation of sulfhydryl groups causing a denaturation of a number of enzymes, and also the peroxidation of cell membrane lipids that causes increased membrane permeability (Kong and Davison 1980). It can also be as a forerunner for the production of bactericidal free radicals such as superoxide (O2-) and hydroxyl (OH.) radicals which can damage DNA. Davidson *et al.* (1983) have reported using H2O2 produced by *Lactobacillus* and *Lactococcus* strains to inhibit *Staphylococcus aureus*, *Pseudomonas* sp. and various psychotropic microorganisms in foods.

This ability of LAB strains and/or their metabolites to inhibit undesirable microorganisms in food is described as bio-preservation (Stiles, 1996). The potential bio-preservative effect of fermenting LAB strains in milk provides an additional processing parameter, contributing to both the food quality and safety while enhancing or at least maintaining its sensorial characteristics. In the process of selection of starter cultures for use in controlled fermentations, this functional property constitutes a key criterion (Holzapfel, 2002).

It is essential that the predominant LAB species involved in milk fermentation for *Nono* production are characterized with respect to their technological functions. The

establishment of a starter culture will need to be developed based on a comprehensive understanding of LAB species that are involved during processing. In this study, the antimicrobial activities of selected LAB against indicators of common foodborne pathogens were investigated.

4.1 Objectives

The general objective of this study was to investigate the antimicrobial activity of seven LAB involved in the traditional fermentation of milk for *Nono* production against selected indicators of foodborne pathogens. To achieve this main objective, specific objectives that include various activities were pursued including:

- Screening the LAB for antimicrobial activity against selected indicator bacteria using the agar spot test, well diffusion assay and spectrophotometric method.

- Characterization of specific antimicrobial compounds produced by LAB e.g., organic acid and/or antimicrobial peptides using a spectrophotometric method.

4.2 Materials and Methods

4.2.1 Microorganisms

Seven out of 11 isolates were selected for this study. The selection of these isolates was based on differences in their rep-PCR profiles described in chapter 2 and they are listed in Table 4.1

Table 4. 1 Lactic acid bacteria studied

Isolate Codes	Species name
52	*Enterococcus thailandicus*
10	*Streptococcus infantarius*
43	*Lactobacillus senioris*
13	*Lactobacillus fermentum*
11	*Lactobacillus delbrueckii subsp indicus*
9	*Leuconostoc pseudomesenteroides*
73	*Streptococcus thermophilus*

4.2.2 Indicator bacteria used

Five indicator bacteria used for this antimicrobial activity test were obtained from the Microbiology Research Unit culture collection at London Metropolitan University (London, UK). These were *Samonella enteritidis serovar Typhimurium* variant DT124, *Escherichia coli* NCTC 12900, *Listeria monocytogenes* NCTC 11994, *Staphylococcus aureus* CMCC 1930 and *Bacillus cereus* LMG 1356.

4.2.3 Antimicrobial activity screening

The spot test and the well diffusion assay described by Ouoba *et al.* (2008a) and Anyogu *et al.* (2014) were used to evaluate the antimicrobial activity of the LAB.

4.2.4 Inoculum preparation

Prior to the experiment, the LAB isolates were sub-cultured several times on deMan-Rogosa Sharpe (MRS) Agar (Oxoid, CM0361 Basingstoke, UK), as well as M17 Agar (Oxoid, CM0785) for *Streptococcus thermophilus* and incubated anaerobically at 37°C for 48 h. The indicator bacteria were sub-cultured on Nutrient agar, Oxoid (NA) and incubated aerobically at 37°C for 24 h.

For the preparation of both of the LAB and indicator bacterium inocula, a stock culture of each organism was prepared by suspending a colony in 1 ml of Maximum Recovery Diluent (MRD, Oxoid CM0733). Aliquots of stock solution were added to 5 ml of MRD until a final concentration of 0.5 McFarland standard (10^7-10^8 CFU/ ml) was achieved by the using a nephelometer (TREK Diagnostic systems, West Sussex, UK).

4.2.5 Inhibition potential of the LAB isolates against indicator of pathogenic bacteria using the spot test

This method was used to investigate the potential antimicrobial activity of the whole cell of the test isolates in term of competition of the nutrient and production of antimicrobial compounds. An inoculum (2 µl) of each isolate was spotted on the surface of an MRS agar plate as well as M17 agar plate for *Streptococcus thermophilus* and allowed to dry at room temperature for 30 min. All cultures were incubated anaerobically at 37°C for 24 h. After the incubation time, 100 µl of each

stock solution of an indicator organism was inoculated into 10 ml TSB + 0.8% (w/v) agar and overlaid on the grown spotted cultures of the LAB isolates. The overlaid plates were left to dry for 1 h at ambient temperature. Control plates were set up by pouring the soft agar + indicator overlay on MRS agars without any test isolates spots. All plates were incubated aerobically for 24 - 48 h at 37°C which is the optimum growth condition for the indicator bacteria. The diameter of the zone of inhibition was measured and recorded in mm.

4.2.6 Inhibition potential of the LAB isolates against indicator of pathogenic bacteria using well-diffusion assay

This method was used to investigate if LAB isolates could produce any primary or secondary antimicrobial metabolites during growth in broth culture. Twenty (20) ml of soft TSA [TSB + 0.8% (w/v) agar] was seeded with 200 µl of inoculum of each indicator bacterium and poured into a petri-dish. The agar was allowed to set and wells were made in each agar plate. The supernatant of each test isolate was obtained by centrifuging 1 ml of a 24 h culture in MRS broth at 10,000 revolutions per min (rpm) for 10 min ((Jouan GR2022). A cell free supernatant (CFS) for each isolate was obtained by filter sterilising the supernatant with a 0.2 µm sterile syringe-driven membrane filter (ThermoFisher, UK). An aliquot of 100 µl of CFS was transferred into each well made in the soft agar plate. The plates were held at 4°C for 3 h to allow diffusion before incubation for 24 - 48 h at 37°C. A negative control was set up by substituting the CFS with MRS broth. The zone of inhibition was determined by measuring the clear zone around the well and was recorded in mm.

4.2.7 Inhibition potential of cell free supernatants of the LAB cultures against indicator bacteria using a spectrophotometric method

4.2.7.1 Screening of the LAB CFS inhibitory activity against indicators of pathogenic bacteria

Antimicrobial activity as a result of a direct antagonism between the CFS and the indicator bacteria in liquid media was tested using the method described by Lash *et al.* (2005) with some modifications. The CFS of each isolate was obtained as described in section 4.2.6 and the pH measured before testing the inhibitory activity as depicted below

An inoculum (2 ml) of CFS of each test isolate was separately transferred into a universal bottle containing a mixture of 2 ml of each indicator bacterium culture and 16 ml of TSB. In the negative control, CFS was substituted with 2 ml of MRS broth or M17. Cultures were incubated aerobically for 24 h at 37°C and the optical density (OD) measured at 540 nm (JENWAY 7315 Staffordshire UK). The spectrophotometer was zeroed using a mixture of 2.6 ml TSB and 0.4 ml MRSB. The inhibitory activity of the LAB isolates was determined by measuring and comparing the OD of the indicator bacteria after the 24 h incubation period in both test and control experiment.

4.2.8 Characterization of antimicrobial activity by LAB isolates

To eliminate acid production as the sole antimicrobial compound, an acid neutralisation test was conducted. The CFS from the test isolates were prepared as previously described and then neutralised with filtered sterilised 0.1M NaOH (Sigma, S8045) to increase the pH of 6.95 ± 0.1(Anyogu *et al.*, 2014). The inhibitory

effect of the neutralised CFS on the indicator bacteria was investigated using the spectrophotometric method as previously described in section 4.2.7.1.

Further characterization of antimicrobial activity examined the possibility that LAB isolates from *Nono* could produce antimicrobial peptides with broad – spectrum activity against indicator bacteria. Neutralised CFS was separately treated with Protease (P5147, Sigma) and Proteinase K (P2308, Sigma) to a final concentration of 1 mg/ml. The treated CFS was incubated at 37°C for 2 h according to manufacturers' instructions. A negative control was set up using non-treated neutralised CFS for comparison. Inhibitory activities were determined using the spectrophotometric method as described previously.

4.2.9 Data Analysis

Each experiment was conducted at least in two occasions. The mean and standard deviation of all measurements were determined and Statistical significant differences set at $P \leq 0.05$ to compare the means using 1 way ANOVA.The results were expressed as mean ± standard deviation of $\log_{10}$ cfu/ml.

4.3 Results

4.3.1 Inhibition potential of the LAB isolates against indicator of pathogenic bacteria using the spot test

Lactic acid bacteria isolated from *Nono* exhibited varying levels of inhibition against common Gram positive and Gram negative foodborne pathogens. In the spot test, inhibitory activities were determined by the diameter of the clear zone around the growth of the spotted cultures of the LAB (Figure 4.1). It was also observed that *Streptococcus thermophilus* did not inhibit any of the indicators screened. On the other hand, *Lactobacillus fermentum* exhibited, in general, a broad spectrum of inhibition against both Gram positive and Gram-negative indicator bacteria (Table 4.2) with inhibition zones between 11 and 40 mm according to the indicator screened. Taking specific indicators into account, *Lactobacillus fermentum* exhibited the strongest inhibitory effect (21 - 30 mm inhibition zone) against *Salmonella enteritidis* while *Streptococcus infantarius* and *Lactobacillus senioris* exhibited the strongest inhibitory effect (21 - 30 mm inhibition zone) against *Escherichia coli*. All the LAB isolates except *Streptococcus thermophilus* showed the same degree of inhibition (11-20 mm inhibition zone) against *Staphylococcus aureus* and *Listeria monocytogenes*. In addition, *Bacillus cereus* was the most susceptible indicator with the largest clear inhibition zones on average of (21 mm – 40 mm, Table 4.2).

4.3.2 Inhibition potential of the LAB isolates against indicator of pathogen bacteria using well-diffusion assay

Initial trials of this method were unsuccessful as results were often difficult to interpret and inconsistent. It is the reason why, the alternative **spectrophotometric**

method adapted from Lash *et al.* (2005) was used to investigate the bacteriostatic effect of the cell free supernatant of LAB isolates against indicator bacteria.

Table 4. 2 Antimicrobial activities of LAB isolates against indicator bacteria using the spot test.

LAB isolates	Indicator organisms				
	Salmonella enteritidis	*Escherichia coli*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Bacillus cereus*
Enterococcus thailandicus	++	++	++	++	++++
Streptococcus infantarius	++	+++	++	++	+++
Lactobacillus senioris	++	+++	++	++	+++
Lactobacillus fermentum	+++	++	++	++	++++
Lactobacillus delbrueckii subsp	++	++	++	++	+++
Leuconostoc pseudomesenteroid	++	++	++	++	+++
Streptococcus thermophilus	-	-	-	-	-

No inhibition: -, 0-10 mm: +, 11-20 mm: ++, 21-30 mm: +++, 31-40 mm: ++++

Data represents the mean of two duplicates (n=2)

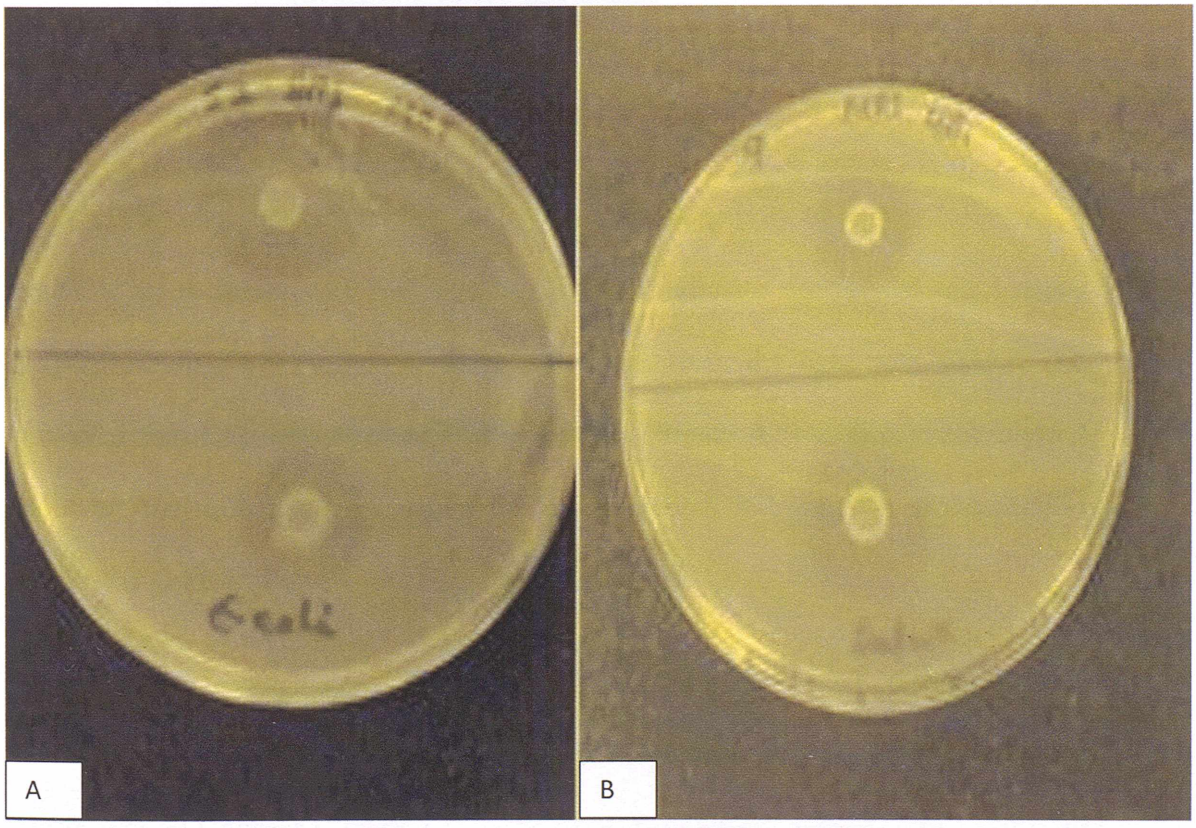

Figure 4. 1 Example of the antimicrobial activity of LAB isolates from Nono against indicators of pathogenic bacteria using the spot test

Zone of inhibition in A: *Enterococcus thailandicus* (52) against *Escherichia coli* and B: *Leuconostoc pseudomesenteroides* (9) against *Salmonella enteritidis*

4.3.3 Inhibition potential of cell free supernatants of the LAB cultures against indicator bacteria using a spectrophotometric method

The pH of the CFS dropped from 6.0 (MRS broth) and 6.8 (M17 broth) to 3.97, 4.14, 4.16, 4.26, 4.29, 4.29 and 6.07 for the CFS of broth cultures of *Lactobacillus delbrueckii subsp indicus,*, *Lactobacillus fermentum*, *Streptococcus infantarius*, *Lactobacillus senioris*, *Enterococcus thailandicus*, *Leuconostoc pseudomesenteroides*, and *Streptococcus thermophiles* respectively

All LAB isolates showed varying range of inhibition activity from 0 to 1.4 mm as a result of direct antagonism between the CFS and indicator bacteria in liquid media (Figure 4.2 to 4.8). Similar to the observations from the agar spot test, it was observed that CFS of *Lactobacillus fermentum* (Figure 4.5) exhibited the highest antimicrobial effect against all indicators screened followed by *Lactobacillus senioris* (Figure 4.4).

Furthermore, *Streptococcus thermophilus* exhibited the least effect on the growth of the indicator bacteria particularly against *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* (Figure 4.8). The weaker inhibitory activity 1 to 1.2 mm of *Enterococcus thailandicus* against most indicator bacteria on agar 10 -20 mm was also noted in broth 1-1.2 mm (Figure 4.1).

The susceptibility of *Bacillus cereus* to most of the LAB isolates observed in the agar spot test was also observed in the CFS experiments except with regards to *Lactobacillus delbrueckii subsp indicus*. In contrast to earlier observations from the spot test, on average, the growth of *Listeria monocytogenes* in TSB was markedly affected by the addition of the CFS of all test isolates screened except *Streptococcus*

thermophilus while *Salmonella enteritidis* was mostly affected by *Lactobacillus fermentum* and *Lactobacillus delbrueckii subsp indicus*.

Except for a few instances, inhibitory activities observed from the CFS of test isolates were removed after neutralisation. For example, neutralised CFS of *Lactobacillus senioris* lost its effect on the growth of all the indicator bacteria screened (Figure 4.4) while, other test isolates when their CFS were neutralised, retained their inhibition effect only against *Bacillus cereus* compared to non – neutralised CFS. The antimicrobial effect of *Streptococcus thermophilus* against *Salmonella enteritidis* was also not observed to be influenced by neutralisation (Figure 4.8).

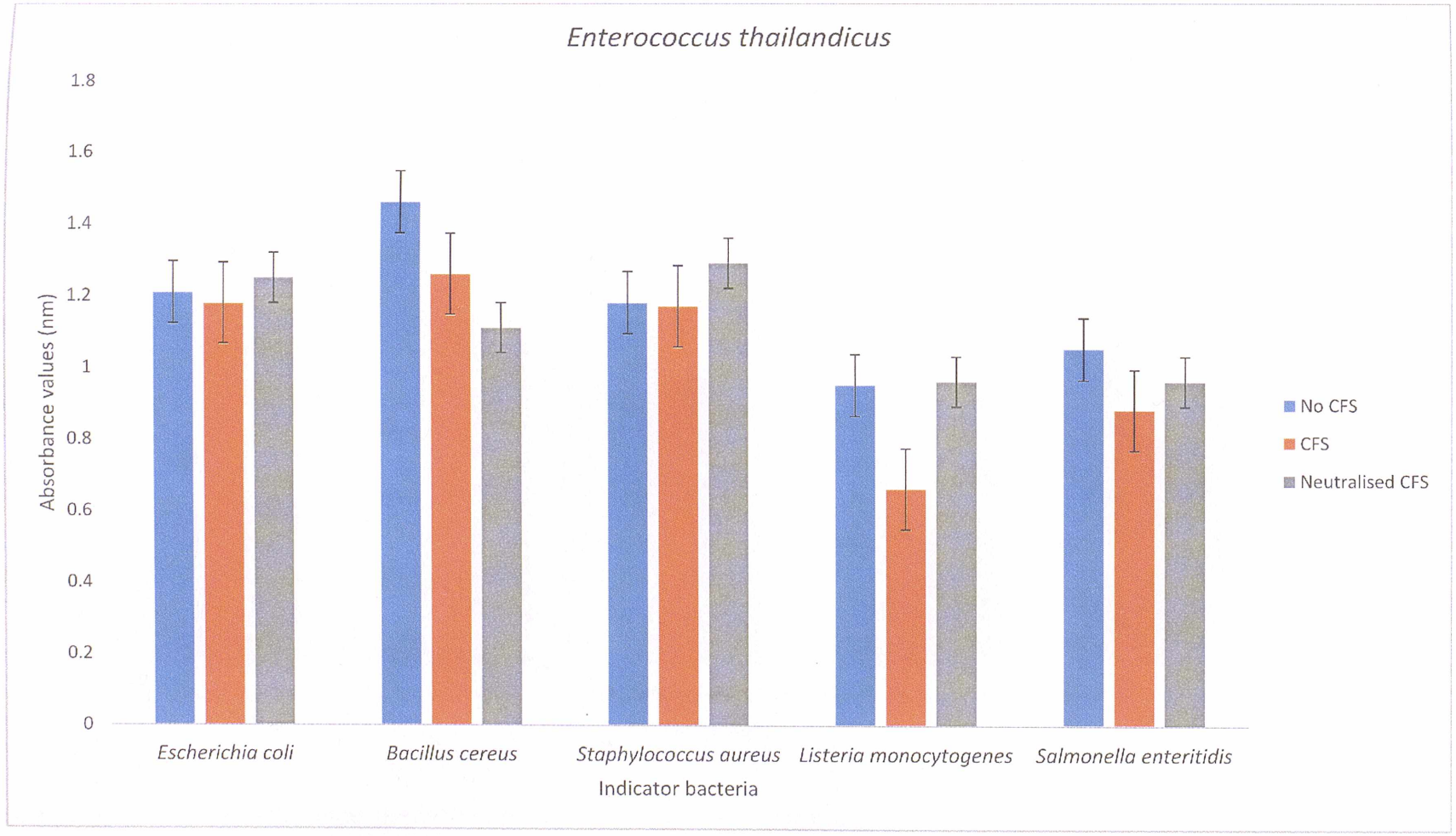

Figure 4. 2 The antimicrobial activity (nm) of CFS and neutralised CFS of *Enterococcus thailandicus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

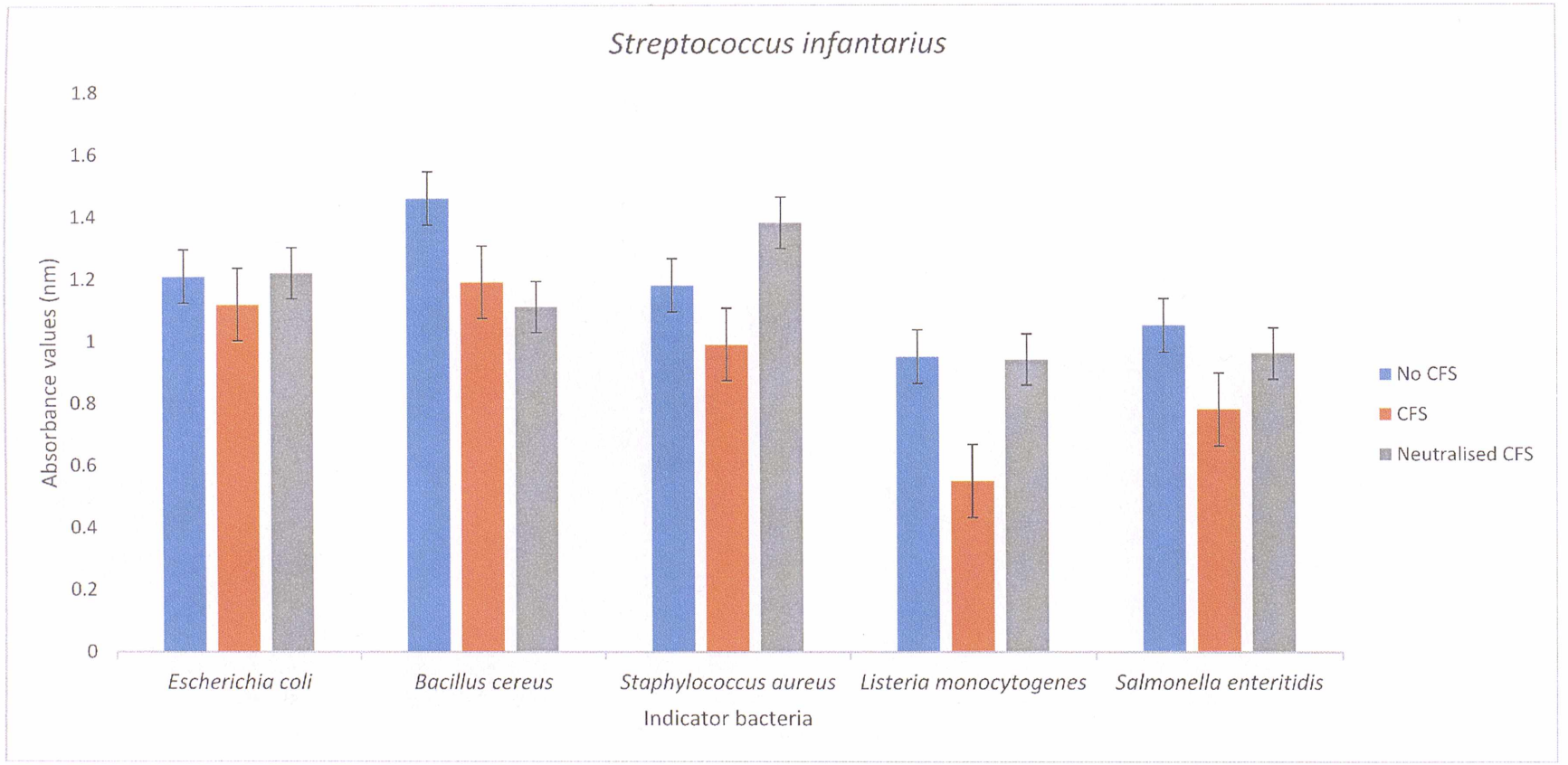

Figure 4. 3 The antimicrobial activity (nm) of CFS and neutralised CFS of *Streptococcus infantarius* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

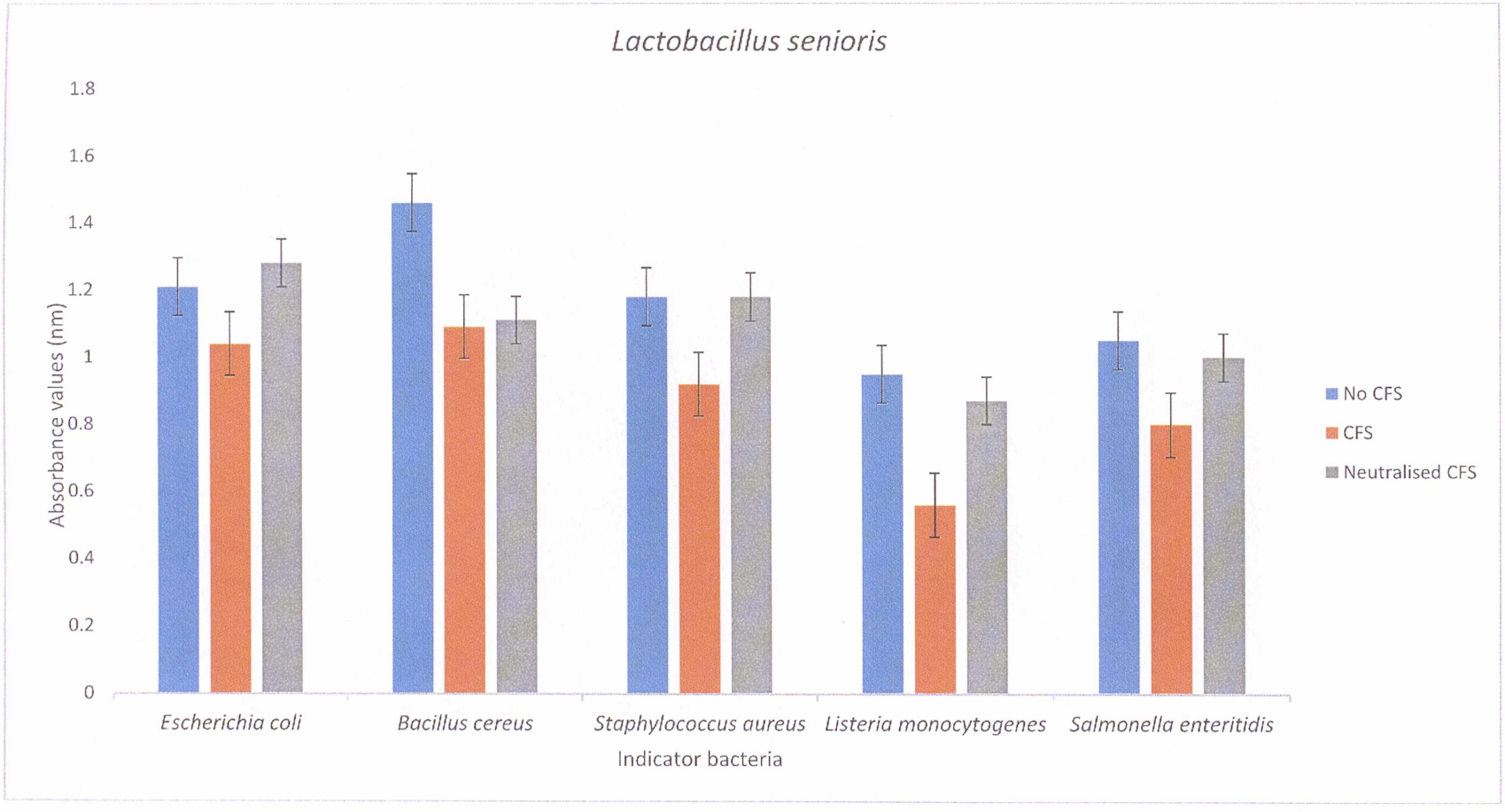

Figure 4. 4 The antimicrobial activity (nm) of CFS and neutralised CFS of *Lactobacillus senioris* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

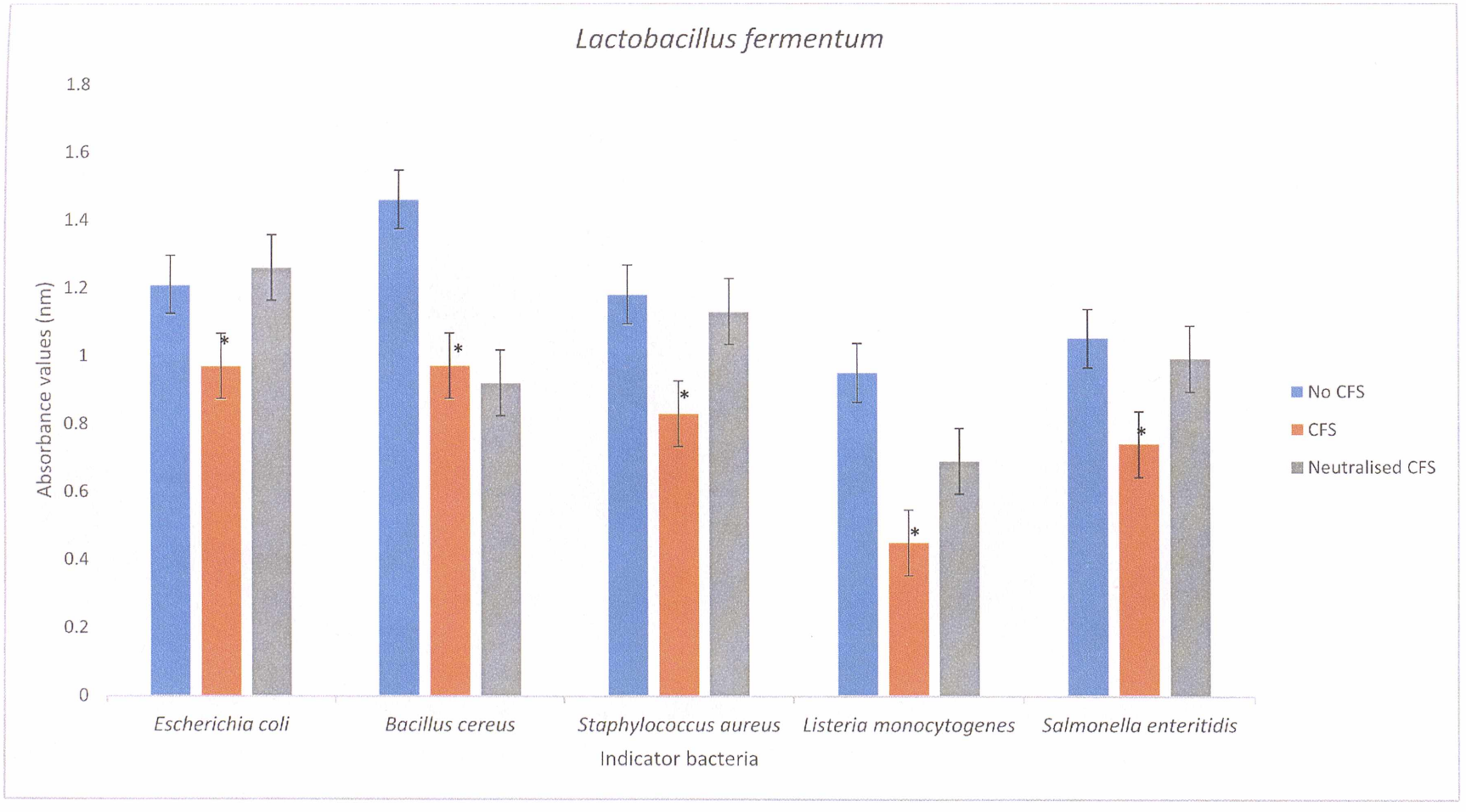

Figure 4. 5 The antimicrobial activity (nm) of CFS and neutralised CFS of *Lactobacillus fermentum* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

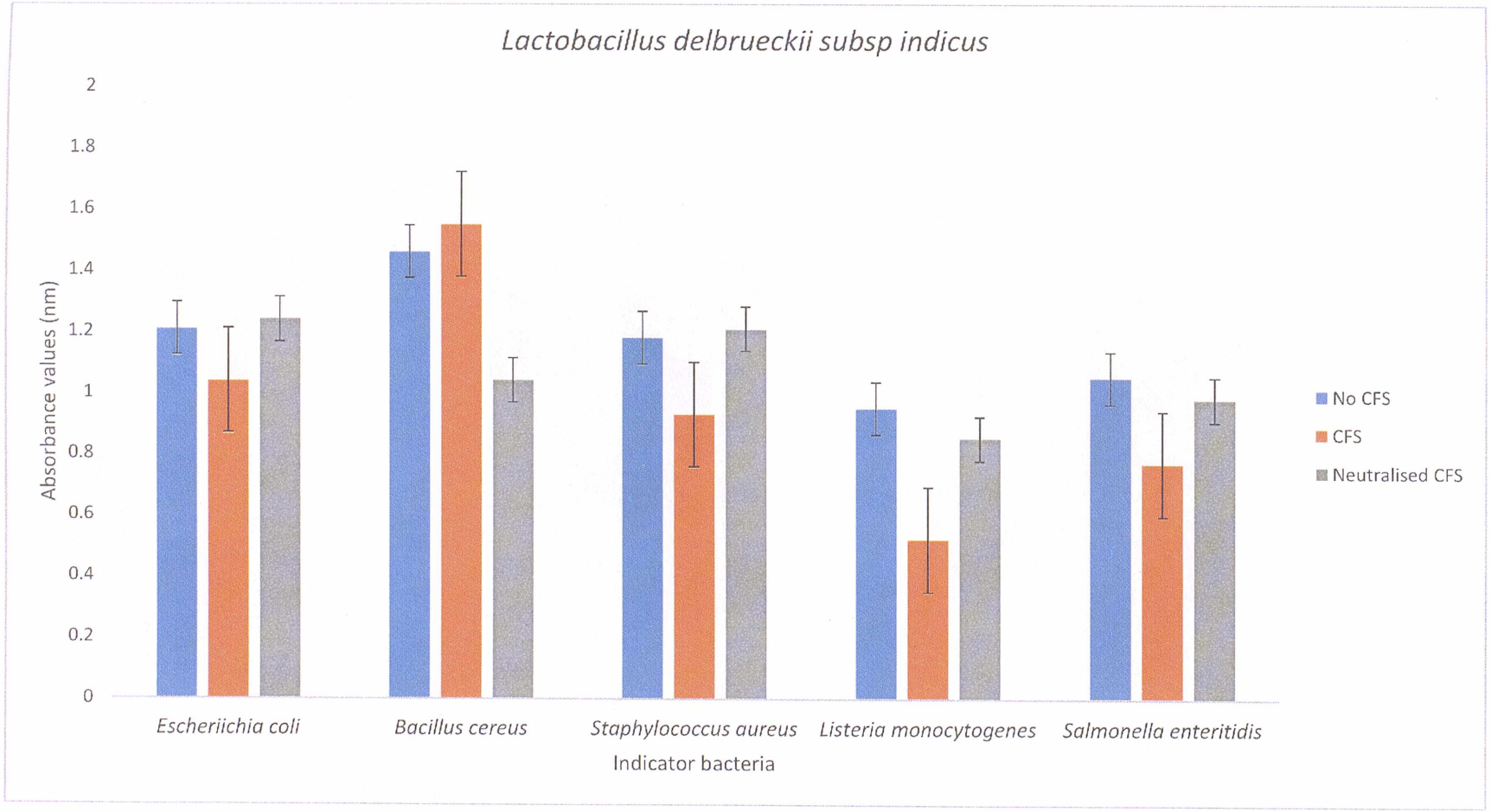

Figure 4. 6 The antimicrobial activity (nm) of CFS and neutralised CFS of *Lactobacillus delbrueckii subsp indicus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

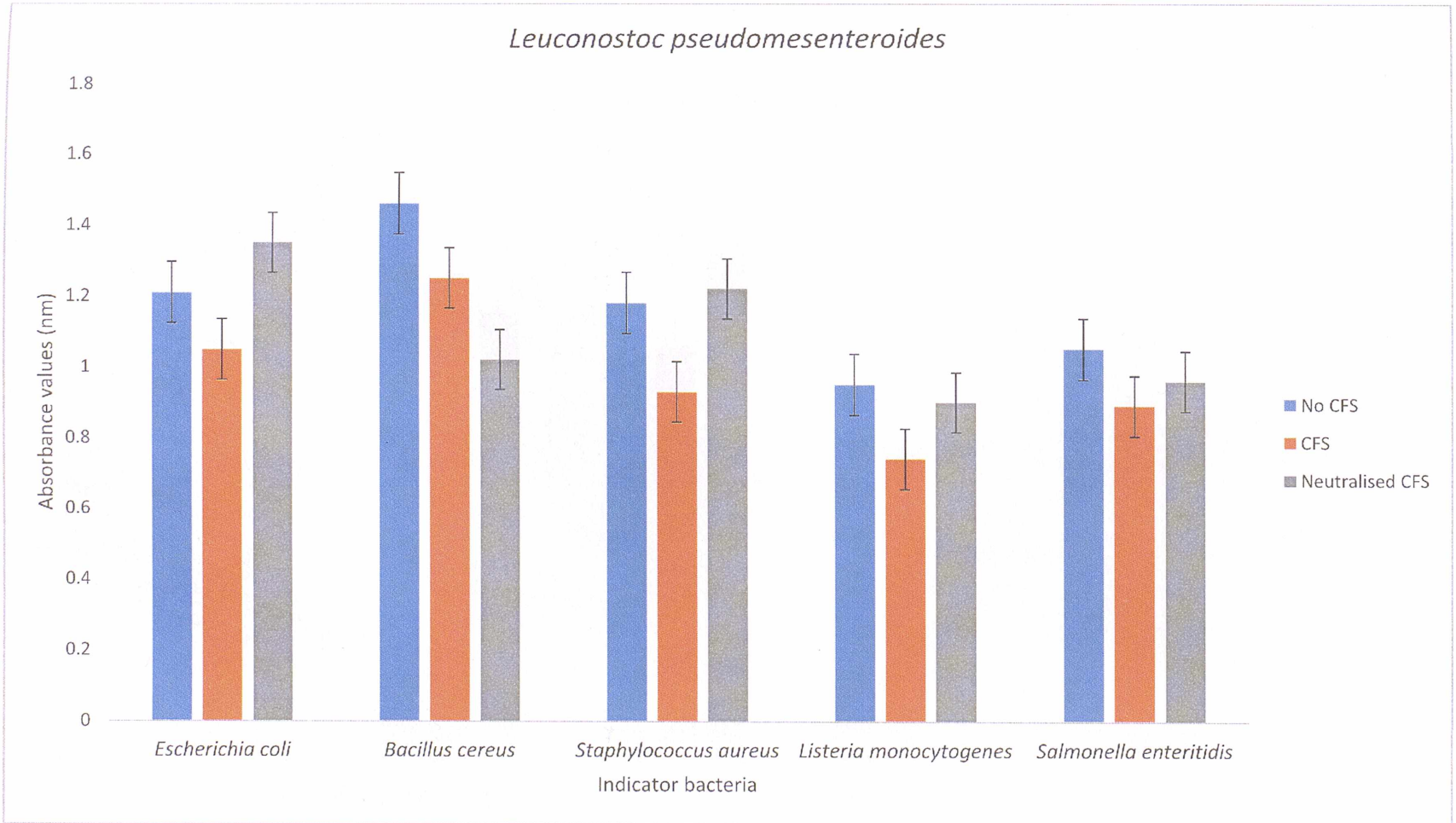

Figure 4. 7 The antimicrobial activity (nm) of CFS and neutralised CFS of *Leuconostoc pseudomesenteroides* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

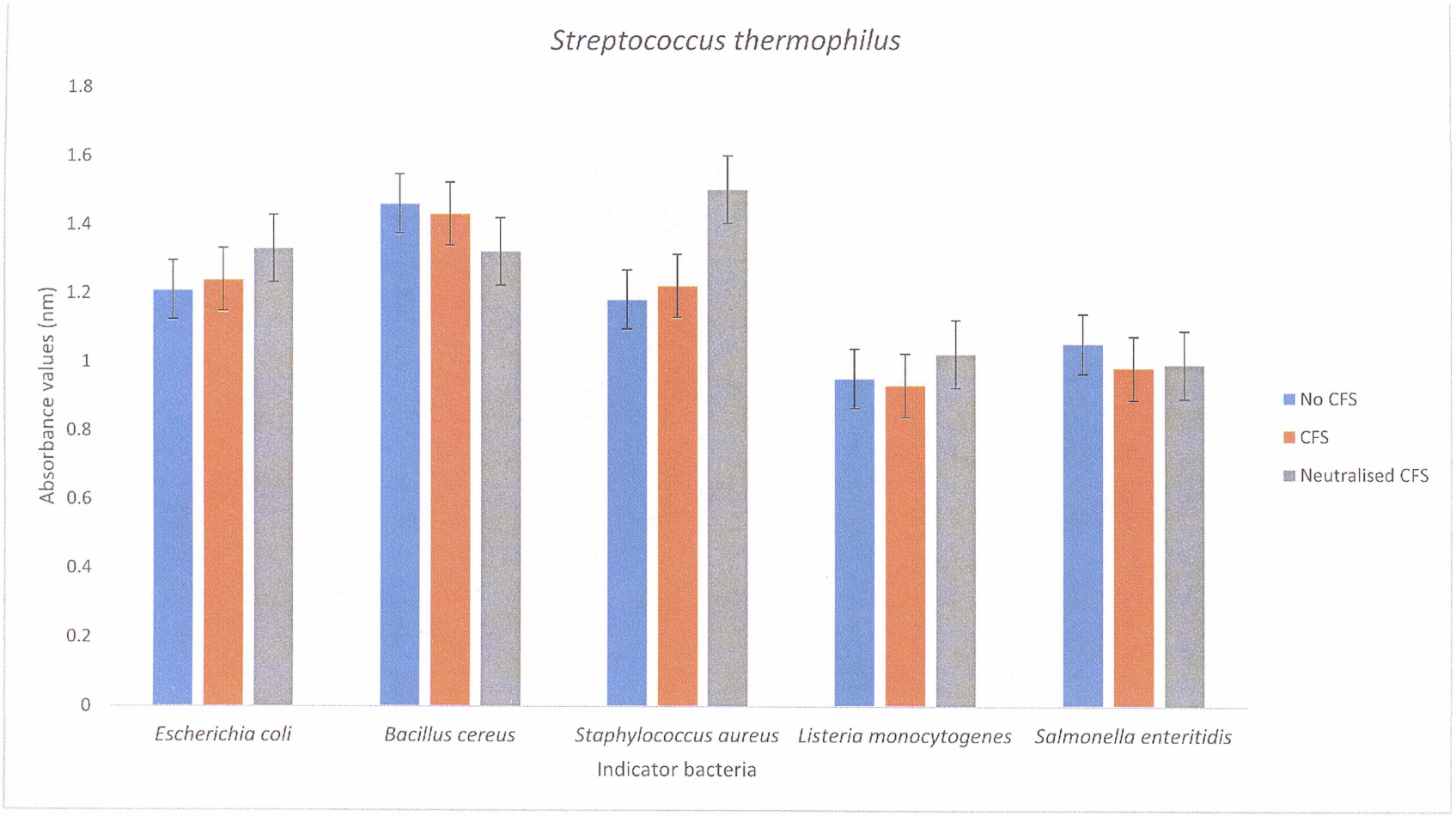

Figure 4. 8 The antimicrobial activity (nm) of CFS and neutralised CFS of *Streptococcus thermophilus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

Further characterization to determine the possibility of the test isolates to produce antimicrobial peptides against the indicators screened showed that the inhibitory activities observed from the neutralised CFS against *Bacillus cereus* were lost after proteolytic enzyme treatments. With the proteinase K, all treated CFS exhibited antimicrobial effect against *Staphylococcus aureus* (Figure 4.9-4.15).The effect of most treated CFS (except those of *Lactobacillus senioris* (Figure 4.11) and *Streptococcus thermophiles* (Figure 4.15) were also observed on *Listeria monocytogenes*.

For the CFS treated with the protease enzyme, the antimicrobial effect against some of the indicators including *Bacillus cereus*, *Escherichia coli* and *Salmonella enteritidis* was lost. CFS of *Leuconostoc pseudomesenteroides* showed inhibition effect against *Escherichia coli* while, *Enterococcus thailandicus* and *Streptococcus thermophilus* showed inhibition effect against Gram positive indicator *Staphylococcus aureus*.

For proteolytic enzymes treated CFS, *Streptococcus infantarius* exhibited more antilisterial activity compared to other test isolates. The isolate inhibition effect on the growth of *Listeria monocytogenes* was higher while, *Lactobacillus senioris* and *Streptococcus thermophilus* showed no inhibition effect on the indicator

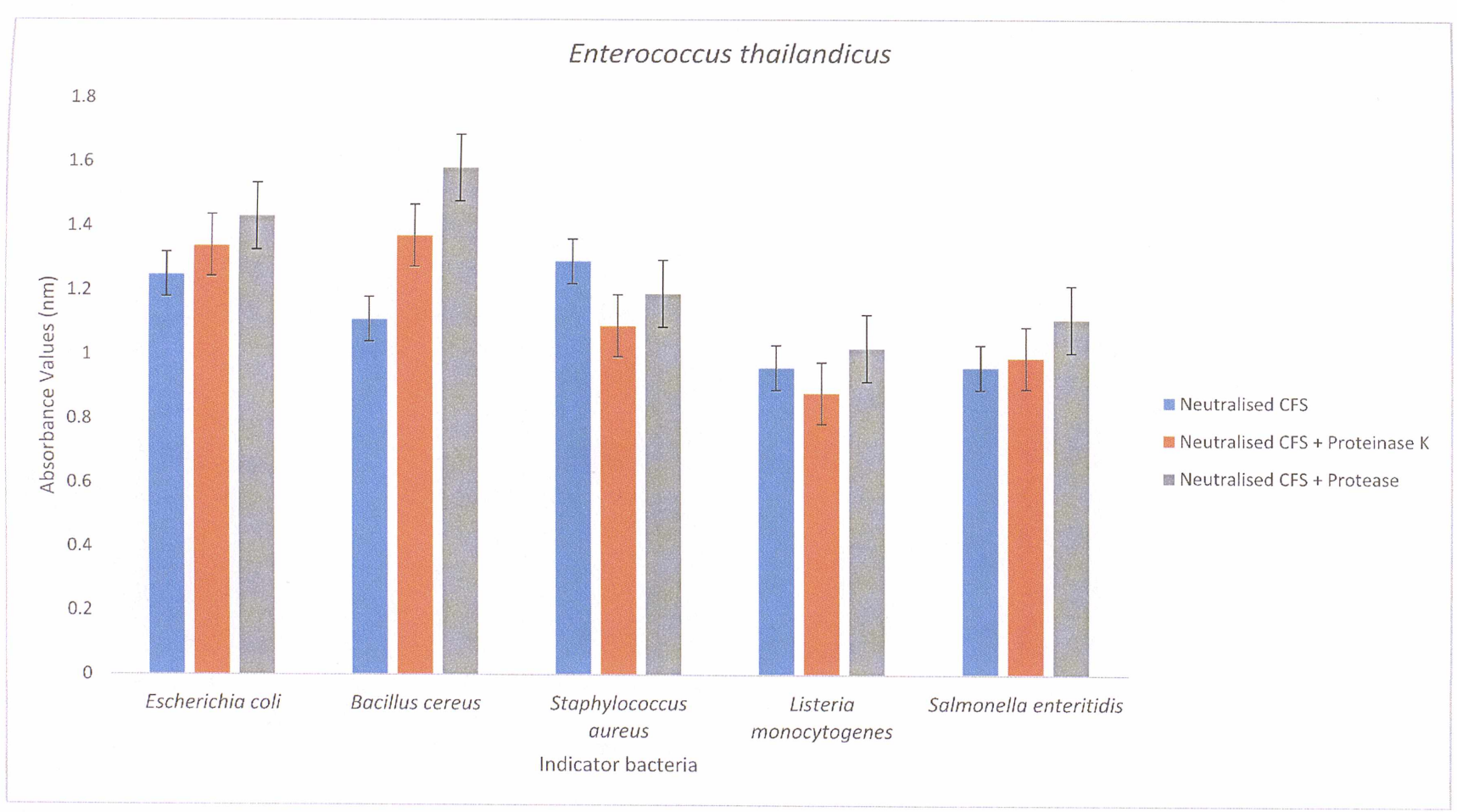

Figure 4. 9 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Enterococcus thailandicus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

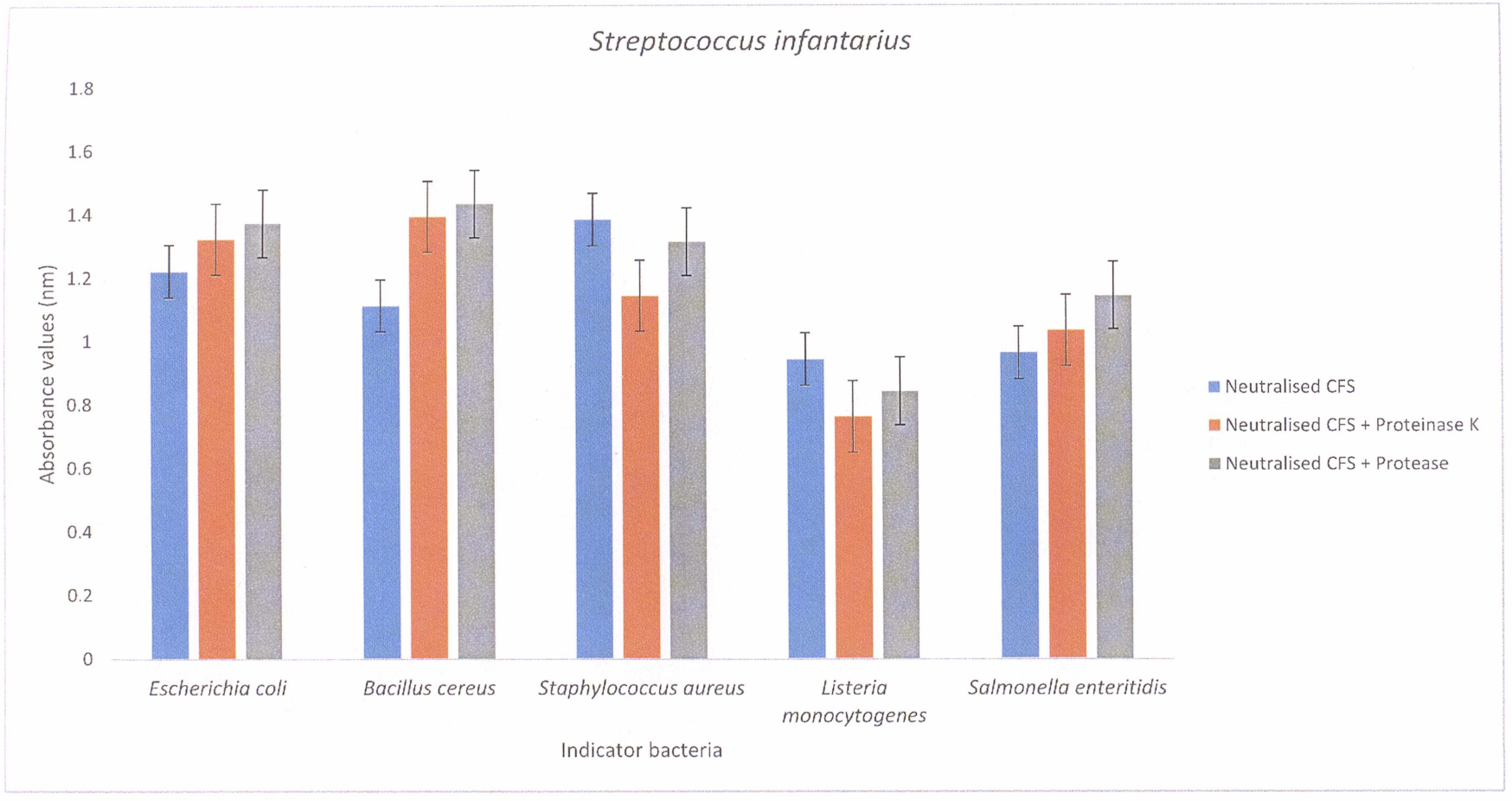

Figure 4. 10 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Streptococcus infantarius* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

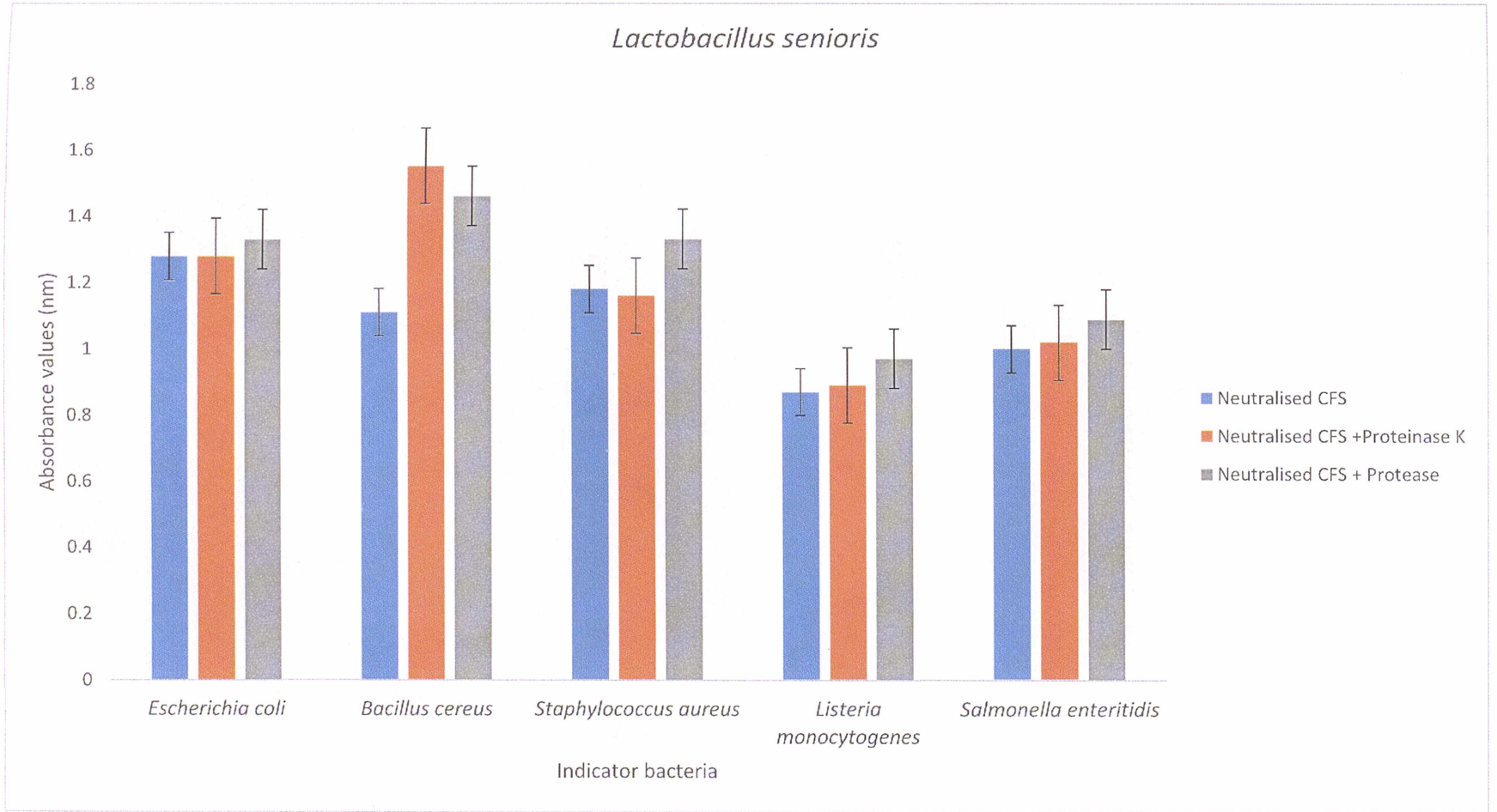

Figure 4. 11 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Lactobacillus senioris* against indicators of the pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

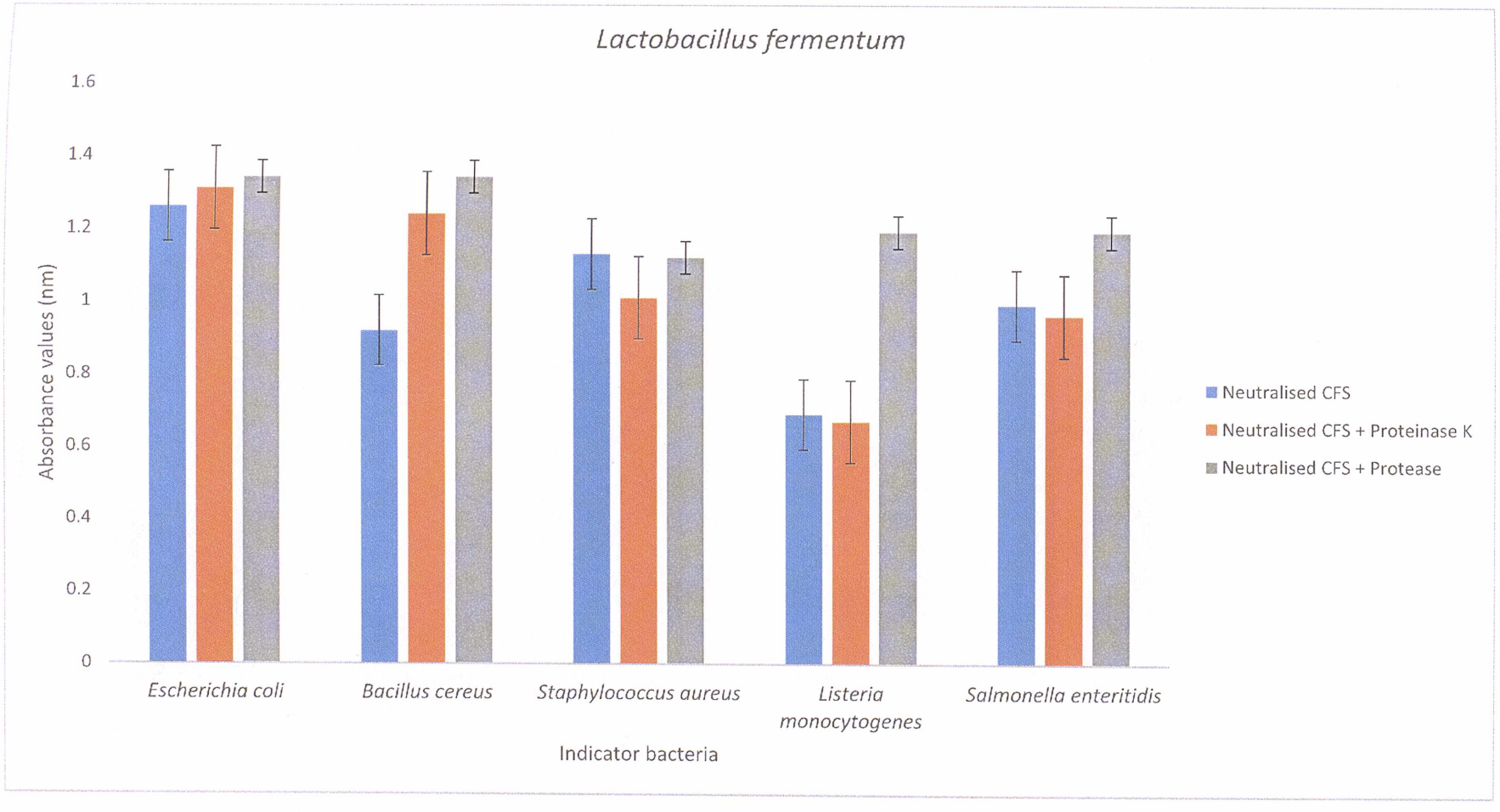

Figure 4. 12 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Lactobacillus fermentum* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

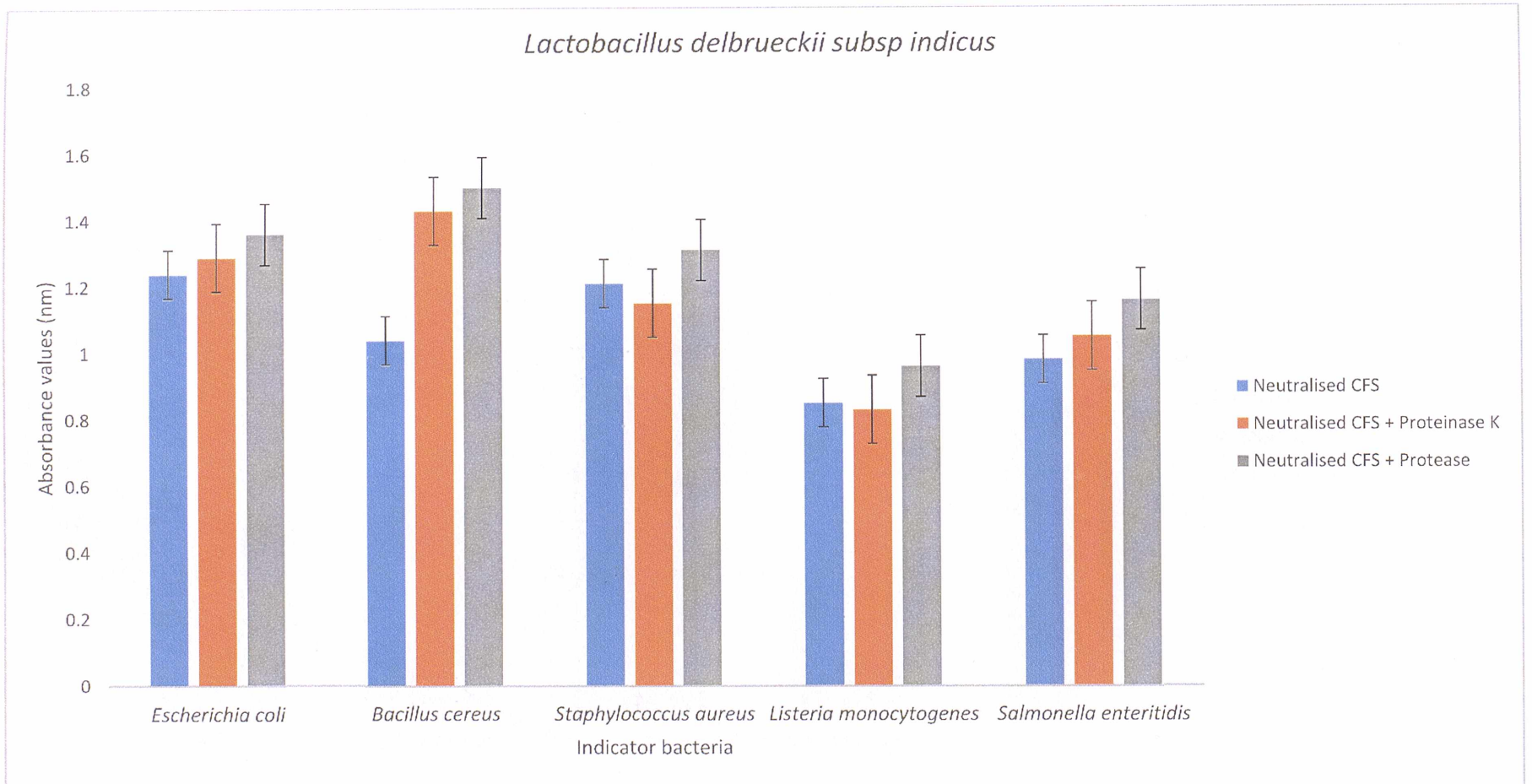

Figure 4. 13 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Lactobacillus delbrueckii* subsp *indicus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

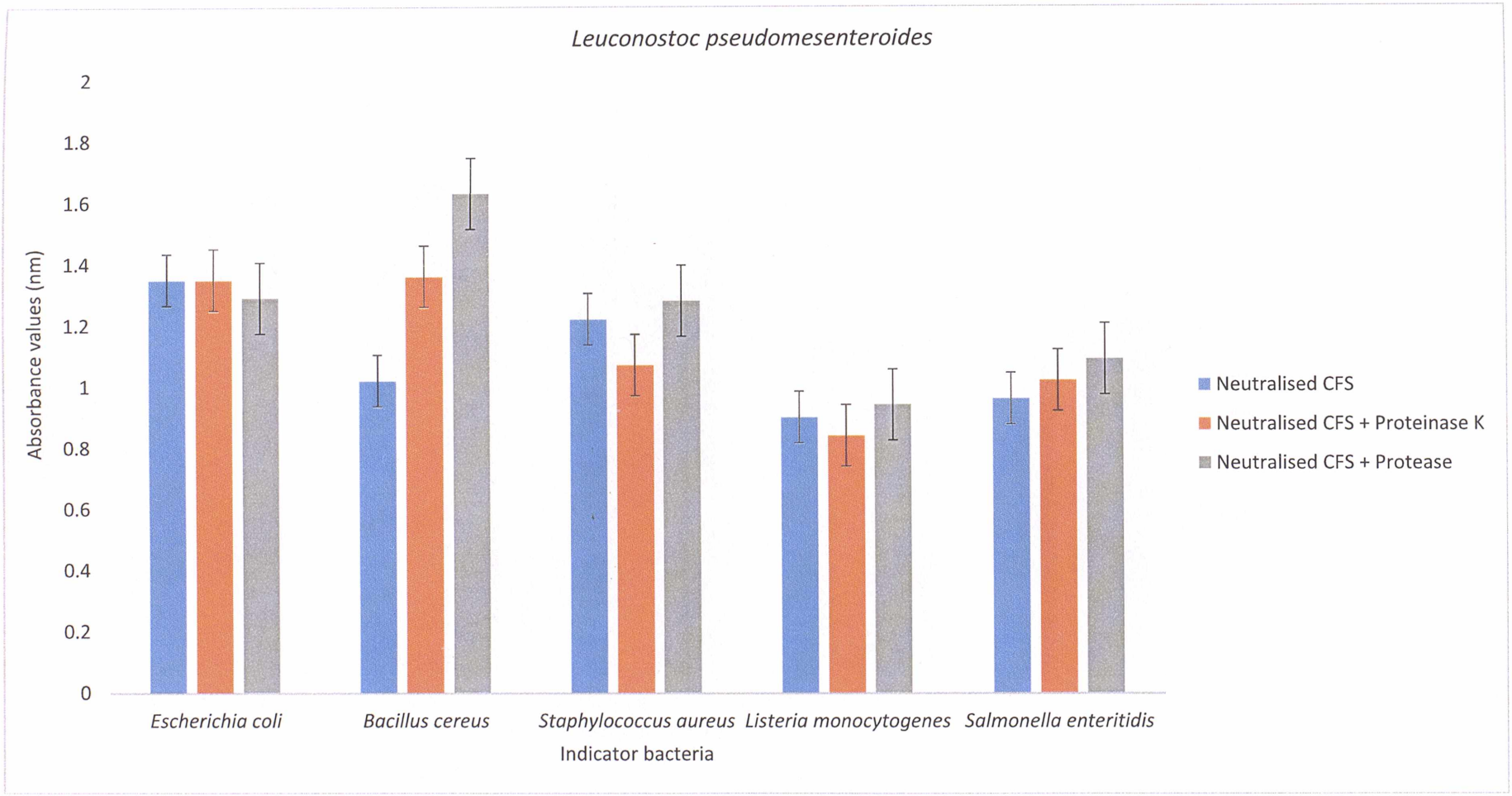

Figure 4. 14 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Leuconostoc pseudomesenteroides* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

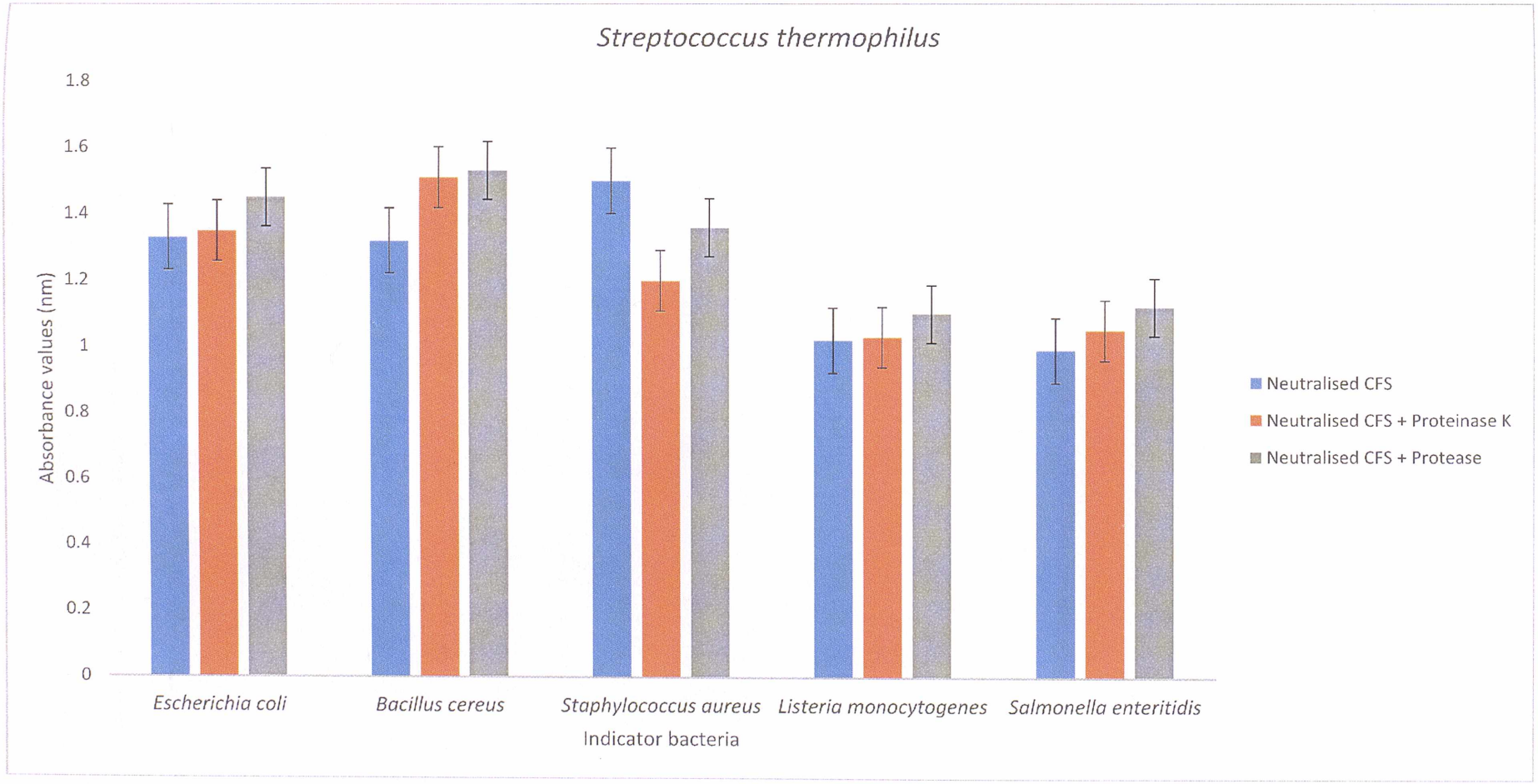

Figure 4. 15 The antimicrobial activity (nm) of the proteolytic enzymes treated neutralised CFS of *Streptococcus thermophilus* against indicators of pathogenic bacteria. Error bars represent standard deviation of the mean (SD). Asterisks represent significant differences ($P \leq 0.05$).

4.4 Discussion

Milk and dairy products harbour a wide variety of bacterial species that compete for both limited nutrients and space. The ability of LAB to produce antimicrobial compounds establishes them as candidates for a good starter culture fermentation process. This chapter describes the characterisation of predominant LAB from *Nono* on the basis of their antimicrobial properties against three Gram positive (*Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*) and two Gram negative (*Salmonella enteritidis, Escherichia coli*) indicators of foodborne pathogens. These LAB had been identified using a combination of phenotypic and genotypic methods as described in Chapter 2.

Further to screening the LAB for potential antimicrobial activities, the study also aimed to accurately attribute antimicrobial properties as being due to one or a combination of competition for nutrients, acid production and production of antimicrobial peptides. Three different approaches were used during this investigation. The agar spot test and well diffusion assays were used with varying success to ascertain the antimicrobial effect of cells and/or metabolites of the test organisms against indicator bacteria. Both methods are well recognised in studies of this type and have been used singly or in combination by other authors (Diop *et al.*, 2008; Anyogu *et al.*, 2014; Princewill and Ojimelukwe, 2014; Owusu-Kwarteng *et al.*, 2015).

A more sensitive assay based on a spectrophotometric method was also used to investigate the in-vitro bacteriostatic effect of the LAB isolates against indicators of pathogenic bacteria in a broth environment. Apart from confirming the observations

recorded from the spot test, the results provided a more accurate quantitative measure of inhibition.

The agar spot test was used to evaluate the inhibitory potential of the test isolates against the indicators based on the ability to compete for nutrients and produce antimicrobial compounds in the medium. Results showed that six out of seven LAB isolates exhibited varying levels of inhibition against common Gram positive and Gram negative foodborne pathogens. Particularly, *Lactobacillus fermentum* exhibited a broad spectrum of inhibition against both types of bacteria. The ability of *Lactobacillus fermentum* strains isolated from fermented milk products to show broad spectrum inhibitory activity has been reported by other authors (Katcham *et al.*, 2011; Heredia-Castro *et al.*, 2015). This ability appears to be strain specific as results contradict observations by Sharma *et al.* (2017). The author observed that CFSs of *Lactobacillus* strains including *Lactobacillus casei*, *Lactobacillus delbrueckii subsp indicus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Lactobacillus pentosus* isolated from curd and human milk when screened for their antagonistic activity against *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *K. pneumoniae* exhibited no zone of inhibition. Other LAB such as *Enterococcus faecium* and *Enterococcus faecalis* associated with food systems are capable of producing antimicrobial compounds with broad spectrum activity (Giraffa, 2003; Banwo *et al.*, 2013). This was not the case in this study as *Enterococcus thailandicus* showed a relatively weak inhibitory effect against the pathogens.

Of special interest was the observation that *Bacillus cereus* was the most sensitive indicator when tested against all LAB isolates that showed antimicrobial activity in

the spot test. This strong antagonistic activity of LAB isolates from fermented milk products against strains of *Bacillus cereus* has been reported by other authors (Banwo *et al.*, 2013; de Lima *et al.*, 2017; Sharma *et al.*, 2017). The high nutrient content of milk products makes them a particularly good environment to support the growth of a variety of organisms and raw milk is a usual source of spore-forming bacteria such as *Bacillus* spp. (Quigley *et al.*, 2013). This result is promising as it is important for any potential starter to be able to inhibit the growth of spore forming bacteria and contribute thereby to improving the safety and quality of the product.

Further evaluation of potential antimicrobial activity of the test isolates using the well diffusion assay was carried out. Based on the results obtained, no interpretations of the inhibition zone from the test isolates could be made. This finding was also reported by Farahmand (2015) when screening probiotic *Lactobacillus* spp. isolated from commercial fermented milks for antimicrobial activity. According to the author, this could be due to the low concentration of the antimicrobial compound in the CFS. This observation was also made by Harris *et al.* (1989) who observed that seven out of 14 bacteriocin-producing LAB that produced bacteriocin in thee strains produced no inhibition zones against *Listeria monocytogenes* by the agar diffusion method. The author explained that this does not necessarily implies lack of antilisterial activity but might be due to low production of antilisterial compound inn CFS and also.

The CFS of most LAB isolates from *Nono* inhibited the growth of the indicators bacteria in broth cultures. The growth of *Listeria monocytogenes* was notably impeded in the presence of CFS. This is of particular interest as starter cultures for fermented milk products that show antilisterial activity are important in the food and

dairy industries. In fact, *Listeria* spp. are commonly associated with dairy products (Banwo *et al.*, 2012) and can cause safety issues. The ability of the CFS to inhibit the growth of the indicators shows that the antimicrobial effect cannot be solely attributed to competition for nutrients. Thus, the exact mechanism of inhibitory activities was further evaluated to ascertain if this was due to factors such as production of acids and/or protein antimicrobials. In general, the bacteriostatic effect of the test isolates on the indicator organisms were disappeared after neutralisation of the CFS indicating that acid production was most likely the main antimicrobial effect for instance, in the case of *Lactobacillus fermentum*, there were significant difference P value = 0.035 between the control (no CFS) vs CFS among all the treatments but, when the CFS was neutralised, there were no significant differences among the control vs neutralised CFS and CFS vs neutralized CFS $P \geq 0.05$. In fact, the MRS and M17 media contain compounds such as carbohydrates (including glucose and lactose) that can be used by the bacteria to produce various acidic compounds and the significant decrease of the pH of the CFS of most cultures support the production of such compounds Also, during milk fermentation, many LAB have the potential to ferment the substrate carbohydrates, allowing thereby a release of acids such as lactic acid and this is beneficial for the inhibition of undesirable microorganisms that are acid sensitive (Agrawal and Prakash, 2013; Mohammed and Ijah 2013).

The observation that neutralisation of LAB CFS significantly reduces antimicrobial activity has been reported in other studies (Mante *et. al.* 2003; Amoa-Awua *et al.*, 2005). More recently, after screening LAB strains for antimicrobial activity, Owusu-

Kwarteng *et al.* (2015) reported that none of the neutralised CFS from LAB strains studied showed antimicrobial activity against any of the Gram negative pathogens tested. This is the case in the current study of, for example, the test isolates except *Lactobacillus senioris* showed antimicrobial activities against *Bacillus cereus* but there were no significant differences among the treatments in all the indicators $P \geq 0.05$. However, the presence of comparable inhibitory activities in neutralised and non-neutralised CFS in some cases such as *Streptococcus infatarius* and *Lactobacillus senioris* against *E.coli* indicates that inhibition cannot be fully attributed to acid production. In addition, the removal or reduction of inhibition after treatment with proteolytic enzymes in many cases suggests that some of the antimicrobial activities observed are likely due to the action of antimicrobial peptides such as bacteriocins or bacteriocin like inhibitory substances (BLIS). As reported by Fricourt *et al.* (1994) and Piard and Desmazeand, (1992), LAB synthesizes bactericidal agents that vary in their spectra of activity and many of the bactericidal agents synthesized by the LAB were bacteriocins with a proteinaceous active moiety while others were non-protein agents. Heredia-Castro *et al.* (2015) in his study on production of bacteriocin-like inhibitory substances (BLS) by *Lactobacillus* spp. isolated from artisanal Mexican cheese observed that 12 out of 18 isolates were able to produce BLS active against *Staphylococcus aureus*, *Listeria innocua*, *Escherichia coli*, and *Salmonella typhimurium*. Also, Mohammed and Ijah (2013) observed that LAB such as *Lactobacillus bulgaricus, Lactobacillus lactis, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus cremoris, Pediococcus halophilus and Pedioccus cerevisiae* from fermented milk products were potential bacteriocin producers against *Staphylococcus* spp, *Salmonella* spp, *Bacillus* spp, *Shigella* spp and *Pseudomonas* spp.

Although the addition of proteolytic enzymes to the neutralised CFS appeared to decrease the inhibitory effect of the neutralised CFS of some bacteria, this was not observed for the CFS of a few isolates such those of *Lactobacillus senioris* and *Leuconostoc pseudomesenteroides* when used against *E. coli*. This could be due to the fact that no proteinaceous compound was involved in the inhibition process and in this case acid production could be the main antimicrobial factor or all conditions were not met to allow a potential proteinacous compound to exhibit the inhibitory effect appropriately. In fact, protein antimicrobials such as bacteriocins will have a maximum activity within a defined pH range and further work can target studying antimicrobial activity over a range of pH values. When characterising bacteriocin production from two *Enterococcus* spp., Banwo *et al.* (2013) observed a moderate effect on pH on the activity of enterocins from *Enterococcus* spp. This finding was corroborated by observations from Cocolin *et al.* (2007). In addition, LAB isolates could also be screened for genes encoding known bacteriocins to confirm any phenotypic observations made. It also worth noting that a part of acid and protein antimicrobial production, antimicrobial properties of bacteria can be related to substance such hydrogen peroxide.

Table 4.3 Summaries of significant findings including % inhibitions of means of Control, CFS and Neutralized CFS of the isolates against indicator bacteria.

Test isolates	Control no CFS vs. CFS		Control no CFS vs. Neutralised CFS		CFS vs. Neutralised CFS	
	Sig. Summary	P Value	Sig. Summary	P Value	Sig. Summary	P Value
Enterococcus thailandicus	ns	0.541	ns	0.902	ns	0.795
Streptococcus infantarius	ns	0.215	ns	0.934	ns	0.354
Lactobacillus senioris	ns	0.079	ns	0.776	ns	0.238
Lactobacillus fermentum	s	0.035	ns	0.419	ns	0.298
Lactobacillus delbrueckii subsp *indicus*	ns	0.452	ns	0.805	ns	0.818
Leuconostoc pseudomesenteroides	ns	0.267	ns	0.789	ns	0.604
Streptococcus thermophilus	ns	0.997	ns	0.884	ns	0.847

ns= No significant difference $P \geq 0.05$

s= Significant difference $P \leq 0.05$

Table 4.4 Summaries of significant findings including % inhibitions of means of Neutralized CFS, Neutralised CFS +Proteinase K and Neutralized CFS + Protease of the isolates against indicator bacteria.

Test isolates	Neutralized CFS vs. Neutralised CFS +Proteinase K		Neutralized CFS vs. Neutralised CFS +Protease		Neutralised CFS +Proteinase K vs. Neutralised CFS +Protease	
	Sig. Summary	P Value	Sig. Summary	P Value	Sig. Summary	P Value
Enterococcus thailandicus	ns	0.987	ns	0.487	ns	0.576
Streptococcus infantarius	ns	0.999	ns	0.784	ns	0.807
Lactobacillus senioris	ns	0.768	ns	0.517	ns	0.906
Lactobacillus fermentum	ns	0.947	ns	0.188	ns	0.300
Lactobacillus delbrueckii subsp *indicus*	ns	0.782	ns	0.315	ns	0.681
Leuconostoc pseudomesenteroides	ns	0.962	ns	0.537	ns	0.695
Streptococcus thermophilus	ns	0.999	ns	0.815	ns	0.799

ns= No significant difference $P \geq 0.05$

s= Significant difference $P \leq 0.05$

4.5 Conclusion and further work

Seven strains of LAB namely *Lactobacillus fermentum, Lactobacillus senioris, Lactobacillus delbruckii* subsp *indicus, Enterococcus thailandicus*, *Leuconostoc pseudomesenteroides, Streptococcus infantarius* and *Streptococcus thermophilus* were tested against five indicator bacteria. The test isolates showed varying inhibitory effect against the indicators screened although some isolates exhibited higher inhibition activity compared to others against specific indicators. For example, in the spot test, *Lactobacillus fermentum* exhibited the highest inhibition activity 11 – 40 mm against both Gram positive and Gram negative indicator bacteria. This could be an advantage in food safety and a potential probiotic property related to the inhibition of intestinal pathogens. *Streptococcus thermophilus* exhibited the least 0-10 mm inhibitions potential. The study also revealed that the antimicrobial activity of the bacteria can be related to various factors such as competition for nutrients, acid production and production of antimicrobial peptides indicating a strong potential of some of the LAB studied to control foodborne pathogens in food by various means.

The CFSs of the test isolates exhibited strong to moderate antimicrobial activities against *Bacillus cereus*. *Bacillus cereus* is a food poisoning causative agent and is frequently found in raw and unprocessed food product such as milk products, meat and vegetables. The ability of the LAB from *Nono* to produce antimicrobial agents against *Bacillus cereus* is beneficial in assuring food safety and biopreservation. Also, the fact that the CFS of most of test strains exhibited antilisterial activities and inhibition potential toward *Staphylococcus aureus* is likely to help control these pathogens in fermented milk products.

Chapter Five: Screening of the antimicrobial resistance profile and genetic basis of LAB from *Nono*

5 Introduction

One of the selection criteria for starter cultures and probiotics is that they are free of transferable antimicrobial resistance genes for safety reasons. This is an important factor to consider as when ingested, the bacteria may transfer their resistance elements to pathogenic bacteria. If pathogenic bacteria acquired one or multiple resistances to antimicrobials, infections caused by such multi-resistant bacteria become difficult to treat and lead to death in many cases.

For various reasons, a microorganism can become resistant to an antimicrobial to which it was originally susceptible to. Development of antimicrobial resistance can be linked to environmental and/or humans factors including e.g. misuse or overuse of antimicrobials, inadequate antimicrobial prescription (not prescribing the required dose), and unnecessary use of antimicrobials in animal production (Macovei and Zurek, 2006; Hummel *et al.*, 2007). Some microorganisms that contain specific resistance genes are able to transfer the genetic elements to other bacteria. Thus, antimicrobial resistance can be acquired. Horizontal transfer or conjugation is one of the mechanisms by which genes are transferred across species or genus via genetic elements such as plasmids, transposons and integrons (Courvalin 1994; Gever *et al.*, 2003; Jahan *et al.*, 2015). Plasmids are circular and non-essential extra-chomosomal fragments of DNA that replicate with different degree of autonomy from chomosomal DNA. They can be composed of series of genetic sequences encoding different functions (Carattoli, 2008; Espinosa *et al.*, 2005). Conjugative transposons are mobile DNA elements that encode all necessary functions for intracellular transposition and intercellular conjugation. They can be located in a wide variety of Gram negative and Gram positive bacteria and are very important in spreading

antibiotic resistance genes (Scot 2002). Horizontal transfer of antimicrobial resistance genes from the gastrointestinal tracts of domestic animals to the gastrointestinal tract of humans is still a controversial subject because the ecology and horizontal transfer of antimicrobial resistance determinants in the environment are poorly understood (Macovei and Zurek 2006). Another way of acquiring antimicrobial resistance is though genetic mutations where one or several base pairs of DNA are substituted by other base pairs or deleted or inserted in the genome (Howden *et al.*, 2006; Patel *et al.*, 2012).

Both phenotypic and genotypic methods are used to screen antimicrobial resistance. Approved standard method by the Clinical Laboratory Standards Institutes (CLSI) is one of the best phenotypic methods for susceptibility testing using e.g. agar dilution, broth micro-dilution and Etest (predefined gradient of antibiotic concentrations on plastic strips). Genotypic methods include different PCR-based methods such as traditional PCR, microarray, southern hybridization and plasmid profiling (Honore and Cole 1994; Sreevatsan *et al.*, 1996; Lipin *et al.*, 2007; Ammor *et al.*, 2008). Phenotypic and genotypic methods work together to give a clear determination of whether a strain is resistant or susceptible to an antimicrobial. However, a bacterium can be genotypically susceptible while it is phenotypically resistant. This is due to the fact that, for example, some genes were not screened during the investigation, or the bacterium possesses an intrinsic resistance to a particular antimicrobial that is not gene related.

With regards to antimicrobial resistance in LAB, many studies have demonstrated the role of beneficial LAB as reservoirs for antibiotic resistance genes and their

potential transferability though, a few data report *in vivo* occurrence of gene transfer from clinical and food isolates (Vignaroli *et al.*, 2011; Jahan *et al.*, 2015).

5.1 Aim and Objectives

The main aim of this study was to investigate and determine the antimicrobial resistance patterns of LAB involved in the traditional fermentation of milk for *Nono* production, especially in relation to transferable antimicrobial resistance genes. To achieve this aim, specific objectives composed of different activities were targeted including:

- Determination of antibiotic susceptibility profile of selected LAB by screening minimal inhibitory concentration (MIC)

- Investigation of the presence of various resistance genes for the most common antimicrobials by PCR and using specific primers

- Determination of the transferability of the resistance genes by *in vitro* conjugation experiments using specific donors and recipients

- Confirmation of the transfer by screening the presence of specific genes in potential transconjugants (recipients that have received a specific gene from the donor) using PCR

- Determination by PCR of genetic elements such as plasmids and transposons that may have facilitated a gene transfer

5.2 Material and Method

5.2.1 Microorganisms

Seven out of 11 isolates were selected for this study. The selection of these isolates was based on differences in their rep-PCR profiles described in chapter 2 and they are listed in Table 5.1.

Table 5. 1 Lactic acid bacteria studied

Isolate Codes	Species name
52	*Enterococcus thailandicus*
10	*Streptococcus infantarius*
43	*Lactobacillus senioris*
13	*Lactobacillus fermentum*
11	*Lactobacillus delbrueckii subsp indicus*
9	*Leuconostoc pseudomesenteroides*
73	*Streptococcus thermophilus*

5.2.2. LAB inoculum preparation

After two consecutive sub cultures of isolates on MRS agar, a single colony was suspended in 1 ml of sterile Maximum Recovery Diluent (MRD) (Oxoid, Basingstoke, UK). The suspension was used to prepare an inoculum (in 5 ml MRD) with a final cell concentration of 10^7-10^8 CFU/ml (equivalent to 0.5 McFarland standard) using a sensitre nephelometer (TREK Diagnostic systems, West Sussex, UK).

5.2.3. Determination of Minimal Inhibitory Concentration (MIC) of the isolates

The MIC for 18 antimicrobials was determined using 96 well Sensititre NARMS plates (TREK Diagnostic Systems Ltd, West Sussex, UK) containing varying concentrations of each antimicrobial. An inoculum of each LAB was prepared as described above and 100 µl of the bacterium suspension was mixed with 20 ml MRS broth (Oxoid). This was followed by the inoculation of 50 µl the later mixture into the antimicrobial sensititre plate which was sealed, labelled and incubated anaerobically for 48 h at 37°C. After the incubation period, the MIC for each antimicrobial was determined using a sensititre magnifier mirror where visible bacteria growth as well as an absence of growth could be observed. The susceptibility of the isolates to each antimicrobial was established using the breakpoints described in Table 5.2.

Table 5. 2 Proposed breakpoints for determining of the antimicrobial susceptibility profile of the LAB (ECHCPDG; 2003; EFSA, 2008; EUCAST, 2018; Florez *et al.*, 2016; Klayraung *et al.*, 2008; Ouoba *et al.*, 2008)

Antibiotics	*Enterococcus thailandicus*	*Streptococcus infantarius*	*Lactobacillus senioris*	*Lactobacillus fermentum*	*Lactobacillus delbrueckii subsp indicus*	*Leuconostoc pseudomesenteriodes*	*Streptococcus thermophilus*
Ampicillin	4	1	4	1	1	2	2
Ceftriaxone	2	2	2	2	2	2	2
Clindamycin	4	1	1	1	1	1	2
Ciprofloxacin	4	4	4	4	4	4	4
Daptomycin	4	1	4	4	4	4	1
Erythomycin	4	1	1	1	1	1	2
Gatifloxacin	≥8	≥8	≥8	≥8	≥8	≥8	≥8
Gentamicin	32	16	16	16	16	16	32
Levofloxacin	4	4	4	4	4	4	4
Linezolid	≥8	≥8	≥8	≥8	≥8	≥8	≥8
Oxacillin+2%NaCL	8	8	8	8	8	8	8
Penicillin	4	4	4	4	4	4	4
Quinupristin+dalphopristin	4	4	4	4	4	4	4
Rifampin	4	4	32	32	32	4	4
Streptomycin	128	64	64	64	16	64	64
Tetracycline	2	8	8	8	4	8	4
Trimethoprim	32	32	32	32	32	32	32
Vancomycin	4	2	2	2	2	2	4

5.2.4 Detection of resistance genes for specific antimicrobials

Antimicrobial resistance in the isolates were further investigated by PCR for the presence of specific genes using PCR and the primers described in Table 5.4. A positive control isolate was included where possible (Table 5.3). The positive control isolates were provided by the National Food Institute, Denmark Technical University (Denmark). For antimicrobials to which a tested bacterium had reduced susceptibility (resistant), PCR was performed for well-known genes for these antimicrobial.

5.2.4.1 Extraction of total DNA

Each isolate was cultured for 48 h on the same medium from which they were enumerated (MRS or M17). A pure colony was sub-cultured on agar and incubated for 48 h anaerobically at 37°C. The DNA of a pure colony was extracted using the Instagene matrix (Bio-Red 732-6030, Hercules, CA, USA) according to the manufacturer's instructions. The extracted DNA was stored in an Eppendorf tube at -20°C until required for further analysis.

5.2.4.2 Amplification of the genes by PCR

For the detection of tetracycline, chloramphenicol, vancomycin, gentamycin, kanamycin and erythomycin resistance genes, the following reaction mixture was used according to Ouoba *et al.* (2008b). High purity water (Sigma,W4502, Poole, UK): 41.4 µl, 10×PCR buffer with (25mmoll^{-1}) $MgCl_2$ (Applied Biosystems, 58002067-01, Paisley, UK): 5 µl, dNTP(1:25 mmol l^{-1}) Promega, U151A, UK): 0.5 µl, Primer 1 (21 pmol/µl): 0.5 µl, Primer 2 (21 pmol/µl): 0.5 µl, Taq DNA Polymerase (5U) (Applied Biosystems, N11912, UK): 0.1 µl, DNA: 2 µl.

For screening streptomycin resistance genes *strA* and *strB* the following mixture was used: high purity water (Sigma, W4502): 39.4 µl, 10×PCR buffer with (25mmoll^{-1}) $MgCl_2$ (Applied Biosystems, 58002067-01) : 5 µl, dNTP (1:25 mmol l^{-1}) (Promega, U151A): 0.5 µl, $MgCl_2$(25mmoll^{-1}) (Applied Biosystems, LP0693, UK): 2 µl, Primer 1 (21 pmol/µl): 0.5 µl, Primer 2 (21 pmol/µl): 0.5 µl, Taq DNA Polymerase (5U) (Applied Biosystems, N11912): 0.1 µl, DNA: 2 µl. The reaction mixture for determination of the streptomycin resistance gene *aadA* was as follows: high purity water (Sigma,W4502): 40.9 µl, 10×PCR buffer with $MgCl_2$ (25mmoll^{-1}) (Applied Biosystems, 58002067-01): 5 µl, dNTP (1:25 mmol l^{-1}) Promega, U151A): 0.5 µl, $MgCl_2$ (25mmoll^{-1}) Applied Biosystems, LP0693) : 0.5 µl, Primer 1 (21 pmol/µl): 0.5 µl, Primer 2 (21 pmo l/µl): 0.5 µl, Taq DNA Polymerase (5U) (Applied Biosystems, N11912): 0.1 µl, DNA: 2 µl. PCR mixture for investigating the resistance gene *aadE* for streptomycin and *Blaz* for penicillin was as followed: high purity water (Sigma,W4502): 38.4 µl, 10×PCR buffer with 15 mM $MgCl_2$ (Applied Biosystems, 58002067-01): 5 µl, dNTP (1.25 mmol l^{-1}) (Promega, U151A): 0.5 µl, $MgCl_2$ (25 mmol l^{-1}) (Applied Biosystems, LP0693) : 3 µl, Primer 1 (21 pmol/µl): 0.5 µl, Primer 2 (21 pmo l/µl): 0.5 µl, Taq DNA Polymerase (5U) (Applied Biosystems, N11912): 0.1 µl, DNA: 2 µl.

All PCR amplifications were performed in a T2700 Thermocycler (GeneAmp PCR 2700 system, Applied Bio system Singapore) using the following cycle condition: Initial denaturation at 94°C for 3 min, 25 or 35 cycles of 94°C for 1 min, annealing (45-65°C) Table 5.3, for 1min, 72°C for 1 min and a final extension step at 72°C for 10 min. Gel electrophoresis was used to evidence the presence of positive amplicons from the PCR by loading 10µl of PCR products on 1.5 % agarose gel. The

gels were staining in ethidium bromide staining, distained in water, visualize under a UV light and photographed.

5.2.4.3 Sequencing of the resistance genes

Positive amplicons for some tetracycline genes including *tet*(S), *tet*(M), *tet*(O) and streptomycin genes including *str*(A) and *aad*(E) were purified using QIAquick PCR Purification kit (Qiagen GmbH, Hilden, Germany) and sequenced (Source Bioscience, Cambridge, UK) using the same primers (Table 5.5) at a concentration of 3.2 pmol/µl. The sequences were analysed in Genebank/BLAST and the identity of the gene confirmed.

5.2.5. Transferability of the resistance genes (*in vitro* conjugation)

When the identity of the gene was confirmed, conjugation experiments were carried out as described by Ouoba *et al.* (2008b) to investigate the ability of the isolates to transfer the genes to other bacteria. The study involved two donors: *Enterococcus thailandicus* (52) which carried both the *tet*(S) and *tet* (M) genes encoding resistance to tetracycline and the *aad*(E) gene encoding resistance to streptomycin and *Streptococcus infantarius* (10) which carried both the *tet*(S) and *tet*(M) genes encoding resistance to tetracycline. For the transferability of the resistance genes, *Enterococcus faecalis* JH2-2 was used as a recipient. Donors and recipients were subcultured twice at 37°C on MRS (donor) or BHI (recipient) agars. A single colony was transferred to MRS broth or BHI and the culture were incubated anaerobically (LAB) and aerobically (*Enterococcus faecalis)* for 6h (mid-exponential growth phase) at 37°C. The cultures (1 ml) were centrifuged (1200 rpm for 3 min) and the pellet re-suspended in 1 ml sterile MRD. The suspension was used to prepare an inoculum at a final concentration of 10^8 cfu/ml, (McFarland standard). Inocula from

donors and recipients were mixed at a ratio of 9:1 (9 ml of the donor and 1ml of the recipient) and filtered though a sterile membrane filter (0.45 μm) (Whatman Laboratory Division, Maidstone, UK) using a filter holder (Whatman) and a vacuum pump (Welch Thomas, Model No. 2522C-02, Skokie, Illinois, USA). Further, the filters containing the mixed bacteria were incubated aerobically on BHI agar at 37 °C for 48h (maximum growth conditions for the recipient). Another method of conjugation was carried out by mixing 9ml of the donor and 1ml of the recipient without filtration: 100 μl of the mixture was inoculated on a BHI agar plate and incubated for 5 days at the optimum growth conditions of the recipient. After 48 h of incubation, colonies were washed off the filters or agar plates with 2 ml of MRD, diluted (up to 10^{-4}) and inoculated on BHI plates containing different combinations of antimicrobials as described below.

Transconjugants (potential recipients that have acquired a resistance gene from a donor) were recovered on agar containing the antimicrobial to which the donor is resistant to but the recipient sensitive to (tetracycline) and antimicrobials to which the recipient is resistant to and the donor sensitive to (rifampicin and fusidic acid). Each dilution (100 μl) was spread onto BHI-RFT [rifampicin (25 μg/ml), fusidic acid (25 μg/ml) and tetracycline (10 μg/ml)], BHI-RF [rifampicin (25 μg/ml) and fusidic acid (25 μg/ ml)] and BHI-T [(tetracycline (10 μg/ml)] agar plates. BHI agar plates without antimicrobials were used as controls.

5.2.6. Confirmation of the transconjugants using MIC determination and Rep-PCR

The MIC of the transconjugants for tetracycline and streptomycin was determined as described above and compared with those of the donors and recipient. A resistance

transfer is characterized by an increased MIC in the transconjugants. Moreover, the DNA fingerprint as determined by rep-PCR was compared to those of the donors and recipients using the GTG5 primer as described by Ouoba *et al.* (2008a).

5.2.7 Determination of the presence and location of the tetracycline and streptomycin genes in the potential transconjugants

5.2.7.1 Extraction of total DNA

Each transconjugant was cultured for 48 h on the same medium from which they were enumerated. A pure colony was sub-cultured on agar and incubated for 24 h aerobically at 37°C. The DNA of a pure colony was extracted using the Instagene matrix (Bio-Red 732-6030, Hercules, CA, USA) according to the manufacturer's instructions. The extracted DNA was stored in an Eppendorf tube at -20°C until required for further analysis.

5.2.7.2 Extraction of plasmid DNA

Each transconjugant was cultured for 48 h on BHI agar. A pure colony was then transferred into 10 ml BHI broth and incubated in shaking water bath (133 s/min Grant OLS 200) at 37°C for 12 h. Plasmid DNA were extracted using the QIAGEN Plasmid miniprep Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted plasmid DNA samples were stored in an Eppendorf tube at -20°C until required for further analysis. Plasmid DNA was extracted and used for the amplification to increase the chance to evidence a potential transfer should the gene be located on a plasmid and present in low number of copies.

5.2.7.3 Amplification and sequencing of the genes

To check if the *tet*(S), *tet*(M) and *aad*E genes was transferred into the potential transconjugants, they were amplified using the methods described in section 5.2.4.2. Total DNA samples were first used for the amplification. But when no amplicon was obtained, the plasmid DNA samples were used. After running a gel electrophoresis to evidence the presence of amplicons, the positive PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and following the manufacturer's instructions. Further, they were sequenced using the corresponding primers at a concentration of 3.2pmol/µl. The identity of the genes was confirmed by comparing the sequences to those in the GenBank database using the BLAST.

5.2.8 Determination of the presence transposons

Similarly to plasmids, transposons are mobile genetic elements that can be involved in antimicrobial resistance gene transfer. Thus, the presence of conjugative transposons Tn916 and Tn1545 known to be involved in tetracycline resistance was screened in the donors, recipient and potential transconjugants using the primers depicted in Table 5.4 and the PCR mixture for tetracycline described in section 5.2.4.2. Gel electrophoresis was run by loading 10µl of PCR products on 1.5% agarose gel. The gel was staining in ethidium bromide, distained in water, visualizes under a UV light and photographed.

Table 5. 3 Reference strains used as positive controls for the detection of antimicrobial resistance genes

Bacteria*	**Related genes**
Staphylococus rissem 7522486-1	*aph*(3'')-I
Entrococus faecalis	*aad*E
Salmonella Typhimurium	*aad*A
Staphylococus aureus RN422	*erm*(C)
Enterococus faecalis JH2-2	*erm*(B)
Staphylococus aureus Tn554	*erm*(A)
Staphylococus aureus PSTS 9-like	*tet*(L)
Staphylococus aureus PT181-like	*tet*(K)
Staphylococus intermedius 2567	*tet*(M)
Escherichia coli	*tet*(Q)
Listeria monocytogenes BM4210/PIP811	*tet*(S)
Escherichia coli K2	*ant*(2'')-I
Enterococcus JH2-1-5	*aph*(3'')-III
Escherichia coli	*tet*(W)
Enterococcus faecium BM4147	*Van* A
*Enterococcus faecalis*V583	*Van* B
Enterococcus faecium UW6605	*Van* X
Enterococcus faecium JH2-2 PSP501, Cat 501	*Cat* 501
Escherichia coli K-B	*aac*(3")IV
No positive control	*tet* (O), *Str* A, *Str* B, *aac*(6')*aph*(2"), cmlA, CatA1

* The bacteria were kindly provided by the National Food Institute, Technical University of Denmark.

Table 5. 4 Primers used for the amplification of the resistance genes (Gevers *et al.*, 2003; Ouoba *et al.*, 2008b).

Antimicrobials	Resistance genes	Primers	Annealing temperature(°C)
Tetracycline	*tet*(M)	5'-GTT AAA TAG TGT TCT TGG AG-3' 5'-CTA AGA TAT GGC TCT AAC AA-3'	45°C
	tet(L)	5'-GTT GCG CGC TAT ATT CCA AA-3' 5'-TTA AGC AAA CTC ATT CCA GC-3'	54°C
	tet(S)	5'-TGG AAC GCC AGA GAG GTA TT-3' 5'-ACA TAG ACA AGC CGT TGA CC-3'	55°C
	tet(Q)	5'-ATG TTC AAT ATC GGT ATC AAT GA-3' 5'-GCG GAT ATC ACC TTG CTT C-3'	55°C
	tet(K)	5'-TTA GGT GAA GGG TTA GGT CC-3' 5'-GCA AAC TCA TTC CAG AAG CA-3'	55°C
	tet(O)	5'-GAT GGC ATA CAG GCA CAG AC-3' 5'-CAA TAT CAC CAG AGC AGG CT-3'	55°C
	tet(W)	5'-GCCATCTTGGTGATCTCC-3' 5'-TGGTCCCCTAATACATCGTT-3'	55°C
Kanamycin	*aph*(3'')-I	5'-AAC GTC TTG CTC GAG GCC GCG-3' 5'-GGC AAG ATC CTG GTA TCG GTC TGC G-3'	68°C
	aph(3'')-III	5'-GCC GAT GTG GAT TGC GAA AA-3' 5'-GCT TGA TCC CCA GTA AGT CA-3'	52°C
Gentamycin	*ant*(2'')-I	5'-GGG CGC GTC ATG GAG GAG TT-3' 5'-TAT CGC GAC CTG AAA GCG GC-3'	67°C
	aac(6')aph(2'')	5'-CCA AGA GCA ATA AGG GCA TA-3' 5'-CAC TAT CAT AAC CAC TAC CG-3'	48°C
	aac(3'')IV	5'-GTG TGC TGC TGG TCC ACA GC-3' 5'-AGT TGA CCC AGG GCT GTC GC-3'	63°C
Streptomycin	*str*A	5'-CTT GGT GAT AAC GGC AAT TC-3' 5'-CCAATCGCAGATAGAAGGC-3'	55°C
	*str*B	5'-ATC GTC AAG GGA TTG AAA CC-3' 5'-GGA TCG TAG AAC ATA TTG GC-3'	56°C

Table 5.4 (contd) Primers used for the amplification of the resistance genes (Gevers *et al.*, 2003; Ouoba *et al.*, 2008b).

Antimicrobials	Resistance genes	Primers	Annealing temperature(°C)
Streptomycin	*aad*A	5'-ATC CTT CGG CGC GAT TTT G-3' 5'-GCA GCG CAA TGA CAT TCT TG-3'	56°C
	*aad*E	5'-ATG GAA TTA TTC CCA CCT GA-3' 5'-TCA AAA CCC CTA TTA AAG CC-3'	50°C
Erythomycin	*erm*(A)	5'-AAG CGG TAA AAC CCC TCT GAG-3' 5'-TCA AAG CCT GTC GGA ATT GG-3'	55°C
	erm(B)	5'-CAT TTA ACG ACG AAA CTG GC-3' 5'-GGA ACA TCT GTG GTA TGG CG-3'	52°C
	erm(C)	5'-CAA ACC CGT ATT CCA CGA TT-3' 5'-ATC TTT GAA ATC GGC TCA GG-3'	48°C
Vancomycin	*Van*(A)	5′-AAC AAC TTA CGC GGC ACT-3′ 5′-AAA GTG CGA AAA ACC TTG -3′	55°C
	Van(B)	5′-GAT ATT CAA AGC TCC GCA GC-3′ 5′-TGA TGG ATG CGG AAG ATA CC-3′	55°C
	Van(X)	5′-TGCGATTTTGCGCTTCATTG -3′ 5′-ACTTGGGATAATTTCACCGG -3′	55°C
Chloramphenicol	*cml*A	5′-TACTCGGATCCATGCTGGCC -3′ 5′-TCCTCGAAGAGCGCCATTGG -3′	65°C
	Cat 501	5′-GGATATGAAATTTATCCCTC -3′ 5′- CAATCATACCCTATGAAT-3′	47°C
	*Cat*1	5′-CGCCTGATGAATGCTCATCCG -3′ 5′- CCTGCCACTCATCGCAGTAC -3′	60°C
Penicillin	*Blaz*	5′-CAGTTCACATGCCAAAGAG -3′ 5′- TACACTCTTGGCGGTTTC -3′	54°C
Transposon	Tn916-1545	5′- GCGTGATTGTATCTCACT-3′ 5′- GACGCTCCTGTTGCTTCT-3′	50°C
	Tn916	5′- GGCTGTCGCTGTAGGATAGAG-3′ 5′- GGGTACTTTTAGGGCTTAGT-3′	50°C

5.3 Results

5.3.1 MIC determination

The MIC values including the susceptibilities of the LAB to various antimicrobial are described in Table 5.5. The isolates were all susceptible to ampicillin, ceftriaxone, quinupristin/dalfopristin, oxacillin+2%NaCl, trimethoprim/sulfamethoxazole and rifampicin. Resistance to the antibiotics was variable according to the isolate and the antimicrobial. For instance, among all the seven isolates tested, four including isolate 52, 10, 43, and 13 were resistant to tetracycline and erythomycin while the other thee were susceptible to the same antibiotics. Also, isolates 9, 11, 13, 43, 10 and 52 showed resistance to daptomycin and levofloxacin and were susceptible to penicillin whereas isolate 73 was susceptible to daptomycin and levofloxacin and resistant to penicillin.

5.3.2 Determination of resistance genes

The determination of resistance genes by PCR revealed positive amplicons for the *tet*(S) and *tet*(M) genes encoding resistance to tetracycline and also the *aad*(E) gene encoding resistance to streptomycin (Figure 5.1 and Table 5.6). No positive amplicon was obtained for the rest of the genes screened. Out of seven isolates, two showed a positive PCR for resistance genes. These include isolates 52 and 10 which both exhibited the *tet*(S) and *tet*(M) genes. In addition 52 also exhibited the *aad*(E) gene. The sequencing of the positive amplicons confirmed the identity of the genes detected (99-100% similarity).

Table 5. 5 Minimal Inhibitory Concentration of lactic acid bacteria from *Nono* for various antimicrobials

Antimicrobial	Isolate/MIC (µg/ml)*						
	Enterococcus thailandicus	*Streptococcus infantarius*	*Lactobacillus senioris*	*Lactobacillus fermentum*	*Lactobacillus delbrueckii*	*Leuconostoc pseudomesenteriodes*	*Streptococcus thermophilus*
Ampicillin	≤0.12s	≤0.12s	0.5s	0.25s	≤0.12s	0.25s	≤0.12s
Ceftriaxone	<8s	<8s	<8s	<8s	<8s	<8s	<8s
Clindamycin	>2r	≤0.12s	2r	≤0.12s	2r	≤0.12s	2s
Ciprofloxacin	4s	8r	≥16r	≥16r	≥16r	8r	2s
Daptomycin	>8r	8r	>8r	>8r	>8r	>8r	1s
Erythomycin	>4r	4r	>4r	>4r	1s	1s	≤0.25s
Gatifloxacin	2s	2s	8r	>8r	8r	≤2s	≤ 1s
Gentamycin	64r	16s	32r	32r	64r	8s	16s
Levofloxacin	8r	8r	>8r	>8r	>8r	8r	2s
Linezolid	4s	4s	4s	8r	2s	2s	2s
Oxacillin+2%NaCL	2s	2s	2s	1s	≤0.25s	0.5s	≤0.25s
Penicillin	≤0.06s	≤0.06s	0.5s	0.12s	≤0.06s	≤0.06s	>8r
Quinupristin/Dalfopristin	4s	≤0.12s	4s	1s	1s	0.5s	2s
Rifampin	2s	≤0.5s	≤0.5s	≤0.5s	≤0.5s	≤0.5s	≤0.5s
Streptomycin	512r	64s	256r	256r	32r	64s	>32s
Tetracycline	64r	32r	16r	16r	≤2s	≤2s	≤2s
Trimethoprim/Sulfamethoxazole	>4/76s	2/28s	>4/76s	>4/76s	>4/76s	>4/76s	>4/76s
Vancomycin	2s	≤1s	>128r	>128r	≤1s	>128r	16r

*MIC (µg/ml):- r: resistant, s: sensitive, according to the proposed breakpoints mentioned in Table 5.1

Table 5. 6 Antimicrobial resistance genes detected in the LAB isolates

Antimicrobial	Isolate/Resistance genes						
	Enterococcus thailandicus	*Streptococcus infantarius*	*Lactobacillus senioris*	*Lactobacillus fermentum*	*Lactobacillus delbrueckii* subsp *indicus*	*Leuconostoc pseudomesenteriodes*	*Streptococcus thermophilus*
Chloramphenicol	-	-	-	-	-	-	-
Erythomycin	-	-	-	-	-	-	-
Gentamycin	-	-	-	-	-	-	-
Kanamycin	-	-	-	-	-	-	-
Penicillin	-	-	-	-	-	-	-
Streptomycin	*aad*(E)	-	-	-	-	-	-
Tetracycline	*tet*(S)/ *tet*(M)	*tet*(S)/ *tet*(M)	-	-	-	-	-
Vancomycin	-	-	-	-	-	-	-

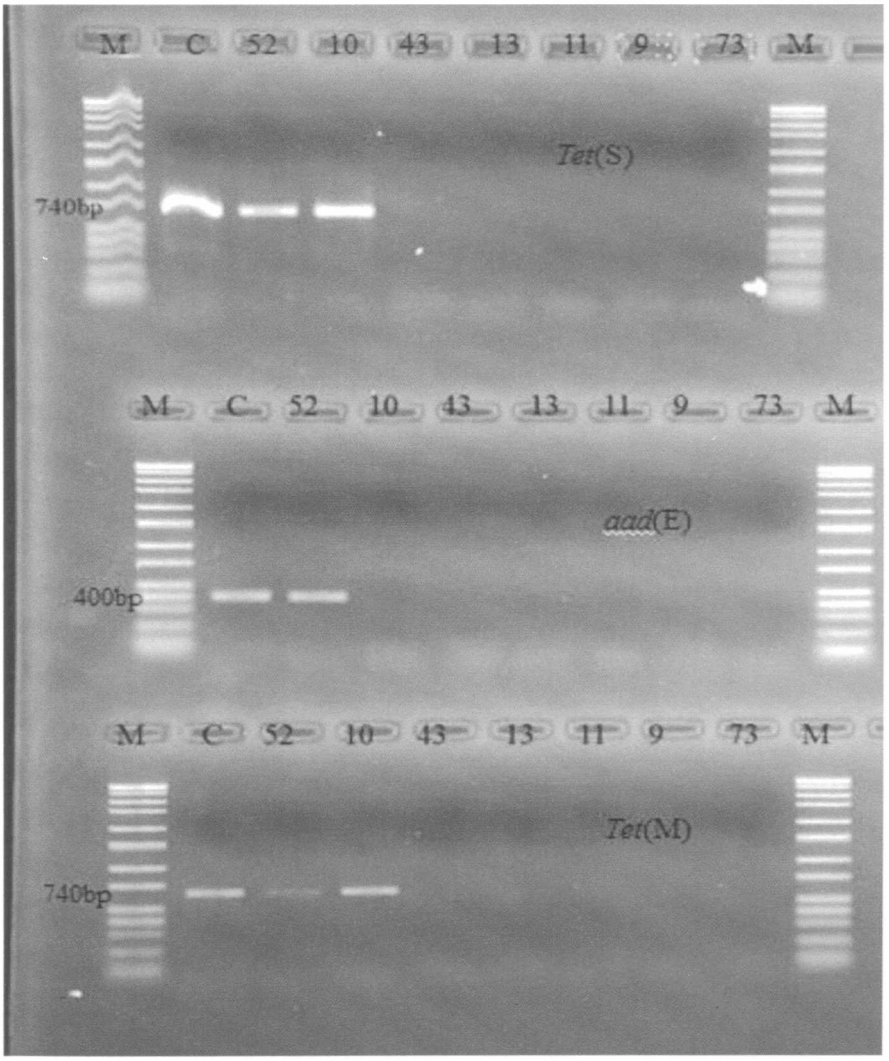

Figure 5. 1 Resistance genes detected by PCR

M: Maker, C: Positive control of the gene screened, 52: *Enterococcus thailandicus*, 10: *Streptococcus infantarius*, 43: *Lactobacillus senioris*, 13: *Lactobacillus fermentun*, 11: *Lactobacillus delbrueckii subsp indicus*, 9: *Leuconostoc pseudomesenteroides* and 74: *Streptococcus thermophilus*

5.3.2 *In vitro* conjugation experiments for the transfer of *tet*(S), *tet*(M) and *aad*(E) genes

A growth of potential transconjugants was observed after thee weeks of incubation on the selective medium (BHI-RF-T). A total of 15 isolates were recovered. Eight transconjugants were obtained from the mating between *Enterococcus thailandicus*

52 and *Enterococcus faecalis* JH2-2 (thee transconjugants from filtered mating and five from unfiltered mating). Seven transconjugants were recovery from the mating between *Streptococcus infantarius* 10 and *Enterococcus faecalis* JH2-2 (two from filtered mating and five from unfiltered mating). It was observed that the MIC for the recipient *Enterococcus faecalis* JH2-2 was $\leq$ 1 μg/ml for tetracycline while the transconjugants exhibited an increased MIC of 8-64 μg/ml which is similar to those of the both donors (32-.64 μg/ml). For streptomycin, the transconjugants exhibited an MIC range of 256-512 μg/ml which similar to those of the donor *Enterococcus thailandicus* (512 μg/ml) and the recipient (512 μg/ml). The rep-PCR profile of the transconjugants showed the same profile as the recipient *Enterococcus faecalis* JH2-2 (Fig 5.2).

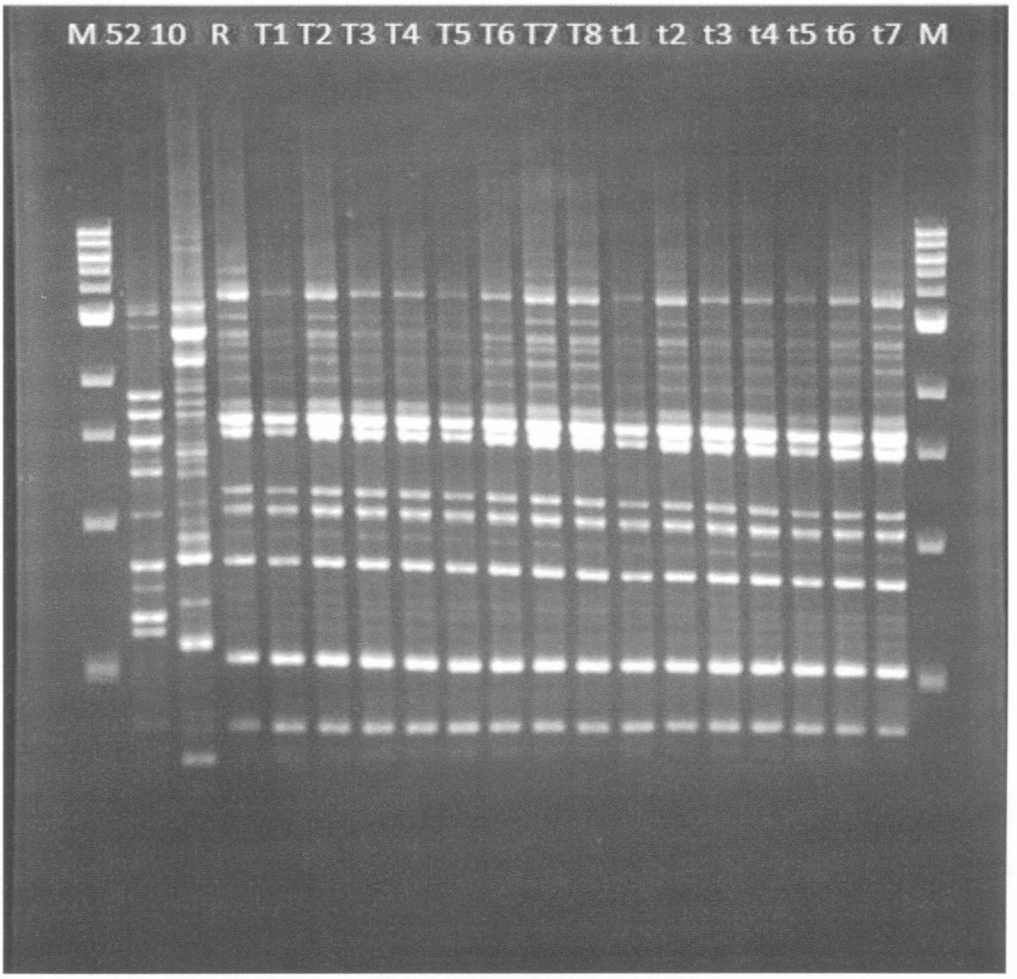

Figure 5. 2 Rep-PCR profile of the transconjugants

M: Maker, 52: *Enterococcus thailandicus*, 10: *Streptococcus infantarius*, R: Recipient (*Enterococcus Faecalis* JH2-2), T1-T8 and t1-t7: *Enterococcus Faecalis* JH2-2 transconjugants from 52 and 10

5.3.3 Determination of the presence of the resistance genes in the potential transconjugants

Using the total DNA samples, positive amplicons for the *tet* (M) and *tet* (S) genes were only obtained for the donors, but not the recipient and the potential transconjugants. Also, the *aad*E gene was amplified in the donor *Enterococcus thailandicus* 52 as expected. However, when the plasmid DNA samples were used, the donors and many potential transconjugants exhibited two of the genes screened, but not the recipient (Fig 5.3 and 5.4). Transconjugants T1, T2, T3, T4, T6, T7 from the mating with *Enterococcus thailandicus* (52) showed positive amplicons for both

the *tet(*S) and *aad*(E) genes. Transconjugant T5 showed a positive amplicon for *tet*(S) only whereas T8 did not exhibit positive amplicons for any of the genes screened. Moreover, the *tet*(M) gene was not evidenced in any of the transconjugants. PCR of the plasmid DNA samples of the transconjugants from the mating with the donor *Streptococcus infantarius* (10) did not generate positive amplicons for the genes screened except for one isolate (t2) where a faint band occurring at the same size as the *tet*(S) gene amplicon was seen (data not shown).

Analysis of the sequences of the positive amplicons for the *tet*(S) and *aad*(E) genes obtained from the transconjugants showed high similarities (98-100%) with sequences of the same genes present in the GenBank database.

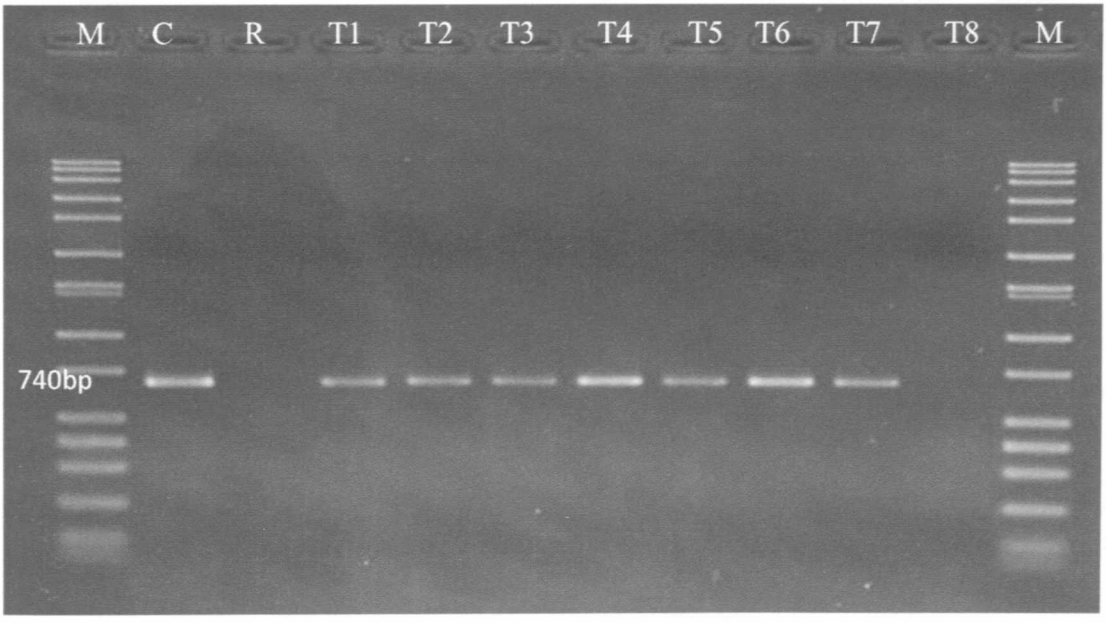

Figure 5. 3 *tet*(S) positive amplicons obtained the donor (*Enterococcus thailandicus)* and *Enterococcus Faecalis* JH2-2 transconjugants (recipients that have received the tet (S) gene)

M: Maker, C: Positive control of the gene screened, D: Donor, R: Recipient (*Enterococcus Faecalis* JH2-2), T1-T8: *Enterococcus Faecalis* JH2-2 transconjugants.

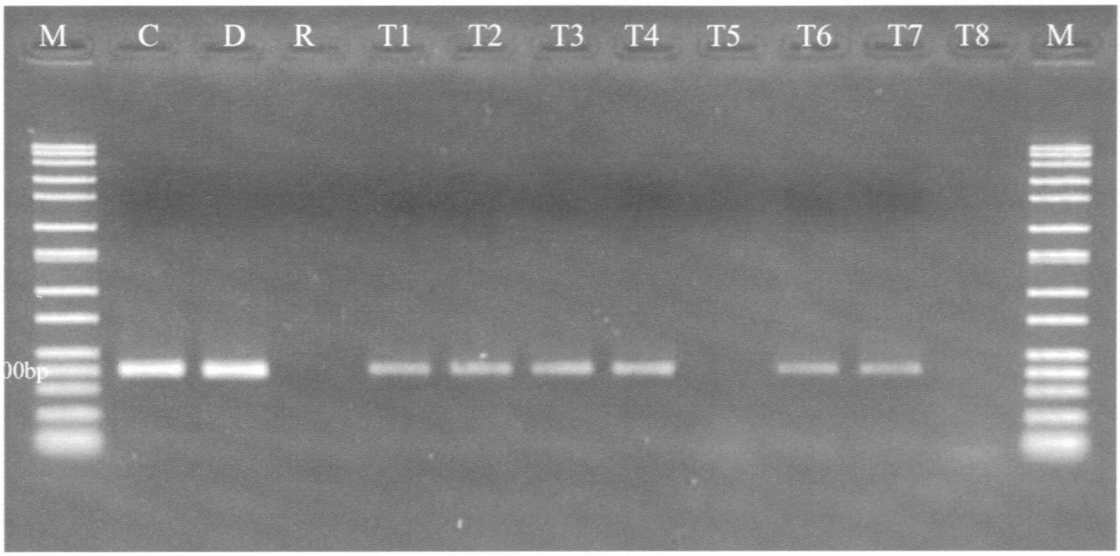

Figure 5. 4 *aad*(E) positive amplicons obtained from the donor (*Enterococcus thailandicus*) and *Enterococcus Faecalis* JH2-2 transconjugants (recipients that have received the aad(E) gene)

M: Maker, C: Positive control of the gene screened, D: Donor, R: Recipient (*Enterococcus Faecalis* JH2-2), T1-T8: *Enterococcus Faecalis* JH2-2 transconjugants.

5.3.4 Determination of the presence of specific transposons in the donors, recipient and transconjugants

The screening of the presence of transposons *Tn916* and *Tn1545* did not yield any positive results whether in the donors, the recipient or the transconjugants. However, the result was positive for the positive control isolate.

5.4 Discussion

Susceptibility of the studied LAB to antimicrobials and their ability to transfer resistance genes to other bacteria were investigated. The variability of the susceptibility observed is common and related to the differences of the LAB genera and species and was previously reported in multiple research works on LAB from foods (Farahmand, 2015; Liu *et al.*, 2009; Ouoba *et al.*, 2008b). All isolates investigated by Farahmand (2015) were also susceptible to ampicillin, quinupristin/dalfopristin, and trimethoprim/sulfamethoxazole as observed for the isolates screened in the current study. Danielsen and Wind (2003) reported that resistance of the *Lactobacillus* species to aminoglycosides such as streptomycin, kanamycin and gentamycin is intrinsic and constitutes a general feature of the genus. This is in agreement with the current results where all species of *Lactobacillus* were resistant to gentamycin and streptomycin. However, Ouoba *et al.* (2008b) reported variable susceptibility of lactobacilli to aminoglycosides with species such as *Lactobacillus paraplantarum*, *Lactobacillus fermentum*, and *Lactobacillus salivarus* exhibiting susceptibility toward gentamycin while species of *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* were resistant to the antimicrobial. Moreover, different strains of the same species such as *Lactobacillus fermentum* exhibited different susceptibility to different aminoglycosides.

The screening of the background of resistances observed for some antimicrobials showed that both intrinsic and genetic factors may be responsible for the resistance. For example, the resistance to erythomycin exhibited by the isolates of *Enterococcus thailandicus* (52), *Streptococcus infantarius* (10), *Lactobacillus senioris* (43) and *Lactobacillus fermentun* (13) may be intrinsic as none of the genes screened and

coding for erythomycin resistance was detected. However, since all erythomycin genes were not screened, the possibility of a gene related resistance cannot be completely rules out. On the other hand, it clearly appeared that the resistance to tetracycline and streptomycin observed in the isolates of *Enterococcus thailandicus*, and *Streptococcus infantarius* is gene related as genes encoding resistance to the antimicrobials was detected. In other studies, genes encoding resistance to tetracycline, streptomycin and other antimicrobials were detected in LAB. For tetracycline, Charpentier *et al.* (1994), Gevers *et al.* (2003) and Huys *et al.* (2004) demonstrated the presence of *tet*(S), *tet*(M), *tet*(L) and *tet*(O) genes in *Enterococcus* and *Streptococcus* species from fermented and other types of foods. The *tet*(M), *tet*(W), *tet(*O) *tet*(Q), *tet*(K) and *tet*(L) have also been detected in various *Lactobacillus* species from foods (Egervarn *et al.*, 2009; Gevers *et al.*, 2003; Huys *et al.*, 2008; Thumu and Halami, 2012). The *tet*(M) gene seems to be most commonly detected tetracycline resistance gene in both Gram negative and Gram positive bacteria including LAB (Gevers *et al.*, 2003). For streptomycin resistance, the detection of the *aad*(E) gene in the current study is similar to the results obtained by Gaglio *et al.* (2016) who demonstrated the presence of the gene in streptomycin resistant enterococci from cheese. Other genes coding for resistances to other antimicrobials such erythomycin and chloramphenicol have been also evidenced in LAB. The studies of Ouoba *et al.* (2008b) and Huys *et al.* (2008) detected the ermB gene in *Lactobacillus reuteri and Lactobacillus paracasei* isolates. Also, Hummel *et al.* (2007) detected the presence of the chloramphenicol resistance Cat gene in *Enterococcus* species isolated from food.

The conjugation experiments carried out in the current study described the possibility of tetracycline and streptomycin resistance gene transfer from

Enterococcus thailandicus (52) and *Streptococcus infantarius* (10) to *Enterococcus faecalis* JH2-2 under laboratory conditions of cell to cell contact. The recovery of transconjugants was possible from both filter mating and unfiltered mating experiments. These are interesting results as most studies reported a recovery from only filter mating. In some studies such as those conducted by Gevers *et al.* (2003) and (Ouoba *et al.*, 2008b), it was even reported that recovery of transconjugants is affected by the size, type of filter and ratio of donor to recipient.

The substantial increase of the tetracycline MIC values in the transconjugants as compared to that of the recipient indicates that the isolates have acquired resistance either by gene transfer or mutations induced by the presence of the donor or the antibiotic in the growth medium. From the results obtained, it appears clearly that the acquired tetracycline resistance is associated at least with the transfer of the *tet*(S) gene absent in the recipient but detected in most of the transconjugants. For streptomycin, the recipient was already resistant to streptomycin with none of the streptomycin gene screened detected. This suggests that the recipient is intrinsically resistant to the antimicrobial. The fact that the transconjugants acquired the *aad*(E) gene simultaneously with the *tet*(S) gene did not increase their resistance potential as the MIC obtained in the transconjugants were similar to that of the recipient.

Interestingly, the experiments demonstrated that both *tet*(S) and *aad*(E) are located at least on plasmids that have mediated the transfer of the genes to *Enterococcus faecalis* JH2-2 because positive amplicons were obtained in the donors and transconjugants by amplification of the gene from plasmid DNA samples. It is well known that plasmids play an important role in the transfer of antimicrobial resistance genes including those of the antimicrobials screened (Huys *et al.*, 2004; Hummel *et*

al., 2007). Transposons *Tn916* and *Tn1545* that are known to be associated with tetracycline resistance genes and their potential transfer were not detected in the donors, the recipient or the transconjuants. This suggests that they were not involved in the gene transfer observed. However, since other transposons such as *Tn6000*, *Tn5387*, *Tn6079*, *Tn919*, *Tn5385*, *Tn5405* that were not screened in the current study are also related to tetracycline and streptomycin resistance gene transfer (Brouwer *et al.*, 2010; Hegstad *et al.*, 2010), the implication of transposons in the transfer process cannot be definitively ruled out.

Similarly to the present study, Charpenter *et al.*, 1994 demonstrated a transfer of *tet*(S) from an *Enterococcus faecalis* isolate to *Enterococcus faecalis* JH2-2 and *L. monocytogenes* L017. However, no gene for another antimicrobial was co-transferred in the transconjugants recovered from the tetracycline resistance selection as observed in the current study for the *aad*E gene. The authors showed that the *tet*(S) was located on the chomosome in this specific isolate of *Enterococcus faecalis* and that its transfer was mediated by an unknown mobile genetic element. In their research work, Lancaster *et al.* (2004) explained that the conjugative transposon *Tn916S* was responsible of the transfer of *tet*(S) from a *Streptococcus intermedius* isolate to *Enterococcus faecalis* JH2-2. A horizontal transfer of *aad*E from a lactic acid bacterium to a Gram-negative intestinal pathogen was observed by Connor *et al.* (2007) in their study and this was mediated by a plasmid as demonstrated in the present research work.

Although not screened, it is also possible that mutations may have also contributed in the acquisition of resistance to the recipient. For tetracycline, it is possible that after conjugations, mutations (e.g. Tyr-58→Asp, Tyr-58→Cys) in the *rpsL* gene

encoding the ribosomal protein S10, which is part of the 30S ribosomal subunit and contains a proposed tetracycline binding site caused an occurrence of tetracycline resistance in *Enterococcus faecalis* JH2-2 (Naghizadeh Raeisi *et al.*, 2018). For streptomycin, it is widely reported that occurrence of resistance to the antimicrobial can be the result of mutations in the *rpsL* (encoding the ribosomal protein S12) and *rrs* (16S rRNA) genes. For instance, in *Mycobacterium tuberculosis* isolates, mutations 43 Lys →Arg (K-43→R) and 88 Lys→Gln (K-88→ Q) in the *rpsL* gene and 516 Cys→ Th (C-516→T) and 513 Ala→ Cys (A-513→C) in the *rrs* gene were reported to cause streptomycin resistance (Lipin *et al.*, 2007; Sreevatsan *et al.*, 1996).

5.5 Conclusion

The antimicrobial resistance assessment of the LAB from *Nono* revealed a variability of resistance patterns according to the isolate and the antimicrobial screened. The resistance observed for some antimicrobials can be intrinsic and/ or gene related. The resistance to tetracycline and streptomycin for *Enterococcus thailandicus* and *Streptococcus infantarius* was shown to be related to the presence of genes encoding resistance to the antimicrobials. Moreover, the isolates hosting the genes were potentially able to transfer the genes to *Enterococcus faecalis* JH2-2. There was a strong indication that the transfers were mediated by plasmids. The study also revealed that the acquisition of a resistance gene in a bacterium exhibiting a potential intrinsic resistance to an antimicrobial does not necessarily increase the resistance strength. Overall, since the isolates of *Enterococcus thailandicus* and *Streptococcus infantarius* contain transferable antimicrobial resistance genes, there is a safety issue that jeopardises their use as multifunctional starter cultures.

Chapter 6 General discussion

Fermentation is considered a means of improving the nutritional quality and safety of food in developing countries. Lactic Acid Bacteria (LAB) play a major role in the fermentation of food by the production of organic acid, which improves the shelf-life of the fermented food and other metabolic processes. Scientifically proven, lactic acid fermented food helped to reduce the incidence of food borne pathogens and other related food borne diseases in Africa and sub-sahara African.

Most of the fermented food products in Africa are based on natural or spontaneous fermentation methods inherited by tradition and passed down from generation to next. *Nono* is one of the fermented milk products that is produced by Fulanis in nomadic Fulani ethnic groups residing in the Northern part of Nigeria. In this study, it has been established that the predominant organisms responsible for the fermentation of *Nono* are LAB. It also revealed that *Nono* contained diversity of species and sub-species of LAB including *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc,* and *Enterococcus*. It was also established that *Nono* has rich microbial biodiversity including potential probiotic organisms such as *Lactobacillus fermentum* that can be used as multifunctional starter culture for the controled fermentation of cow milk into *Nono*.

6 Scope of the study

The aim of this research was to isolate and determine the dominant lactic acid microflora of *Nono* produced in different locations at different production sites. From this study, strains with technological and probiotic potential were selected to be used as starter cultures for controlled fermentation in *Nono* production. In achieving this aim, characterization of the selected isolated LAB was carried out.

The fermentation of this milk product was natural and based on indigenous microflora from the raw milk or calabash used for the fermentation.

Lactic acid bacteria were isolated from *Nono* to select potential starter cultures for controlled fermentation. Diversity of the microflora was achieved by the use of three different media including MRS agar, MRSL and M17 agar. The results showed that use of more than one medium led to isolation of a variety of LAB associated with *Nono*. In addition, it was observed that more diverse range of organisms were harvested from MRS agar than those on M17 and MRSL agars. This is most possibly because MRS is an elective medium for LAB. Screening selected bacteria by phenotypic and genotypic methods were able to identify the bacteria to genus, species and sub-species level. It was observed that genotypic identification method was more reliable in terms of identification at species and sub-species level using 16S rRNA, *phe*S and *rpo*A gene sequencing. Rep-PCR was also used to differentiate the isolates at inter and intra-species level (Anyogu *et al.*, 2004; Ouoba *et al.*, 2008a).

Genotypic identifications allowed the identification of four genera of LAB including *Lactobacillus*, *Streptococcus, Leuconostoc*, and *Enterococcus* and seven species including *Lactobacillus fermentum* (40%), *Lactobacillus senioris* (2%), *Lactobacillus delbrueckii* (23%), *Streptococcus thermophilus* (22%) *Streptococcus infantarius* (10%), *Leuconostoc pseudomesenteriodes* (2 %) and *Enterococcus thailandicus* (1%). It was also noted that *Lactobacillus fermentum* and *Lactobacillus delbruckii* were predominant species irrespective of sample locations and production sites. Screening the isolates with the Rep-PCR technique in this study revealed the diversity of these predominant species at all production site in all sample locations.

Further characterization of the isolates was based on their ability to survive acidic pH and the presence of bile salts to assess their resistance to upper gastrointestinal tract conditions. This is one of the functional criteria in selection of potential probiotic starter cultures. The isolates studied indicated no viability at pH 2 after half hour incubation. Though, according to Holzapfel *et al.*, (1998) the pH of the stomach generally ranges from pH 2.5 to pH 3.5. However, it has been recommended that probiotic microorganisms in fermented food product must reach pH 4.5 to survive for long periods even during refrigerated storage (Jia *et al.*, 2010). All strains tested showed increase in viability as the pH increases to pH 4. The acid tolerance of the tested strains in this study varied; it showed that some of the strains possess strong acid resistance properties compared to others. *Lactobacillus fermentum* followed by *Lactobacillus senioris* were found to be more tolerant to acidic pH than the other species of lactic acid bacteria assessed.

The resistance of tested isolates to different concentrations of bile salts, showed viability at different bile salts concentration during 0 – 3 h incubation time. As reported by Wang *et al.*, (2010) tolerance to bile is considered as one of the essential properties required for probiotic bacteria to survive in the small intestine. In this study, some of the strains tested including *Lactobacillus fermenntum* and *lactobacillus senioris* may be able to survive in stomach and intestinal juice, which signifies their potential to meet probiotics criteria.

Inhibitory activities of the LAB strains against food borne pathogen due to the possession of antimicrobial compounds such as organic acid, bacteriocin and hydrogen peroxide was also evaluated. Investigating the isolates for inhibitory activities against certain pathogens attributed their antimicrobial activities to a

number of factors, including; acid production by the isolates at reduced pH levels, competition for substrate, and the production of substances with bacteriocidal or bacteriostatic action including bacteriocin which enhances the safety and quality of the fermented milk products. In this study, the inhibition activity varied among isolates against screened indicator bacteria. Though, some of the isolates such as *Streptococcus thermophilus* had no or very little inhibititory activity against the indicator organisms even using the standard spot test and/or well diffusion test, respectively. However, most of LAB isolated from *Nono* showed not only good antimicrobial activities against most of selected common food pathogenic bacteria but also demonstrated very good antilisterial activity, which is an important contaminating pathogen in food and dairy industries (Banwo *et al.*, 2012). Among all the tested strains, *Lactobacillus fermentum* showed a wide range of inhibitory activity against both Gram negative and Gram positive indicator bacteria screened, though it was obvious that the inhibitory activities was due to acid production and/or protein nature metabolites as the inhibition was reduced following neutralising the acid or treating the CFS with proteolytic enzymes. It was interesting to note that after the proteolytic enzymes treatment, *Lactobacillus fermentum* still retained its inhibition activity with some indicator bacteria including *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*. Among all the test isolates used, *Lactobacillus fermentum* exhibited the highest rate of inhibition which is an important characterisctic for selecting bacteria as starter culture.

Further to this study was investigation of antimicrobial susceptibility patterns of the isolated LAB. Screening of the isolates for resistance genes as well as their ability to transfer the resistance genes and finally evaluation of the genetic element that facilitate the gene transfer were also studies.

The MIC values varied among the tested isolates and antimicrobials. A higher expression of the tetracycline resistance gene *tet*(S) and *tet*(M) was detected in two of the tested isolates (52 and 10) and streptomycin resistance gene *aad*(E) was detected in test isolate 52. The MIC results obtained in this study further explained the possibility of tetracycline and streptomycin resistance gene transfer from *Enterococcus thailandicus* (52) and *Streptococcus infantarius* (10) to *Enterococcus faecalis* JH2-2 under laboratory conditions of cell to cell contact by *In vitro* conjugation and these isolates hosting the genes were potentially able to transfer the genes to *Enterococcus faecalis* JH2-2 through a genetic element plasmid. This current experiment revealed that, since the isolates of *Enterococcus thailandicus* and *Streptococcus infantarius* contain transferable antimicrobial resistance genes, they were not qualified to use as multifunctional starter cultures.

6.1 Significant and limitation of the study

This study was considered substantial due to the gaps in knowledge about the establishment of the methods of production of *Nono* in Nigeria and to determine the diversity and dominant lactic acid microflora present in *Nono*. It also set the platform for the selection of starter culture and optimum conditions for the production of a safe, consistent product with enhanced shelf-life.

Nono have been in existence though, the processing condition have been characterized unhygienic even the fermentation time is unreliable due to non-standardized production conditions (Okonkwo, 2011; Savadogo *et al.*, 2014). This present research has revealed the diversity of microflora in *Nono* and the result of this study made some suggestions for some of these isolates to be used as starter culture for a controlled fermentation of *Nono*. However, there are many more studies

on the probiotic properties of LAB isolated from *Nono* to be investigated for instance, to screen the type of bacteriocin produced as antimicrobial compound. Also, to conduct the antimicrobial activity test on milk instead broth media.

There were some challenges involved when the probiotic properties of these isolates were examined. For instance, when screening the isolates for antimicrobial resistance, there could have been a greater chance of achieving more potential if resources were available for whole genome sequencing of all the isolates. In addition, only few genes were investigated. Therefore, exploring more genes could have led to discovery of possible transfer of genes. Studying other possible methods could have also shed more lights on the survival of the isolates

6.2 General Conclusion and further work

This study investigated the diversity of LAB associated with naturally fermented cow milk product from Nigerian origin. In other word, selecting multifunctional starter culture for development of controlled fermentation of milk was the main aim of this current study, diversity of LAB strains isolated from this milk product (*Nono*) were characterised. The influence of acid tolerance and % bile salt tolerance on the strains was investigated. Further charaterisation involving antimicrobial activities of the test strains against food pathogenic bacteria which revealed significant production of antimicrobial compounds of the LAB isolates against the indicator tested also, antimicrobial resistance of the isolated LAB was studies which when screened for selected genes, it revealed the ability of gene transfer through a genetic element plasmid.

Presently, information on the development of starter culture of LAB from fermented food products have been in view but none or few have been reviewed from *Nono*, naturally Nigerian fermented milk product. This study revealed useful information for the development of potential multifunctional starter cultures in terms of technological and functional properties. This study revealed the variations that characterised the natural fermentation of *Nono*, it also revealed the time and temperature influence on the pH and % bile salt, survival, microbial growth and development of antimicrobial compounds.

Potential probiotic and functional properties of the test isolates must be considered for multifunctional starter culture development. This study revealed the potential probiotic properties of some of the test isolate including the antimicrobial and antilisterial activities, tolerance to acid pH and % bile salt concentration and susceptibility to antimicrobials. For the selection of multifunctional starter culture, these and other suggested work will be investigated to study mixed culture interaction.

- In vitro Screening of LAB isolated from *Nono* for their acidification rate using batch culture fermentation, to determine if their technological properties are successfully expressed in mixed cultures. This will give a good insight on the mixed culture interaction.
- Further investigation on the effects of these LAB fermentation on the content of essential fatty and amino acid using Gas Chromatography mass spectrometry (GC-MS) or gas chromatography with flame ionization detector (GC-FID).

- *In vitro* screening of the LAB isolates for cholesterol degradation and exopolysaccharides production.

Appendix 1 Sequence analysis results of *Enterococcus thailandicus* (isolate 52)

Enterococcus thailandicus strain a523 chromosome, complete genome
Sequence ID: CP023074.1Length: 2646250Number of Matches: 1
Related Information
Range 1: 1579503 to 1579872GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
662 bits(358)	0.0	366/370(99%)	0/370(0%)	Plus/Minus

```
Query  19       TTTTGATTCGTACACACACTTCACCTGTTCAAGCGCGAACAATGGAAAAACATGATTTTT  78
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1579872  TTTTGATTCGTACACACACTTCACCTGTTCAAGCGCGAACAATGGAAAAACATGATTTTT  1579813

Query  79       CAAAAGGTGCATTACGGATGATCTCACCTGGGAAAGTTTTCCGTCGTGATACTGATGATG  138
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1579812  CAAAAGGTGCATTACGGATGATCTCACCTGGGAAAGTTTTCCGTCGTGATACTGATGATG  1579753

Query  139      CTACTCATAGCCATCAATTCCATCAAATTGAAGGTCTTGTGATTGATAAAAATGTAACAA  198
                 |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1579752  CTACTCATAGCCATCAGTTCCATCAAATTGAAGGTCTTGTGATTGATAAAAATGTAACAA  1579693

Query  199      TGGGTGACTTGAAAGGGACACTGGAAGTTGTTATGAAGAAAATGTTTGGAGAAGATCGTA  258
                 |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  1579692  TGGGTGACTTGAAAGGGACACTGGAAGTTGTTATGAAGAAAATGTTTGGGGAAGATCGTA  1579633

Query  259      AAATTCGTTTACGTCCAAGCTATTTCCCATTTACTGAACCTTCAGTGGAAGTAGATGTTA  318
                 |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1579632  AAATTCGTTTACGTCCAAGCTATTTCCCATTCACTGAACCTTCAGTGGAAGTAGATGTTA  1579573

Query  319      GCTGTTTCAAATGCGGAGGCAAGGGTTGTAATGTCTGCAAATATACTGGCTGGATTGAAA  378
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1579572  GCTGTTTCAAATGCGGAGGCAAGGGTTGTAATGTCTGCAAATATACTGGCTGGATTGAAA  1579513

Query  379      TCTTAGGGGC  388
                 ||||||| ||
Sbjct  1579512  TCTTAGGTGC  1579503
```

Appendix 2 Sequence analysis results of *Streptococcus infantarius* (isolate 10)

Streptococcus infantarius strain ICDDRB-NRC-S5, complete genome
Sequence ID: CP013689.1Length: 1818293Number of Matches: 9
Related Information
Range 1: 5816 to 6193GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
665 bits(360)	0.0	371/379(98%)	1/379(0%)	Plus/Minus

```
Query  120   GCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGNCGTGTCTCAG  179
             ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  6193  GCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAG  6134

Query  180   TCCCAGNGTGGCCNATNACCCTCTCAGGTCGGCTATGTATCGTCGCCTTGGTGAGCCGTT  239
             |||||| |||||| || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6133  TCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTTGGTGAGCCGTT  6074

Query  240   ACCTCACCAACTAGCTAATACAACGCAGGTCCATCTACTAGTGAAGCAATTGCTCCTTTC  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6073  ACCTCACCAACTAGCTAATACAACGCAGGTCCATCTACTAGTGAAGCAATTGCTCCTTTC  6014

Query  300   AAGCATCTAACATGGGTTAAATGCTGTTATGCGGTATTAGCTATCGTTTCCAATAGTTAT  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  6013  AAGCATCTAACATGGGTTAAATGCTGTTATGCGGTATTAGCTATCGTTTCCAATAGTTAT  5954

Query  360   CCCCCGCTAGTAGGCAGGTTACCTACNCGTTACTCACCCGTTCGCAACTCTTCCAACTTT  419
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  5953  CCCCCGCTAGTAGGCAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCTTCCAACTTT  5894

Query  420   AGCAAACTAAAGTCTTCAGCGTTCTACTTGCATGTATTAGGCACGCCCGCCNGCGTTCGT  479
             ||||| ||||||||||||||||||||||||||||||||||||||||| ||| ||||||||
Sbjct  5893  AGCAAGCTAAAGTCTTCAGCGTTCTACTTGCATGTATTAGGCACGCC-GCCAGCGTTCGT  5835

Query  480   CCTGAGCCAGGATCAAACT  498
             ||||||||||||||||||
Sbjct  5834  CCTGAGCCAGGATCAAACT  5816
```

Appendix 3 Sequence analysis results of *Lactobacillus delbrueckii* subsp *indicus* (isolate 11)

Lactobacillus delbrueckii subsp. indicus strain JCM 15610, complete genome
Sequence ID: CP018614.1Length: 1967220Number of Matches: 1
Related Information
Range 1: 357107 to 357869GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
1367 bits(740)	0.0	756/766(99%)	3/766(0%)	Plus/Plus

```
Query  25      GTTACGGTAAGTTTGTCGTTGAACCGCTGGAGCGTGGGTTTGGTACTACCTTGGGTAATT  84
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357107  GTTACGGTAAGTTTGTCGTTGAACCGCTGGAGCGTGGGTTTGGTACTACCTTGGGTAATT  357166

Query  85      CACTGCGTCGGGTTTTGCTGACTTCTGTCCCGGGGACCGGTTTGGTGAAGGTGAAGATCG  144
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357167  CACTGCGTCGGGTTTTGCTGACTTCTGTCCCGGGGACCGGTTTGGTGAAGGTGAAGATCG  357226

Query  145     ATGGTATCTTGNACGAATTCNCTACTGTTCCCGGTGTTAAAGAAGACGTAACCAAGATCA  204
               |||||||||||| |||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  357227  ATGGTATCTTGCACGAATTCACTACTGTTCCCGGTGTTAAAGAAGACGTAACCAAGATCA  357286

Query  205     TCTTGAACCTGAAGAAGCTTGAACTCCGGGCCTACACTGAAGAAGTAAAGACGATCGAAC  264
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357287  TCTTGAACCTGAAGAAGCTTGAACTCCGGGCCTACACTGAAGAAGTAAAGACGATCGAAC  357346

Query  265     TCGACGTTGAAGGTCCAGCTACGGTAACTGCTGAAGATTTGAAGGCTGATGCTGATGTTG  324
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357347  TCGACGTTGAAGGTCCAGCTACGGTAACTGCTGAAGATTTGAAGGCTGATGCTGATGTTG  357406

Query  325     AAGTCTTGAATCCTGACCAATACATTTGTACCATCGCTCAAGGTGGCCACCTGCACATGT  384
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357407  AAGTCTTGAATCCTGACCAATACATTTGTACCATCGCTCAAGGTGGCCACCTGCACATGT  357466

Query  385     GGATTGATGTCTGCAACGGCCGGGGCTACGTACCAGCCAGCGAAAACAAGACTGCTGAAA  444
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357467  GGATTGATGTCTGCAACGGCCGGGGCTACGTACCAGCCAGCGAAAACAAGACTGCTGAAA  357526

Query  445     TGTCCATCGGCGACATTCCAGTTGACTCACTTTTCTCACCAATCGAAAAGGTCAACTACC  504
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357527  TGTCCATCGGCGACATTCCAGTTGACTCACTTTTCTCACCAATCGAAAAGGTCAACTACC  357586
```

Appendix 3 (Contd) Sequence analysis results of *Lactobacillus delbrueckii* subsp *indicus* (isolate 11)

```
Query  505     AAGTTGAATCAACCCGGGTTGGTAAGAGAGAAGACTTTGACAAGCTTACCCTGGAAATTT  564
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357587  AAGTTGAATCAACCCGGGTTGGTAAGAGAGAAGACTTTGACAAGCTTACCCTGGAAATTT  357646

Query  565     GGACAAATGGTTCAATCGCTCCGAATGACGCCCTCAACTTTGCCGCCCGTGTTCTGGTCG  624
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357647  GGACAAATGGTTCAATCGCTCCGAATGACGCCCTCAACTTTGCCGCCCGTGTTCTGGTCG  357706

Query  625     AACACTTCAAGGCCTTCGAATCAGCTGACGCTGCTGCCGAAATCGGCGAAGTTATGGTAG  684
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357707  AACACTTCAAGGCCTTCGAATCAGCTGACGCTGCTGCCGAAATCGGCGAAGTTATGGTAG  357766

Query  685     AACAGGAGAACGACCAAAAGGAAAAGAAACTCGAAATGACTATCGAGGANCTGGNCCTTN  744
               |||||||||||||||||||||||||||||||||||||||||||||||| |||| ||||
Sbjct  357767  AACAGGAGAACGACCAAAAGGAAAAGAAACTCGAAATGACTATCGAGGACCTGGACCTT-  357825

Query  745     TCAGTTCGTTCATACAACTGCTTGAAGCGCGTCNGGCNATCANCAC  790
               |||||||||||||||||||||||||||||| | | ||| |||| |||
Sbjct  357826  TCAGTTCGTTCATACAACTGCTTGAAGCGGG-CTGGC-ATCAACAC  357869
```

Appendix 4 Sequence analysis results of *Leuconostoc pseudomesenteroides* (isolate 9)

Leuconostoc pseudomesenteroides strain 22663 DNA-directed RNA polymerase alpha chain (rpoA) gene, partial cds
Sequence ID: JF411978.1Length: 780Number of Matches: 1
See 1 more title(s)
Related Information
Range 1: 32 to 187GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
289 bits(156)	3e-74	156/156(100%)	0/156(0%)	Plus/Plus

```
Query  21   GCTATGGCAAGTTTGTCATAGAGCCTCTTGAACGAGGATATGGAACGACATTAGGTAACT  80
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32   GCTATGGCAAGTTTGTCATAGAGCCTCTTGAACGAGGATATGGAACGACATTAGGTAACT  91

Query  81   CTTTGCGTCGTATCTTATTATCCTCGCTTCCAGGCGCAGCCGTTAACACGGTACAGATTG  140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92   CTTTGCGTCGTATCTTATTATCCTCGCTTCCAGGCGCAGCCGTTAACACGGTACAGATTG  151

Query  141  ACGGCGTAGTTCACGAGTTTTCAACTGTGGACGGCG  176
            ||||||||||||||||||||||||||||||||||||
Sbjct  152  ACGGCGTAGTTCACGAGTTTTCAACTGTGGACGGCG  187
```

Appendix 5 Sequence analysis results of *Lactobacillus fermentum* (isolate 13)

Lactobacillus fermentum strain CBA7106 chromosome, complete genome
Sequence ID: CP021964.1Length: 2042277Number of Matches: 10
Related Information
Range 1: 278418 to 278918GenBankGraphicsNext MatchPrevious Match

Score	Expect	Identities	Gaps	Strand
907 bits(491)	0.0	497/501(99%)	1/501(0%)	Plus/Minus

```
Query  21      ATACCGTC-ACGTATGAACAGTTACTCTCATACGTGTTCTTCTTTAACAACAGAGCTTNA  79
               |||||||| ||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  278918  ATACCGTCAACGTATGAACAGTTACTCTCATACGTGTTCTTCTTTAACAACAGAGCTTTA  278859

Query  80      NNAGCCGAAACCCTTCTTCACTCACGCGGTGTTGCTCCATCAGGCTTGCGCCCATTGTGG  139
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278858  CGAGCCGAAACCCTTCTTCACTCACGCGGTGTTGCTCCATCAGGCTTGCGCCCATTGTGG  278799

Query  140     AAGATTCCCTACTGCTGCCTCCCGTAGGAGTATGGGCCGTGTCTCAGTCCCATTGTGGCC  199
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278798  AAGATTCCCTACTGCTGCCTCCCGTAGGAGTATGGGCCGTGTCTCAGTCCCATTGTGGCC  278739

Query  200     GATCAGTCTCTCAACTCGGCTATGCATCATCGCCTTGGTAGGCCGTTACCCCACCAACAA  259
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278738  GATCAGTCTCTCAACTCGGCTATGCATCATCGCCTTGGTAGGCCGTTACCCCACCAACAA  278679

Query  260     GCTAATGCACCGCAGGTCCATCCAGAAGTGATAGCGAGAAGCCATCTTTTAAGCGTTGTT  319
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278678  GCTAATGCACCGCAGGTCCATCCAGAAGTGATAGCGAGAAGCCATCTTTTAAGCGTTGTT  278619

Query  320     CATGCGAACAACGTTGTTATGCGGTATTAGCATCTGTTTCCAAATGTTGTCCCCCGCTTC  379
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278618  CATGCGAACAACGTTGTTATGCGGTATTAGCATCTGTTTCCAAATGTTGTCCCCCGCTTC  278559

Query  380     TGGGCAGGTTACCTACGTGTTACTCACCCGTCCGCCACTCGTTGGCGACCAAAATCAATC  439
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278558  TGGGCAGGTTACCTACGTGTTACTCACCCGTCCGCCACTCGTTGGCGACCAAAATCAATC  278499

Query  440     AGGTGCAAGCACCATCAATCAATTGGGCCAACGCGTTCGACTTGCATGTATTAGGCACAC  499
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  278498  AGGTGCAAGCACCATCAATCAATTGGGCCAACGCGTTCGACTTGCATGTATTAGGCACAC  278439
```

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