CHAPTER ONE

INTRODUCTION

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1.0 Introduction

1.1 Background to the Study

Traditional fermented vegetable protein seeds constitute a significant proportion of the dietary requirements of large human populations in West Africa; they are added to various dishes to supplement protein intake, enhance nutritive value and impact sensory attributes (Achi, 2005). *Iru* (in Yoruba language), also known as *daddawa* (in Hausa language), is an alkaline fermented food condiment produced from African locust bean [*Parkia biglobosa* (Jacq. Benth)] (Aderibigbe and Odunfa, 1990). It is the most significant fermented condiment in the W. African sub-region, consumed by over 150 million people, particularly popular in Nigeria, and used as both flavouring and thickening agents, as well as low cost meat substitute in varieties of meals and diets (Dakwa *et al*., 2005; Achi *et al*., 2007).

Traditionally, *iru* is prepared by boiling *P. biglobosa* cotyledons for 24 h followed by dehulling in mortar with pestle or foot pressing, to remove the seed coats. The cotyledons obtained are again boiled for 4 h with optional addition of *iku iru*, a softening agent made from ground seeds of sunflower (*Hibiscus sabdariffa*) and native potash (*kaun*) (Odunfa and Oyewole, 1998). These are then drained using raffia sieve, spread into wide calabash trays while still hot, covered with jute bags and left to ferment for 3-5 days. Salt may be added at the end of the fermentation process as a temporary means of preservation. Two forms of *iru* are thus produced in Nigeria; *iru pete* obtained as a result of the addition of a softening agent, *iku iru* during the second boiling thereby containing partly mashed beans, and *iru woro* that is devoid of *iku iru* having distinct cotyledons. While *iru pete* is used in soups, *iru woro* is used in cooking leafy vegetables and stews.

Iru production is an alkaline solid-state fermentation process, unlike most other fermented foods in Africa that are usually acidic in liquid menstrum (Oyewole, 2001). Its production is

by spontaneous or natural inoculation enhanced by the raw materials, fermentation vessels, processors and environmental microflora, resulting into competitive adaptation and activities of autochthonous, spoilage and pathogenic microorganisms. Processing is carried out using rudimentary household and cottage facilities under varying unhygienic and poor sanitary conditions, devoid of good manufacturing practices (GMPs) (Oguntoyinbo, 2014). Consequently, *iru* produced lack consistency in terms of safety, quality attributes and organoleptic properties; necessitating the need to develop controlled fermentation system, using appropriate starter cultures that can initiate fermentation, dominate the process and rapidly ferment the substrate *in situ* for large-scale industrial production (Oguntoyinbo *et al*., 2007).

The initial efforts on *iru*, focused on microbiological characterization and biochemical changes identified *Bacillus* species as the predominant fermenting organisms (Odunfa, 1981a; Odunfa and Oyewole, 1986). However, these studies failed to provide information on the microbial profile, genomic strains diversity and sub-types of the dominant *Bacillus* species. Another limitation is failure to determine and harness probiotic functions of bacilli endospores in *iru* consumed in large populations.

The sub-Saharan Africa is the region of the world with the highest rate of malnourished people and incidence of diarrhoeal diseases (OECD-FAO, 2011). Traditional fermented foods mostly carry beneficial microorganisms, such as lactic acid bacteria, bacilli and yeasts; specific strains with health-promoting benefits have been tested for *in situ* production of metabolites that inhibited food-borne pathogens (Ogunbanwo *et al*., 2004). Development of microbial strains with probiotic health features and tested safety properties, well adapted to substrates, can be used for food fermentations, especially during household and small-scale food processing in sub-Saharan Africa. This could play an important role in reducing the high risk of gastrointestinal tract (GIT) disorders such as diarrhoea, towards achieving the United

Nations Sustainable Development Goals (SDGs) of promoting healthy living and well-being for all ages.

FAO/WHO (2002) defined probiotics as live microorganisms, which when administered in adequate amounts confer a health benefit on the host. Their beneficial effects include production of antimicrobial substances against pathogenic and spoilage microorganisms, modulation of immune functions, adhesion to intestinal mucosa by site competition for the exclusion of pathogenic bacteria, positive influence on the composition and metabolic activities of resident host microflora (Aragon *et al*., 2010; Maccaferi *et al*., 2012). Other benefits are: establishment of a well-balanced, indigenous intestinal microflora in neonatal and adults (He *et al*., 2008; Salazar *et al*., 2009), suppression of tumor cells and protection against colon cancer (Lam *et al*., 2007; Russo *et al*., 2007; Suzuki *et al*., 2008).

The genera *Bacillus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Bifidobacterium*, *Propionibacterium*, *Escherichia* and yeasts (*Saccharomyces cerevisiae* and *S. boulardii*) are licensed probiotic microorganisms for human and veterinary applications (Sanders *et al*., 2010). Bacilli in this group are not normal flora of the GIT, but have been described as bacteria with frequent transient in the GIT, and the endospores consumed in large populations in fermented vegetable protein seeds could germinate and proliferate to establish adhesion and colonization for probiotic functions (Casula and Cutting, 2002; Sorokulova *et al*., 2008). The heat-stable nature of their spores offers an advantage over other probiotic microorganisms that are not spore-formers; products containing *Bacillus* spores can be maintained at room temperature indefinitely in a desiccated form without refrigeration and deleterious effects on such products (Barbosa *et al*., 2005). *Bacillus*-based products such as *natto*, a popular fermented soybean food used as condiment in Japan, obtained using starter culture containing *B. subtilis* var. *natto* has been confirmed to stimulate immune responses,

and also introduced to the international market as probiotic product (Hosoi and Kiuchi, 2004).

1.2 Statement of the Problem

Previous investigations on the microbiological analysis during fermentation of *P. biglobosa* for *iru* production were based on traditional isolation and cultivation techniques that are tedious, biased, unreliable, and lack detailed information of the microbial composition (Odunfa, 1985a; Omafuvbe *et al*., 2004). Studies on the identification of the microorganisms in *iru* have been poorly described due to reliance on phenotypic and biochemical characteristics that are discriminatorily low, non-reproducible and laborious (Enujiugha, 2009).

Molecular typing techniques were used to characterize, identify and study the diversity of dominant *Bacillus* species during vegetable protein seeds fermentation in Africa and Asia (Jeyaram *et al*., 2008; Parkouda *et al*., 2010), but information of such on *iru* are sparsely available. Culture-independent microbial techniques such as PCR-DGGE have proven reliable and economical in studying the bacterial community profile of fermented vegetal protein seeds e.g. *doenjang* and *meju* in Asia (Lee *et al*., 2010). However, there is dearth of information on the application of these techniques to profiling microbial community of fermented condiments in Africa.

Due to poor refrigeration facilities in many developing countries, there is need to study and develop next generation probiotic products that can survive ambient temperature storage conditions, and significantly support the health and well-being of the people. Wild *Bacillus* species from fermented condiments may be developed as multifunctional strains for maximum beneficial properties. This could be applicable as starter cultures to predominate and bring about controlled and predictable fermentation of *P. biglobosa* during household to

small and medium enterprise condiments production in Nigeria, as well as maintaining probiotic functions and positively impacting gastrointestinal health of the consumers.

1.3 Aim and Objectives

1.3.1 Aim

This study aimed to determine the bacterial diversity in *iru* and investigate *in vitro* probiotic functions of the dominant bacterial strains, to aid development of *iru* as a functional food that can significantly improve consumers' health.

1.3.2 Specific Objectives

The specific objectives of this study were to:

(i) Characterize the bacterial community profile of *iru* using culture-independent molecular methods.

(ii) Analyze the bacterial population density of *iru* obtained from different producers and retail markets.

(iii) Evaluate the phenotypic features and identification of bacterial spore-formers isolated from *iru* using traditional culture-dependent techniques.

(iv) Sub-type the bacterial species associated with *iru* for strains differentiation.

(v) Investigate some *in vitro* probiotic properties of *Bacillus* strains isolated from *iru* that can be applied as probiotic-starter cultures for process optimization during small and intermediate scale industrial condiments production.

1.4 Significance of the Study

This study anticipates generation of expanded knowledge and information of the bacterial composition and diversity of *iru*, applying current state of the art molecular biology assessment techniques; such would form basis for geographical comparison of the microbiota of similar fermented vegetable protein seeds in Asia and Africa. It is important to properly determine the genomic diversity and sub-type of *Bacillus* species in *iru*, to differentiate

bacteria with pathogenic traits, as well as functional properties that are strain-specific. Hence, results emanating from this study will be useful in identifying *Bacillus* strains that can be adapted as cheap, stable and multifunctional starter cultures during fermentation of *P. biglobosa* for *iru* production, which is hoped to positively contribute to the health status of the consumers. These data will also support the promotion of *iru* as carrier of potential probiotic *Bacillus* strains, thereby corroborating existing knowledge in Europe and other parts of the world on the successful applications of bacilli as probiotic microorganisms.

1.5 Operational Definition of Terms

Terms	Definition
Autochthonous microorganisms:	microbial flora that naturally colonize a particular substrate, habitat or environmental niche
Good Manufacturing Practices:	practices required in order to conform to guidelines recommended by agencies that control authorization and licensing for manufacture and sale of food and pharmaceutical products
***in situ*:**	examining a phenomenon exactly in the original or appropriate position
***in vitro*:**	experiments conducted in a test-tube, cultured dish, or elsewhere outside a living host
Sustainable Development Goals:	these are new, universal set of goals, targets and indicators relating to international development, that were adopted at the UN Sustainable Development Summit in 2015
Natural fermentation:	an uncontrolled fermentation process, occurring without deliberate introduction of known microbial cultures

Starter cultures: a preparation containing high number of viable microorganisms, which may be added to a food substrate to bring about fermentation with desirable changes and features

Abbreviation	Definition
ARDRA	Amplified Ribosomal DNA Restriction Analysis
CFU	Colony Forming Units
DNA	Deoxyribonucleic acid
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
MLST	Multi-Locus Sequence Typing
OECD	Organization for Economic Co-operation and Development
PCR-DGGE	PCR-Denaturing Gradient Gel Electrophoresis
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
rep-PCR	repetitive-Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
WHO	World Health Organization

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

2.0 Literature Review

2.1 Fermented foods

Fermented foods comprise products made from plants and animals, processed under natural conditions or by deliberate addition of microorganisms that modify the constituents of the substrates biochemically into food substances that are nutritious, wholesome and palatable to the consumers (Tamang, 2010). They form an important component in the diets of human populations around the world and significantly in Africa (Holzapfel, 1997). Many of them are part of main course meals; some are beverages while some others are food condiments (Odunfa and Oyewole, 1998). African fermented foods have been categorized based on the substrates used in producing them; they include cassava products (*gari*, *fufu*), cereals (*ogi*, *kunun-zaki*), legumes (*iru*, *ugba*), alcoholic beverages (palmwine, *burukutu*) and fermented milk products (*nono*, *warankashi*) (Olasupo, 2006).

Fermented foods harbour diverse forms of microorganisms mostly from the environment, plant or animal sources, utensils, and are reported to be selected through competition and adaptation to the substrates (Steinkraus, 1997; Tamang, 1998). These microorganisms bring about biological transformation of the constituents of food raw materials during fermentation thereby enhancing the nutritive value of the final products; impacting sensory and organoleptic attributes; improve digestibility as a result of reduction of undesirable and antinutritional factors; fortification of food products with essential amino acids, vitamins and other health promoting bioactive compounds, including biopreservatives (Teniola and Odunfa, 2001; Ouoba *et al*., 2003a; Oboh, 2006; Oguntoyinbo *et al*., 2007).

2.2 Fermented vegetable protein seeds of Africa and Asia

Seeds of legumes may account for up to 80% dietary protein and may be the only source of protein for low income people, especially in Africa where protein/calorie malnutrition is a

major problem (Achi, 2005). Their cooked cotyledons are eaten as meals and commonly used in fermented form as food flavouring condiments (Odunfa, 1985b; Aidoo, 1986; Oniofiok *et al*., 1996).

In Nigeria, many names are used to describe multitude of traditional fermented food condiments produced using different legumes, and these include *ogiri-igbo* (or *ogiri-agbor*) from castor oil seeds [*Ricinus communis* (Schrad)] (Odunfa, 1985b), *ogiri-nwan* (also known as *ogiri-ugwu*) from fluted pumpkin seeds [*Telfaria occidentale* (Hook.)] (Odibo and Umeh, 1989; Barber *et al*., 1992), *ugba* (or *ukpaka*) from African oil beans [*Pentaclethra macrophylla* (Benth.)] (Oyeyiola, 1981; Obeta, 1983; Sanni *et al*., 2002), *owoh* from cotton seeds [*Gossypium hirsitium* (L.)] (Sanni and Ogbonna, 1991; Sanni *et al*., 1998) or from African yam bean [(*Sphenostylis stenocarpa* (Harms)] (Ogbonna *et al*., 2001), *okpiye*/*okpehe* from mesquite seeds [*Prosopis africana* (Tuab)] (Sanni and Onilude, 1999; Oguntoyinbo *et al*., 2001), *ogiri-ijebu* from melon seeds [*Citrullus vulgaris* (Schrad)] (Odunfa, 1981b), *dawadawa* from soybeans [*Glycine max* (L.)] (Popoola and Akueshi, 1984; Ogbadu and Okagbue, 1988) and *iru* (or *daddawa*) from African locust bean [*Parkia biglobosa* (Jacq. Benth)] (Odunfa, 1981a).

Although, the vegetable seeds used for the production of these condiments differ considerably from one region to another (Achi, 2005), the production is characterized with similar biochemical processes with increase in pH as a result of extensive hydrolysis of the proteins into peptides, amino acids and ammonia, which favours the dominance of *Bacillus* spp. as the fermenting organisms (Kiers *et al*., 2000; Sarkar *et al*., 2002; Ouoba *et al*., 2003b; Achi, 2005).

In Africa, other fermented vegetable protein seeds used as condiments include *soumbala* (Burkina Faso); *afitin*, *iru* and *sonru* (Benin Republic); *nététou* (Senegal) and *kinda* (Sierra Leone) all from African locust bean [*Parkia biglobosa* (Jacq. Benth)] (N'dir *et al*., 1994;

Azokpota *et al*., 2006; Ouoba *et al*., 2008; Ouoba *et al*., 2010), *bikalga* (Burkina Faso); *dawadawa botso* (Niger Republic); *datou* (Mali); *mbuja* (Cameroon) and *furundu* (Sudan) all from roselle seeds [*Hibiscus sabdariffa* (Linn.)] (Ouoba *et al*., 2008), *maari* (Burkina Faso) and *tayohounta* (Benin Republic) from seeds of baobab tree [*Adansonia digitata* (L.)] (Parkouda *et al*., 2010; Chadare *et al*., 2011).

Similarly, in Southeast Asia, various fermented alkaline food condiments produced mainly from soybeans [*Glycine max* (L.)] have been reported. These include *kinema*, consumed by the people of eastern Himalayan regions of the Darjeeling hills and Sikkim in India, Nepal and Bhutan (Tamang and Nikkuni, 1996; Sarkar *et al*., 1997; Dahal *et al*., 2005); *hawaijar*, popular among the Manipuris of northeast India (Jeyaram *et al*., 2008); Japanese *natto*, which has been commercialized (Kiuchi, 2004); *thua nao* from northern Thailand (Leejeerajumnean *et al*., 2001); Chinese *douchi* (Peng *et al*., 2004) and Korean *chungkook-jang* (Kim *et al*., 1996).

2.3 Nutritional values of *iru* and biochemical changes during production

Iru is the most important soup flavouring condiment in many W. African countries, which nutritionally serves as protein source, essential amino acids and fatty acids, including B vitamins in many poor rural families (Odunfa, 1985b, 1986). Studies have also shown high levels of free amino acids, non-protein and soluble nitrogenous compounds produced during *iru* production (Azokpota *et al*., 2006; Gernah *et al*., 2007). Ouoba *et al*. (2003b) reported increase in the concentration of essential amino acids, especially methionine, phenylalanine, leucine, isoleucine and lysine during alkaline fermentation of African locust bean. The predominant biochemical change was protein hydrolysis, due to the high proteinase activity, which results in rapid production of polypeptides, amino acids, ammonia, and polyglutamic acid (PGA) and other volatile compounds that contribute to the product's characteristic pungent smell and ammoniacal flavour (Odunfa, 1985a; Aderibigbe and Odunfa, 1990;

Leejeerajumnean *et al*., 2001; Omafuvbe *et al*., 2004). Other important biochemical activities reported include degradation of oligosaccharides to simple sugars (Omafuvbe *et al*., 2000; Ouoba *et al*., 2007b). Evidently, *iru* contributes significantly to the nutritional requirements and status of the consumers' diets.

2.4 Microorganisms associated with fermented condiments

Microorganisms isolated from different fermented vegetal protein seeds in W. Africa include genera of *Bacillus*, *Stapylococcus*, *Micrococcus*, *Brevibacillus*, *Proteus*, *Pseudomonas*, *Brevibacterium*, *Aerococcus*, lactic acid bacteria (LAB), some yeasts and moulds (Ouoba *et al*., 2004; Jeff-Agboola, 2007; Ouoba *et al*., 2010; Okorie and Olasupo, 2013). However, *Bacillus* spp. have been found to dominate the fermentation process owing to their growth in exothermic environment, large protease production that hydrolyse the proteins in legume into amino acids, as well as easy sporulation and re-germination (Ouoba *et al*., 2003b).

2.5 Microbiological methods for assessment of microbiota in fermented foods

2.5.1 Traditional culture-dependent methods

Conventional microbiological procedures of selective isolation, enumeration and identification have been employed to analyse the microflora of different indigenous traditional fermented foods; challenges associated with these techniques include tedium, labour and time consumption, and often times, misleading results (Rantsiou and Cocolin, 2006). The techniques include cultural morphology, microscopic, biochemical/physiological characteristics and other phenotypic features such as carbohydrate fermentation profiles that are used in the detection, identification and differentiation of bacterial species associated with various African fermented foods (Odunfa and Adeyele, 1985; Sanni *et al*., 1995; Oyewole, 2001; Uzeh *et al*., 2006; Obadina *et al*., 2008). Microbial examinations of some fermented condiments in W. Africa, including *iru*, have been studied using classical culturing systems

that are tendentious, inaccurate and do not produce unequivocal results (Jeff-Agboola, 2007; Okorie and Olasupo, 2013).

2.5.2 Culture-dependent molecular techniques

The availability of different molecular fingerprinting techniques based on analysis of DNA or RNA used in recent years has enabled better comprehension and detail investigations of the microbial flora composition and quantification, taxonomic identification and biodiversity of food matrices (Quigley *et al*., 2011). These approaches are known to be generally fast, more specific, sensitive, accurate, reproducible, and easily automated and permit precise microbial study of any given ecological system (Charteris *et al*., 1997; Justé *et al*., 2008). They have also been found useful in determining the microbiological processes occurring during food fermentation for the purpose of assuring quality and safety of the end products as required by the consumers (Giraffa and Carminati, 2008).

In the light of this, several culture-dependent molecular typing methods have been applied to study the diversity and dynamics of various microbial fermented food ecosystems. Amplified ribosomal DNA restriction analysis (ARDRA) with *Mbo*II or *Hha*I was used to distinguish *Lactococcus lactis* strains, and to also differentiate *Lactococcus* and *Enterococcus* species isolated from Spanish starter-free cheeses. In the same study, randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) by pulsed field gel electrophoresis (PFGE) further revealed genetic variability in both lactococci and enterococci strains (Delgado and Mayo, 2004). Species-specific PCR and RAPD-PCR with M13 primer were respectively employed to identify and typed about a thousand LAB strains isolated from dairy products. Cluster analysis of the RAPD-PCR profiles enabled grouping of the bacterial species into 11 fingerprinting library units identified as *Lactobacillus casei*/*paracasei*, *L. plantarum*, *L. rhamnosus*, *L. helveticus*, *L. delbrueckii*, *L. fermentum*, *L. brevis*, *E. faecium*, *E. faecalis*, *Streptococcus thermophilus* and *L. lactis* (Rossetti and

Giraffa, 2005). Closely related strains of *L. lactis* obtained from cheese made from unpasteurized milk were also distinguished using a combination of RAPD-PCR, plasmid profiling and PFGE (Corroler *et al*., 1998; Psoni *et al*., 2007).

Diversity among *Lactobacillus* species isolated from fermented caper berries was determined using RAPD-PCR with different banding patterns in the dendrogram obtained (Pulido *et al*., 2005). Venturi *et al*. (2012) developed a rapid and reproducible molecular method that combined conventional and multiplex RAPD-PCR to differentiate strains of *L. sanfranciscensis* isolated from traditional sourdoughs of northern Italy. De Vuyst *et al*. (2002) also investigated the biodiversity of LAB isolates from Greek wheat sourdough using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of total cell protein, RAPD-PCR (M13 primer), DNA-DNA hybridization and 16S rRNA gene sequencing, with a view to understanding their technological functions that will aid the development of starter strains for the optimization of sourdough processes. Among the LAB strains found dominant include *L. sanfranciscensis, L. brevis, L. paralimentarius* and *Weissella cibaria*. PCR primers specific for each of *L. sakei, L. curvatus* and *L. plantarum* were used for identification of 338 LAB strains significantly present in 3 Greek traditional fermented sausage plants (Rantsiou *et al*., 2006). Further characterization by RAPD-PCR implied a plant-specific distribution of strains.

Studies involving repetitive-PCR (rep-PCR), DNA-DNA hybridization, RAPD-PCR, PFGE and 16S rRNA gene sequencing have been conducted to identify the predominant LAB associated with fermented cassava food products such as *gari* and *lafun*, and to also evaluate the diversity of lactobacilli strains that are of technological and functional relevance (Kostinek *et al*., 2005; Oguntoyinbo, 2007a; Huch *et al*., 2008; Padonou *et al*., 2009). *Candida* species also isolated from *gari* were differentiated and identified base on 18S rRNA gene sequences (Oguntoyinbo, 2007b). Hayford *et al*. (1999) earlier used polymorphism of

DNA fragments generated from RAPD-PCR to characterize and identify dominant *Lactobacillus* species isolated from Ghanaian fermented maize dough.

Bacillus species obtained from diverse sources have been typed using various PCR-based genotypic methods for fingerprinting DNA fragment patterns necessary for strains differentiation and identification (Giraffa and Carminati, 2008). Wu *et al*. (2006) developed a *Bacillus*-specific PCR-ARDRA pattern combined with 16S rRNA gene group-specific primer to identify *Bacillus* strains and other related genera isolated from various environmental samples. Matarante *et al*. (2004) used RAPD-PCR and fluorescent amplified fragment length polymorphism (fAFLP) to characterize *Bacillus* species from traditional and industrially processed cured ready-to-eat sausages. Both RAPD-PCR and fAFLP revealed wide strain heterogeneity of *B. subtilis* resulting into 56 different biotypes. RAPD proved effective in the characterization and epidemiological sub-typing of *B. licheniformis* and other *Bacillus* species that are of medical significance (Stephan *et al*., 1994). Daffonchio *et al*. (1998) used internal transcribed spacers (ITS) between the 16S and 23S rRNA genes to discriminate the two groups of *Bacillus* species, *B. subtilis* and *B. cereus*, including the different species of *B. subtilis* group except closely related members of *B. cereus* and *B. subtilis* (*B. amyloliquefaciens* and *B. licheniformis*). *Bacillus* species associated with *gergoush*, a spontaneously fermented traditional Sudanese bread snack were identified using RAPD-PCR and ITS-PCR combined with 16S rRNA, *gyrA* and *gyrB* genes sequences (Thorsen *et al*., 2011).

Molecular characterization, identification and diversity analyses of *Bacillus* species isolated from fermented condiments in Asia and Africa have been reported. Inatsu *et al*. (2006) studied the diversity among *B. subtilis* strains isolated from *thua nao*, a traditional fermented soybean food of northern Thailand using RAPD-PCR. Oguntoyinbo *et al*. (2010) used a combination of ARDRA and RAPD-PCR fingerprinting techniques to successfully

differentiate between *Bacillus* species isolated from *okpehe*, a traditional fermented soup condiment from Nigeria. These authors submitted that ARDRA alone was not sufficient to differentiate between the *Bacillus* species; except for distinguishing *B. cereus* from *B. subtilis*. Ouoba *et al*. (2004) characterized the potential starter cultures, *B. subtilis* and *B. pumilus* obtained from the fermentation of African locust bean to produce *soumbala*, a fermented food condiment of Burkina Faso, using ITS-PCR, ITS-PCR- restriction fragment length polymorphism (ITS-PCR-RFLP). ITS-PCR and ITS-PCR-RFLP only allowed typing of *Bacillus* at species level while PFGE allowed intraspecies differentiation. Rep-PCR and PCR analysis of the internal transcribed spacers were also used to distinguish bacilli from *bikalga*, a product of alkaline fermentation of *H. sabdariffa* (Ouoba *et al*., 2008).

RAPD-PCR protocol and species-specific primer were developed for the rapid identification of *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens*, the dominant *Bacillus* strains associated with *cheonggukjang*, a Korean fermented soy food (Kwon *et al*., 2009). Sarkar *et al*. (2002) used RAPD-PCR to study the diversity of *B. subtilis* strains isolated from two spontaneously fermented alkaline foods, *kinema* and *soumbala*. Identification of the dominant *Bacillus* species associated with *hawaijar*, a traditional non-salted fermented soybean condiment of Manipur, northeast India, has been reported, including species and strains differentiation using a combination of ARDRA, ITS-PCR, ITS-PCR-RFLP and RAPD-PCR (Jeyaram *et al*., 2008).

Bacillus species responsible for the initial fermentation of baobab seeds for *maari* production, a fermented food condiment from Burkina Faso were characterized and identified using rep-PCR fingerprinting and 16S rRNA gene sequencing respectively (Parkouda *et al*., 2010). Ahaotu *et al*. (2013) identified 49 *Bacillus* species isolated during fermentation of African oil beans for *ugba* production by sequencing of 16S rRNA, *gyrB* and *rpoB* genes; species and strains diversity were also conducted using ITS-PCR and rep-PCR. Phylogenetic analysis of

16S rRNA and *gyrB* genes were used to identify and evaluate the diversity of 26 *Bacillus* species isolated from 12 samples of *mbuja*, a fermented condiment commonly consumed among the Cameroonians. *B. subtilis* and other related species were found most abundant, including genetic strains variability that can contribute to selection of starter cultures for *mbuja* production (Mohamadou *et al*., 2013). The diversity of aerobic endospore-forming bacteria occurring in *yanyanku* and *ikpiru*, two additives commonly used during fermentation of *P. biglobosa* seeds for condiments production in Benin Republic was studied using genetic fingerprinting markers, which includes rep-PCR, M13-PCR, 16S rRNA, *gyrA* and *gyrB* PCR genes amplification (Agbobatinkpo *et al*., 2013). From the results obtained, it was evident that *B. subtilis* is the dominant organism involved in the spontaneous fermentation of *H. sabdariffa* seeds for *yanyanku* and *ikpiru* production followed by *B. cereus*, *B. amyloliquefaciens*, *B. licheniformis*, *B. safensis* and *B. altitudinis*. Other bacterial species identified were *B. aryabhattai*, *B. flexus*, *B. circulans*, *Lysinibacillu*s spp., *Paenibacillus dendritiformis*, *Brevibacillus laterosporus* and *Aneurinibacillus aneurinilyticus*. However, information using molecular fingerprinting techniques for studying the microbial species associated with *iru* in Nigeria is unavailable.

2.5.3 Culture-independent molecular analyses

In spite of their widespread applications, culture-dependent genotyping methods still suffer some drawbacks. They analyse only the microorganisms recovered from cultured plates, using synthetic media that resemble the conditions of the system from which they were isolated (Cocolin *et al*., 2013). In addition, microbial cells that are numerically low in population ($\leq 10^4$ CFU/g), stressed or injured or weakened, whose growth requirements are unknown, and for which enrichment culture is necessary, are not detected by traditional plating schemes (Cocolin and Ercolini, 2008). It has also been demonstrated that about 25-50% of the active microbial community cannot be cultured *in vitro* using laboratory media

(Ampe *et al*., 1999). These viable but yet to be cultured (VBYC) cells may not be able to undergo normal cellular division required for growth on both selective and non-selective media that will generally support them (Oliver, 1993); a phenomenon which can be better described as a surviving strategy and response to adverse environmental situations such as nutrient depletion, low temperature and acid stress that are easily found in food fermentation and processing (Rowan, 2004). The VBYC microbial cells in this state is of concern when associated with food-borne pathogens and spoilage microorganisms, hence, it cannot be ruled out that such cells will emerge from this condition and cause diseases after gaining entrance into the human body. They may also be responsible for various complex biochemical reactions involved in the formation of aromatic substances and other organic compounds that contribute to the final characteristics of the products (Cocolin *et al*., 2013).

The above limitations prompted the development of culture-independent molecular assessment technologies, which directly characterize microorganisms as low as 10^4 CFU/g present in a particular habitat without the need for enrichment or isolation (Head *et al*., 1998). These methods have been increasingly used to determine the composition of complex microbial communities by examining the total DNA or RNA derived from mixed microbial populations (Hugenholtz and Pace, 1996). Culture-independent molecular approaches also monitor microbial population dynamics and structure, and enable thorough characterization of entire ecosystems as well as accurate detection and reliable identification of many species and strains (Quigley *et al*., 2011).

Molecular techniques to assess microbial community structures, functions and dynamics have been classified into two major categories. The PCR-based methods, which are the most predominant include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP) and clone libraries while fluorescence *in*

situ hybridization (FISH) represents one of the few methods that do not rely on PCR (Yang *et al*., 2001; Cocolin and Ercolini, 2008). However, PCR-DGGE is perhaps the most popular and commonly used among the culture-independent fingerprinting techniques. It is based on the electrophoretic separation of PCR-generated double stranded DNA in a polyacrylamide gel containing a gradient of chemical denaturants (urea and formamide); temperature being the denaturing gradient in TGGE (Rantsiou and Cocolin, 2006). As the DNA molecules encounter an increasing denaturant concentration, a sequence-dependent partial denaturation of the double strand occurs (Cocolin *et al*., 2013). The changes in the conformation of the DNA structures cause a reduction in the migration rate of the molecules such that DNA fragments of same size but different in nucleotide sequences are separated according to their melting properties (Ercolini, 2004; Cocolin *et al*., 2013). PCR-DGGE can likewise detects DNA molecules that differ by a single or a few base pairs (Myers *et al*., 1987) and hence capable of evaluating variations in any target gene that can enhance comparison of structural changes, microbial genetic diversity and monitoring of population dynamics of fermented foods, including microbiological and commercial food quality assessment (Ercolini, 2004; Sun *et al*., 2004; Camu *et al*., 2007). It is highly suitable for amplification of both major and minor constituents of microbial communities with a detection limit of 10^3 cfu/ml or g (Cocolin *et al*., 2001a; Rantsiou and Cocolin, 2006).

As an applied molecular microbial community analysis technique, DGGE results can be easily interpreted and at the same time supports identification of large number of species and strains recovered from the gels (Cocolin *et al*., 2001b; Cocolin *et al*., 2004a). The first study to exploit its potential was by Muyzer *et al*. (1993) who characterized the microbial ecosystems of mats taken from different depths and bacterial biofilms isolated from aerobic and anaerobic wastewater treatment reactors. PCR-DGGE was also first applied in food microbiology as a molecular ecology tool to investigate the spatial distribution of

microorganisms in pozol balls, Mexican fermented maize dough (Ampe *et al*., 1999). Since then, it has been extensively utilized to analyse sundry spontaneously fermented foods, such as milk and dairy products, fermented meats, sourdoughs, olives and wines, fermented vegetables, fermented fish products, cereals, cocoa, roots and tubers, legumes etc.

Ercolini *et al*. (2003) characterized the complex bacterial flora and structure of Stilton cheese by amplification of the variable V3 and V4-V5 regions of the 16S rRNA gene. PCR-DGGE band sequences based on DNA extracted directly from cheese and bulk microbial species, from a number of cultured plate media were identified as close relatives of *L. lactis*, *E. faecalis*, *L. plantarum*, *L. curvatus*, *Leuconostoc mesenteroides*, *Staphylococcus equorum* and *Staphylococcus* sp. Flórez and Mayo (2006) examined the dynamics and diversity of dominant prokaryotic and eukaryotic populations, during the manufacture and ripening of different batches of traditional fermented Cabrales cheeses by PCR-DGGE analysis of V3 region of the bacterial 16S rRNA gene and D1 region of the fungal 26S rRNA gene. DGGE detected variation in microbial profiles between different producers and similarity in batches from same producers, predicting dominance of particular group of microorganisms associated with each farmhouse of cheese making.

Bacterial community and populations of raw milk and fresh curds used for traditional production of Fontina cheese were analysed by PCR amplification of hypervariable V3 region of 16S rRNA gene and DGGE. DGGE band patterns showed microbial diversity for milk and curds, while the sequence data confirmed *E. faecium*, *E. faecalis* and *S. thermophilus* as the major LAB species, including spoilage bacterial genera such as *Macrococcus caseolyticus*, *Rothia* spp., *Pantoea* spp., *Pseudomonas* spp. and *Chryseobacterium* spp. (Giannino *et al*., 2009). Milk, curd and Castelmagno cheese at different manufacturing and maturation times were subjected to PCR-DGGE and reverse transcription (RT)-PCR-DGGE to evaluate the persistence of *S. agalactiae*, *L. lactis* and *L.*

lactis subsp. *cremoris* (Dolci *et al*., 2010). Cocolin *et al*. (2009) determined the microbial species of Gorgonzola cheese rinds and swabs from maturing shelves to be *Arthrobacter* sp., *Carnobacterium* sp., *Staphylococcus* sp., *Brevibacterium linens* and *Debaryomyces hansenii* using PCR-DGGE. Cluster diagram of DGGE gel profiles highlighted a cellar-specific microflora, attributed to the influence of environmental factors. An optimized PCR-DGGE protocol was devised by Cocolin *et al*. (2004b) to confirm the occurrence of *Clostridium* spp. in spoiled cheeses with late blowing symptoms. Ongol and Asano (2009) reported the participation of acetic acid bacteria (AAB) during Ugandan *ghee* fermentation and ripening, apart from LAB and yeasts, which were earlier noted as the significant microflora.

In fermented meat products, analysis of DGGE gel fingerprints and bands sequences established the constant presence and technological roles of *Staphylococcus* spp., *L. sakei* and *L. curvatus* in Italian fermented sausages. Other bacterial groups found were *L. paracasei*, *Bacillus* sp., *Ruminococcus* sp. and *M. caseolyticus*, including yeasts belonging to *D. hansenii*, *Candida* spp. and *Willopsis saturnus* (Rantsiou *et al*., 2005; Villani *et al*., 2007). Also, the bacterial ecology and population dynamics during natural fermentation and ripening of sausages were monitored by PCR-DGGE and RT-PCR-DGGE of the DNA and RNA extracted respectively (Cocolin *et al*., 2001b). LAB (*L. sakei* and *L. curvatus*) were the most prominent and active bacterial group, responsible for acidification and proteolysis that contributed to organoleptic properties of the fermented sausages. Others include *Micrococcaceae*, *S. xylosus* and natural meat contaminants such as *Brochothrix thermosphacta*, *Enterococcus* sp., *L. mesenteroides* and *Brevibacillus* sp. as detected by (RT)-PCR-DGGE. In another related study, PCR-DGGE was found useful in profiling the microbial composition and diversity of *alheira*, an artisanal Portuguese fermented sausage (Albano *et al*., 2008). Tu *et al*. (2010) identified LAB and coagulase-negative-cocci (CNC)

staphylococci as the major microbial flora naturally occurring in Taiwanese traditional fermented ham based on PCR-DGGE.

Meroth *et al*. (2003) used LAB species-specific PCR-DGGE to monitor changes in bacterial populations and development in sourdoughs inoculated with starter formulations, and found *L. sanfranciscensis*, *L. mindensis*, *L. crispatus*, *L. pontis*, *L. crispatus*, *L. panis*, *L. frumenti*, *L. johnsonii* and *L. reuteri* predominated. During wines fermentations PCR amplification of 26S rRNA gene and DGGE analysis qualitatively revealed yeasts populations of 10^3 cfu/ml and their activities at different fermentation stages, apart from distinguishing the yeasts species (Cocolin *et al*., 2000; Mills *et al*., 2002). Yeasts flora and diversity during tapping and fermentation of palm wine in Cameroon were selectively dominated by *S. cerevisiae*, as shown by PCR-DGGE; including other species like *Hanseniaspora uvarum*, *C. parapsilopsis*, *C. fermentati* and *Pichia fermentans* not isolated on cultured plate media (Stringini *et al*., 2009). Abriouel *et al*. (2011) assessed the microbial constituents of commercial Aloreña table olives using non-culturable molecular approach based on PCR-DGGE, with a view to differentiating and identifying the various microbial species. DNA samples of bacterial species from healthy and *Botrytis*-infected grapes were amplified targeting V1-V3 region of 16S rRNA gene. The DGGE band sequences and phylogenetic dendrogram indicated wider bacterial diversity against previous reports (Nisiotou *et al*., 2011). The bacterial flora (*L. delbrueckii*, *L. fermentum* and *L. plantarum*) of *sunki*, an unsalted fermented vegetable product of Japan, studied with PCR-DGGE was stable throughout the fermentation period (Endo *et al*., 2008). PCR-DGGE targeting 16S and 23S rRNA genes of the microbial composition during various types of *kimchi* fermentation expressed population changes as reflected in DGGE banding patterns (Chang *et al*., 2008).

Fermented non-alcoholic cereal food products, which are routinely consumed in W. and Central Africa, including Mexico and Brazil have been studied using PCR-DGGE

fingerprinting technique of total community DNA. Analysis of DGGE patterns of Mexican fermented maize dough, *pozol*, demonstrated successive microbial community shift and higher biodiversity. The initial microflora at the beginning of fermentation was dominantly streptococci, followed by heterofermentative LAB (*L. fermentum* and *Leuconostoc* spp.), progressively homofermentative LAB (*L. plantarum*, *L. casei* and *L. delbrueckii*), yeasts and moulds (ben Omar and Ampe, 2000). Comparison of DGGE profiles of *pozol*, *poto-poto* and *ogi* from Mexico, Congo and Benin Republic respectively showed significant differences in bacterial communities and ecological indices (Ampe and Miambi, 2000). Specifically, group clustering of microbial fingerprints of the fermented foods displayed interspecific variation, perhaps due to variant processing methods, equipment and environmental influences. However, intraspecies divergence was found within specific food groups, suggesting that different microbial association can yield similar food products. Also, DGGE band sequences corresponding to *L. plantarum*, *L. delbrueckii* and *L. fermentum* were found in most food samples analysed, confirming their adaptation to maize fermentation. Similarly, *ogi* and *kunun-zaki* in Nigeria were reported to possess diverse bacterial composition and species (Oguntoyinbo *et al*., 2011). DGGE amplicons of Tibetan kefir grains reportedly consist of two major group of microorganisms, LAB and yeasts, where the latter was less rich and complex than the former (Jianzhong *et al*., 2009).

Yoshikawa *et al*. (2010) used PCR-DGGE to identify the succession of halotolerant starter cultures during production of fermented fish sauce. The consistency of these starters proved effective in preventing unpleasant flavour contributed by wild yeasts. Oguntoyinbo (2011) profiled the yeasts population and identities of fermented cassava roots for *gari* production by amplification of eukarya 18S rRNA gene combined with DGGE. Yeasts species prominent were *C. rugopelliculosa*, *C. maritime*, *Zygosaccharomyces rouxii*, including *Issatchenkia sculata* and *Galactomyces geotrichum* not previously detected by culture-based practices.

Nested PCR-DGGE and PCR-DGGE were successfully applied to understand the variety of microbiomes in Korean traditional fermented soybean foods, *doenjang*, *meju* and *chungkookjang* (Kim *et al*., 2009; Lee *et al*., 2010; Hong *et al*., 2012).

As reported above, DGGE appears to be broadly implored in virtually all areas of food microbiology, such as starter cultures development, food spoilage and safety control. Nevertheless, it is not without some inherent weaknesses. Foremost, DNA extracted directly from any natural ecosystem contains mixture of microbial species, resulting in competition between target and non-target DNA templates, mostly for PCR reaction components like primers, dNTPs etc. When this happens, very low populated organisms, even at cfu $>10^4$ are always masked, leading to omission of their bands on the DGGE gel (Lopez *et al*., 2003). Second, DGGE bands with low intensity, are not easily excised in most cases, hampered by small fragment sizes of their PCR products, which may not contain enough sequences, thereby making their taxonomic classification and identification unachievable (Øvreås, 2000). In addition, different sequences may possess identical melting behaviour and electrophoretic mobility, resulting in co-migration of DNA fragments that cannot be separated on DGGE gels (Sekiguchi *et al*., 2001). But the fact that masked microflora in DGGE can sometimes be accounted for in culture-based techniques (Randazzo *et al*., 2002; Gala *et al*., 2008); highlights the importance and benefits of a polyphasic strategy to effectively and fully describe the complex microbial ecology of naturally fermented foods. Hence, studies involving the combination or otherwise simultaneous use of culture-dependent and culture-independent molecular methods to evaluate dominant and diverse bacterial species; accurate determination and thorough investigation of microbial contents of various kinds of traditional fermented food products have been documented (Oguntoyinbo and Dodd, 2010; Vilela *et al*., 2010; Kesmen *et al*., 2012; Greppi *et al*., 2013 a,b).

2.6 *Bacillus cereus* phylogeny: characteristics and identification

The *Bacillus cereus sensu lato* cluster comprises clonally related Gram-positive, spore-forming bacterial species, which includes *B. cereus sensu stricto*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. cytotoxicus* (Ehling-Schulz *et al*., 2005; Lindbäck and Granum, 2006; Ehling-Schulz and Messelhäusser, 2013). *B. cereus*, the first species in this group, is responsible for causing two forms of food poisoning, diarrhoeal heat-labile enterotoxins, leading to abdominal pain and diarrhoea after incubation period of 8-16 h of the vegetative growth of the bacterial cell in the small intestine, a condition referred to as food infection (Granum, 1994; Granum and Lund, 1997; Chaves *et al*., 2011). This diarrhoeal type consists of four different enterotoxins: two protein complexes, haemolysin BL (*Hbl*) and non-haemolytic enterotoxin (*Nhe*), and two enterotoxic proteins, enterotoxin T (bc-D-ENT) and cytotoxin (Agata *et al*., 1995; Beecher *et al*., 1995; Granum *et al*., 1999; Lund *et al*., 2000). The other type of food poisoning is a form of food intoxication, emetic food poisoning, caused by a small cyclic heat-stable peptide (cereulide), resulting in nausea and vomiting after 0.5-6 h ingestion (Ehling-Schulz *et al*., 2004; Rajkovic *et al*., 2008). *B. cereus sensu stricto* can also cause non-gastrointestinal disease conditions, such as local eye or wound infection, including non-pulmonary infections such as bacteraemia and endocarditis (Bottone, 2010).

B. thuringiensis, the second species, is distinguished from other members of the *B. cereus* group by the formation of a crystal parasporal inclusion body upon sporulation (Tourasse *et al*., 2006). This crystal protein coding for the insecticidal toxin of *B. thuringiensis* is plasmid borne and the loss of it makes *B. thuringiensis* no longer distinguished from *B. cereus* (Thorne *et al*., 1993). The plasmid has also been successfully transferred to *B. cereus*, making it a crystal toxin producer (Gonzáles *et al*., 1982). *B. thuringiensis* often exhibits specific insecticidal activities that are used industrially as biological pesticide for the control of insect

pests in agriculture, forestry and also for the control of insect vectors in human and animal diseases (Schnepf *et al*., 1998; Glare and O'Callaghan, 2000). The bacterium has occasionally been implicated in human infections similar to those caused by *B. cereus*, leading to outbreak of food poisoning (Jackson *et al*., 1995; Damgaard *et al*., 1997). Some strains have been reported to produce *B. cereus*-type enterotoxin and also possess genes known to be involved in *B. cereus* pathogenicity (Carlosn and Kolstø, 1993; Hansen and Hendriksen, 2001). In addition, *B. thuringiensis* has been implicated as the cause of gastroenteritis among workers using it as biopesticide in horticultural farming (Jensen *et al*., 2002a, b).

B. anthracis is a pathogen that infects human and other mammals, causing acute and often lethal anthrax disease and has been used as a bioterrorism agent (Jernigan *et al*., 2001; Mock and Fouet, 2001; Hughes and Gerberding, 2002). *B. mycoides* and *B. pseudomycoides* can be differentiated from other species by their distinctive rhizoid colony morphology and are not considered human pathogens (Cardazzo *et al*., 2008). Strains of *B. mycoides* with conifer roots that possess plant growth promoting activities necessary for the improvement of plant production have been reported (Petersen *et al*., 1995). The sixth species, *B. weihenstephanensis* are the psychrotolerant strains and are characterized by the presence of specific signature sequences in two genes, the 16S rRNA gene and *cspA*, which codes for the major cold shock protein (Lechner *et al*., 1998). *B. cytotoxicus*, the last species, is a thermotolerant member of the group that was recently described, and reportedly caused food poisoning outbreaks (Guinebretière *et al*., 2013).

From the foregoing, it thus becomes imperative to accurately identify and discriminate *B. cereus sensu lato* to species and strains level in order to ascertain the safety of food products, as few species of this group, especially *B. cereus sensu stricto* and *B. thuringiensis* both possess the potential to cause food borne diseases, apart from the use of *B. thuringiensis* as

biopesticides. Identification techniques capable of rapidly and correctly differentiating *B. cereus* and *B. thuringiensis* would assist greatly in understanding the epidemiological mapping, prevalence and distribution of these bacteria in foods and their various roles in food poisoning and food borne disease outbreaks (La Duc *et al*., 2004; Manzano *et al*., 2009; Thorsen *et al*., 2010).

Several methods for typing the *B. cereus* phylogeny have been attempted based on food safety and food quality requirements, as well as the contributions of *B. cereus* and *B. thuringiensis* to human infections (Ehling-Schulz and Messelhäusser, 2013). Thorsen *et al.* (2010, 2011) employed phenotypic parameters of *B. cereus sensu lato* namely: rhizoid growth; growth at 6^{o}C; crystal protein formation; β-haemolysis on sheep blood agar and genotypic characteristics such as PCR-amplifications of toxigenic and virulent genes to identify *B. cereus sensu stricto* and other species of the *B. cereus* group isolated from Beninese traditional fermented food condiments. However, the *B. cereus* group members are phenotypically related, possessing close homology, hence, cultural and biochemical features may not be sufficient in differentiating them. In addition, some phenotypic attributes may be easily lost by one species or strain and acquired by another through horizontal transfer of plasmids, thereby creating taxonomy difficulties in distinguishing species of the *B. cereus* group (Wilcks *et al*., 1998; Helgason *et al*., 2000; Manzano *et al*., 2010).

High level of lateral gene transfer in the *Bacillus* genus also makes virulence genes-based methods not always reliable in determining species identities of the *B. cereus* group (Jensen *et al*., 2005). Earlier, combined analyses of PFGE and multilocus enzyme electrophoresis (MEE) based on 15 chromosomal genes encoding enzymes failed to distinguish between *B. cereus* and *B. thuringiensis* (Carlson *et al*., 1994). Their results strongly indicated that both *B. cereus* and *B. thuringiensis* should be regarded as one species. Furthermore, PFGE and PCR detection of toxin gene *hblA* could not differentiate strains of the *B. cereus* group (Hansen

and Hendriksen, 2001). Helgason *et al*. (2000) also used MEE and sequence analysis of nine chromosomal genes to study the genetic relationship of *B. anthracis, B. cereus* and *B. thuringiensis* and found close genetic similarity among them, suggesting that they should be considered as one and same species.

B. cereus and *B. thuringiensis* isolated from diverse sources were analysed using temporal gradient gel electrophoresis (TTGE), M13 RAPD-PCR and rep-PCR to evaluate possible differentiation in these two homologous species of the *B. cereus* phylogeny; no clear discrimination was obtained using these methods (Manzano *et al*., 2009). ITS-PCR fingerprinting and analysis of ITS sequence polymorphisms by SSCP and restriction analysis could not effectively distinguish the *B. cereus* group, especially *B. mycoides, B. cereus* and *B. thuringiensis* (Daffonchio *et al*., 1998). According to Daffonchio *et al*. (2000), dendrogram constructed based on ITS-homoduplex-heteroduplex polymorphisms (ITS-HHP) analysis by polyacrylamide gel electrophoresis and silver staining showed two main clusters, with *B. cereus* and *B. thuringiensis* belonging to one cluster, and *B. anthracis* appearing as a lineage of *B. cereus*. Phylogenetic analysis of tRNA containing long ITS sequences conducted on *B. cereus* group revealed that *B. anthracis* diverged from related species (*B. cereus* and *B. thuringiensis*), thereby representing a separate phylogenetic clade (Cherif *et al*., 2003). However, sequence analysis of *gryB*, a virulence-independent, highly evolving, single-copied housekeeping gene was confirmed effective and precise phylogenetic marker in differentiating various species and serotypes of the *B. cereus* group, compare to phenotypic traits, 16S rRNA gene sequence-based analysis, DNA-DNA hybridization and virulent factors (La Duc *et al*., 2004).

Bavykin *et al*. (2004) demonstrated the potential of *gyrB* sequences (a conserved single-copied gene with more nucleotide variations than 16S rRNA gene) for identification of members of the *B. cereus* group, including differentiation of *B. anthracis* from other close

relatives. Also, PCR-amplified products of *gryB* gene digested with *Rsa*I, *Sau*3AI and *Eco*RI restriction endonucleases were used to discriminate between *B. mycoides*, *B. cereus* and *B. thuringiensis* obtained from various food samples, including reference and type strains (Manzano *et al*., 2003). Oliwa-Stasiak *et al.* (2010) developed a rapid and sensitive identification PCR technique for species of the *B. cereus* group based on unique conserved sequence of the *motB* gene, encoding flagella motor protein. Apart from species differentiation, studies involving *B. cereus* strains typing have also been reported. Cluster analysis of DNA fingerprints generated by repetitive element sequence polymorphism-PCR (Rep-PCR) showed seven major clusters with high genetic diversity among 97 food borne *B. cereus sensu stricto* strains isolated from various food substances in Brazil (Chavez *et al*., 2011). *B. cereus* strains recovered from two food poisoning outbreaks in Italy and reference *B. cereus* and *B. thuringiensis* strains were identified and typed using a combination of RAPD-PCR and multiplex RAPD-PCR (Ghelardi *et al*., 2002). Other molecular typing methods such as PFGE and amplified fragment length polymorphism (AFLP) have also been used to discriminate *B. cereus* strains obtained from different sources (Liu *et al*., 1997; Ripabelli *et al*., 2000).

PCR amplification of housekeeping genes and DNA sequence analyses otherwise known as multilocus sequence typing (MLST) is rather becoming popular and choice method for molecular sub-typing and population genetic studies of strains of the *B. cereus* group. This technique has been described as lucid, reproducible and portable tool for the understanding of bacterial genomic relatedness both at interspecies and intraspecies level (Soufiane *et al*., 2012). It depends on the comparative analysis of DNA sequences of mostly seven evolutionary conserved housekeeping genes among bacteria, where the sequence of a housekeeping gene of a particular bacterial isolate is assigned distinct alleles and the alleles of each of the seven loci define the allelic profile or sequence types (STs) (Gevers *et al*.,

2005). Though, MLST is a direct adaptation of multilocus enzyme electrophoresis (MLEE), it differs from the latter in the assignation of alleles by nucleotide sequencing rather than the electrophoretic mobility of their gene products (Selander *et al*., 1986).

MLST schemes have been successfully explored for a number of applications, including population structure of bacteria; evolution of bacterial virulence properties; identification of antibiotic-resistant strains and epidemic clones; affirmation of sources of an infection and detection of bacterial isolates that may be potential human pathogens (Helgason *et al*., 2004; Priest *et al*., 2004). MLST based on concatenated sequences of *clpC*, *dinB*, *gdpD*, *panC*, *purF* and *yhfL* loci was used by Sorokin *et al*. (2006) to characterize the phylogenetic relationship and separation of a total of 134 strains of *B. cereus sensu lato* isolated from forest soil near Versailles, Paris, into three major strain clusters of *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* designated as C, T and W based on the comparison of individual gene sequences. Their results also showed differentiation of psychrotrophic *B. cereus* and *B. thuringiensis* strains.

MLST of seven chromosomal housekeeping gene sequences (*adk*, *glpT*, *glpF*, *panC*, *pycA*, *ccpA* and *pta*) were also used to investigate clonal relationship among clinical isolates of the *B. cereus* group (Tourasse *et al*., 2006). Daffonchio *et al*. (2006) studied the phylogenetic relationship and diversity of *B. cereus*, *B. thuringiensis* and *B. anthracis* strains, the three most closely related *B. cereus* group members, applying MLST and found close genetic association. The genomic relationship and evolutionary studies of pathogenicity of 667 strains of *B. cereus* group obtained from diverse sources have been conducted; results showed that most of the *B. cereus* isolates from human infections were distributed throughout the population with no clear delineation of clonality (Didelot *et al*., 2009). Their findings further confirmed the three clade structure of the *B. cereus sensu lato* population comprising *B. cereus* (clade 1), *B. thuringiensis* (clade 2) and *B. mycoides*/*B. weihenstephanensis* (clade 3).

Barker *et al*. (2005) examined the phylogenetic origins of eight *B. cereus* strains from cases of bacteremia and soft tissue infections by MLST and observed that the *B. cereus* involved in opportunistic infections are genetically diverse and do not belong to a single clone or lineage within *B. cereus*. Phylogenetic analysis of MLST sequences proved to be an accurate molecular approach to understanding the evolutionary history of the *B. cereus* complex; identification of occurrence of horizontal gene transfer (HGT); strain genetic typing suitable for epidemiological studies and tracking of the spread of food-borne diseases caused by *B. cereus* (Cardazzo *et al*., 2008).

2.7 Probiotics

2.7.1 History and definitions of probiotics

During the last three decades, there have been increasing scientific attention and focus on the search for potential probiotic microorganisms and their subsequent applications as starter cultures in various food products. The concept of probiotics actually evolved more than one century ago, when Elie Metchnikoff suggested that the prolonged life of the Bulgarian peasants results from the consumption of fermented milk containing *L. bulgaricus*, which is currently known as *L. delbrueckii* subsp. *bulgaricus* (Metchnikoff, 1907). The term 'probiotic' was believed to have been earlier mentioned by Vergin in 1954 and used as an antonym of antibiotic (Vergin, 1954). It was also thought to have originated from two Greek words, *ρπο* and *βοϛi*, which literally means 'for life' (Hamilton-Miller *et al*., 2003).

Over the years, many definitions of probiotics were proposed. Parker (1974) defined probiotics as organisms and substances that contribute to intestinal microbial balance. Fuller (1991), being the first to point out the microbial nature of probiotics, redefined it as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal balance. In 2002, a joint FAO/WHO committee defined probiotics as live microorganisms which, when administered in adequate amounts confer health benefits on the host

(FAO/WHO, 2002). Taken together, probiotics are defined as preparation of live/viable microorganisms, which when consumed by humans or animals in adequate amounts (10^7-10^9 cfu/g or ml), induce beneficial effects by qualitatively or quantitatively influence gut microflora, modify immune status and contribute to general well being, beyond basic nutrition (Madsen *et al*., 1999; Fuller, 2004; Botić *et al*., 2007; Ivec *et al*., 2007; Pipenbaher *et al*., 2009).

2.7.2 Criteria for selecting good probiotic strains

For a probiotic microbial strain to exert health benefits and other positive desirable effects on the host when administered, it is expected to fulfil several selection criteria, consisting of evaluation guidelines and basic requirements. These detailed prerequisites and conditions have been collated from numerous published reports and recommendations, and summarily presented in Table 2.1.

2.7.3 Mechanisms of actions of probiotic microorganisms

The precise mechanisms by which probiotics exert their functions on the host have not been fully elucidated. Sanders (2009) highlighted some proposed manners of probiotic actions such as: production of bacteriocins and short chain fatty acids (SCFA); up-regulation of immune response (e.g. secretion of IgA) towards pathogens or vaccines; down regulation of inflammatory responses; improving gut mucosal barrier function; enhance stability and promote recovery of commensal microbiota when disturbed; modulation of host gene expression and delivery of functional proteins (e.g. lactase).

Table 2.1: Selection criteria for probiotic strains[‡].

Appropriateness and safety	(i) proper and accurate taxonomic characterization, identification and typing up to genus, species and strain level, including strain designation, using polyphasic approach, combining phenotypic techniques and molecular methods [such as DNA-DNA hybridization and 16S rRNA gene sequencing (for species identification); PFGE, RAPD, AFLP, RFLP and plasmid profiling (for strain typing)]. Deposition of defined probiotic strain in an internationally recognized culture collection. (ii) human origin; isolated from healthy human GIT or autochthonous of the targeted species. (iii) long history of being non-pathogenic (including production of enterotoxins, cytotoxins and haemolysin); non-toxic and GRAS (generally recognized as safe). (iv) inability to deconjugate bile salts; bile salt deconjugation or dehydroxylation is a negative trait in the small intestine. (v) lack of transmissible antibiotic resistance genes. (vi) potential probiotic strain should not degrade gastrointestinal mucin *in vitro* or get it translocated; this makes them non-invasive to the mucosal interface.
Functionality and performance	(i) capable of survival, proliferation and metabolic activity at the target site *in vivo*. (ii) resistance to gastric acidity of the stomach and tolerance to bile salts of the small intestine. (iii) production of antimicrobial substances (bacteriocins, organic acids, hydrogen peroxide and other inhibitory substances) against potentially pathogenic bacteria. (iv) ability to compete with the normal microflora in the host, including the same or closely related species; potentially resistant to bacteriocins, acids and other antimicrobials produced by resident microbial flora. (v) adhesion to human mucosal and epithelial cell linings, as well as persistence and colonization of the GIT, both *in vitro* (using human colon carcinoma cell lines, such as HT-29, HT-29-MTX and Caco-2) and *in vivo* using animal models and human subjects. Also, ability to inhibit adhesion of pathogens and colonization, by exclusion, competition and displacement assays. (vi) validated and clinically documented health benefits [e.g. immunostimulatory and immunomodulation; reduction of serum cholesterol and cancer prevention; treatment of acute diarrhoeal diseases and prevention of antibiotic-associated diarrhoea; prevention of inflammatory bowel disease (IBD); prevention and reduction of symptoms of rotavirus; alleviation of lactose intolerance; treatment and prevention of allergy. (vii) antimutagenic and anticarcinogenic properties. (viii) production of bioactive compounds (e.g. enzymes, vitamins, peptides, vaccines etc.). (ix) nutritional benefits and production of important digestive enzymes (e.g. β-galactosidase).
Technological requirements	(i) amenable to mass production and viability at high populations (10^7-10^9 cfu/g or ml) during processing, transportation, storage, shelf-life of the product and following consumption. (ii) stability of desired characteristics during culture preparation, and eventual inclusion in the final product. (iii) provides desirable organoleptic properties when included in foods or fermentation process. (iv) genetically stable and amenable strains. (v) phage resistance.

[‡]Compiled from Huis in't Veld *et al*. (1994), Marteau *et al*. (1995), Vandamme *et al*. (1996), Klaenhammer and Kullen (1999), Saarela *et al*. (2000), Holzapfel *et al*. (2001), Zhou *et al*. (2001), FAO/WHO (2002), Holzapfel and Schillinger (2002), Vuaghan *et al*. (2005), Kolida *et al*. (2006), Fric (2007), Vasiljevic and Shah (2008), Huys *et al*. (2013).

2.7.4 Significant benefits of probiotics

Probiotics are increasingly gaining scientific and commercial interest as functional foods in this era of self-care and complementary medicine (De Vecchi and Drago, 2006). They are included in various food products as supplements or as a therapy for several infectious diseases (Saarela *et al*., 2000; Hammerman *et al*., 2006). Probiotic-containing food products are available for human nutrition, as animal feed supplements, and also for aquaculture (Tournot, 1989; Rowland, 1999; Rolfe, 2000; Verschuere *et al*., 2000). In some countries probiotics are taken as prophylactic agents (for example, to prevent childhood diarrhoea, mainly rotavirus infections), while in Southeast Asia they are used as therapeutics (Mazza, 1994). Given the potential health promoting effects of probiotics coupled with the fact that they are relatively inexpensive to produce, transport and store, these microbes may herald a new epoch in preventive health care delivery, particularly for the developing world (Sleator and Hill, 2007).

Probiotic microorganisms in use, licensed for human and veterinary applications as prescription and non-prescription products, comprise the genera *Bacillus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Bifidobacterium*, *Propionibacterium*, *Escherichia* and yeasts (*S. cerevisiae* and *S. boulardii*) (Sanders *et al*., 2010).

The heat-stable nature of *Bacillus* spores offers them a number of uniqueness and long-term advantages over others that are non-spore bearers. First, products containing *Bacillus* spores can be maintained at room temperature indefinitely in a desiccated form, without refrigeration, and the entire dose of ingested spores will reach the small intestine in viable form; thus extending the shelf-stability of such products (Mazza, 1994). Consequently, probiotic products of this nature will be more promising and appropriate in under-developed and developing nations of the world, particularly, sub-Saharan Africa, where infrastructure for adequate power supply that is needed for refrigeration is lacking. Second, spores are

metabolically dormant (i.e. inactive), but resilient to environmental stresses and capable of passing through alive and surviving the low pH of the gastric barrier (Spinosa *et al*., 2000; Barbosa *et al*., 2005), which is not same for all *Lactobacillus* spp. (Tuohy *et al*., 2007). These features constitute an attractive attribute for commercial applications of bacterial spore-formers as probiotics. Unlike other probiotic microorganisms, spore-formers present in commercial products are consumed as spores and not as vegetative cells (Sanders *et al*., 2003).

Though, *Bacillus* species are allochthonous microbes, not normal inhabitants of the human intestinal tract (Sanders *et al*., 2003), yet some studies evidently claimed germination of *Bacillus* spores (including *B. subtilis* var. *natto*) in the GIT of chickens, mice and pigs, and adaptation to intestinal ecosystems as part of their natural life cycle (Hosoi *et al*., 1999; Hoa *et al*., 2001; Casula and Cutting, 2002; Duc *et al*., 2003a; Jensen *et al*., 2003; Tam *et al*., 2006; Cartman *et al*., 2008; Leser *et al*., 2008). Jadamus *et al*. (2001) observed the germination of *B. cereus* var. *toyoi* spores (the commercial probiotic strain present in Toyocerin®) in the GIT of broiler chickens and suckling piglets, and concluded that germination of spores was necessary for possible probiotic effects. *Bacillus* spores have also been found in the human GIT and other different mammals base on analysis of faeces and ileum biopsies, suggesting colonization rather than transient the intestinal tract that can enhance intimate interaction with the host cells or microflora for efficient potential probiotic functions (Barbosa *et al*., 2005; Guo *et al*., 2006; Fakhry *et al*., 2008; Hong *et al*., 2009a, b). It is important to note that *Bacillus* species being assessed for probiotic functionality includes *B. subtilis, B. licheniformis, B. pumilus, B. clausii, B. coagulans* and *B. cereus* (Hong *et al*., 2005).

Studies regarding the positive impact of *Bacillus* spores on indigenous microflora have been conducted. *B. subtilis* var. *natto* spores dosed with mice promoted the growth of *Bacteroides*

and *Lactobacillus* species under some dietary conditions (Hosoi *et al*., 1999). Increased counts of aerobic and anaerobic spore-formers were recorded in the feaces of piglets when fed *B. coagulans* probiotic strain CNCM I-1061 (Adami and Cavazzoni, 1999). *Bacillus* strains have in addition been reported to produce dozens of antimicrobial substances, considered to be a pathogen-inhibiting mechanism, exhibiting antagonism against broad spectrum of microbes (Sanders *et al*., 2003). Germinated *Bacillus* spores (that is the vegetative cells) produced bacteriocin-like inhibitory substances for competitive exclusion of pathogens (Duc *et al*., 2004a). Coagulin, an inhibitory compound that was plasmid-mediated, heat-stable, protease-sensitive and produced by *B. coagulans* I_4, exhibited both bacteriocidal and bacteriolytic action against related and unrelated bacterial species (Hyronimus *et al*., 1998).

B. subtilis and *B. licheniformis* strains isolated from a number of traditional fermented condiments in W. Africa were found to produce bacteriocins that possess antagonistic activity against array of Gram-positive and -negative bacteria, including yeasts (Oguntoyinbo *et al*., 2007; Kaboré *et al*., 2012; Compaoré *et al*., 2013). *B. subtilis* var. *natto* from Japanese *natto* inhibited the growth and activities of *Candida albicans* (Ozawa *et al*., 1979). *B. polyfermenticus* contained in the South Korean probiotic product, Bispan, was reported to produce a heat-labile, proteinase K sensitive bacteriocin (polyfermenticin SCD), which inhibited closely related *Bacillus* species (Lee *et al*., 2001). Pinchuk *et al*. (2001) described the characterization of an isocourmarin antibiotic (aminocoumacin A), produced by *B. subtilis* strain (*B. subtilis* 3) found in the commercial probiotic product (Biosporin®, from Ukraine), and shown to inhibit the growth of *Helicobacter pylori*. Enterogermina®, an Italian product, carrying *B. clausii* strains, has been shown to produce antimicrobial compounds against Gram-positive bacteria (Duc *et al*., 2004a; Urdaci *et al*., 2004). Promarine and BioPlus® 2B, available products in Taiwan and Denmark respectively, containing *B. subtilis*

strains, have been documented to produce antimicrobials (Urdaci and Pinchuk, 2004). Biocontrol potentials of *B. subtilis*, *Paenabacillus* spp. and *B. cereus* were evaluated against *Vibrio* spp., using both *in vitro* and *in vivo* inhibitory assays (Vaseeharan and Ramasamy, 2003; Ravi *et al*., 2007). Oral administrations of *B. subtilis* spores reduce infection by *Salmonella enterica* serotype Enteritidis, *Clostridium perfringens* and *E. coli* 078:K80 in poultry studies (La Ragione *et al*., 2001; La Ragione and Woodward, 2003). *B. subtilis* spores also significantly suppressed enteric infection and enteropathy of *Citrobacter rodentium* in a mouse model (D'Arienzo *et al*., 2006).

Modulation of immune function has been postulated as a mechanism of action of probiotic bacteria, including spore-formers (Sanders *et al*., 2003). In view of this, Muscettola *et al*. (1992) confirmed that Enterogermina® containing different strains of *B. clausii* spores administered to mice increased interferon production *ex vivo* by stimulating peritoneal and spleen cells. Duc *et al*. (2004b) examined the immunogenicity and intracellular reactions of *B. subtilis* spores in murine model. Mice dosed orally with spores developed systemic anti-spore IgG and mucosal secretory IgA (sIgA) responses, while analysis of cytokine mRNA in gut-associated lymphoid tissue (GALT) and lymphoid organs showed early induction of IFN-$_{\gamma}$, a Th1 cytokine, as well as the pro-inflammatory cytokine TNF-α. *B. subtilis* var. *natto* stimulated the secretion of interleukin-6 (IL-6) and/or IL-8 when cultured with Caco-2 cell monolayers (Hosoi *et al*., 2003). Feed supplemented with probiotic *B. cereus* var. *toyoi* enhanced modulation of the systemic immune responses of piglets (Schierack *et al*., 2009). Here, blood samples of probiotic-treated piglets gave a significantly lower frequency of $CD8^{high}$/CD3+ T cells and $CD8_{low}$/CD3+ T cells and a significant higher CD4+/CD8+ ratio. IL-4 and IFN-$_{\gamma}$ production of polyclonally stimulated peripheral blood mononuclear cells (PBMCs) was on the average higher in the probiotic group than control. The immune parameters, specifically, proliferative responses of PBMCs to influenza vaccination antigens

were significantly higher and antibody titres against H3N2 *Influenza* and *Mycoplasma* vaccination antigens were averagely higher in the probiotic group.

As potential vaccine vehicles in the production of next generation probiotics, *B. subtilis* spores induced the tetanus toxin fragment C (TTFC) antigen when delivered orally and intra-nasally into mice, displaying mucosal and systemic immune responses (Duc *et al*., 2003b). In another related study, *B. subtilis* endospores were developed and used to vaccinate against *C. perfringens* alpha toxin, to protect against gas gangrene in humans and necrotic enteritis in poultry (Hoang *et al*., 2008). Killed spores of *B. subtilis* bound to H5N1 virions (NIBRG-14; clade 1), and intra-nasally administered into mice, enhanced both humoral and cell-mediated immune responses compared to immunization with the virion alone, thus providing rationale for the use of *Bacillus* spores as a mucosal adjuvant for H5N1 vaccine (Song *et al*., 2012).

A double-blind, randomized, placebo-controlled clinical trial of Colinox®, a probiotic-based product containing *B. coagulans* spores, in combination with simethicone (an inert antifoaming agent), efficiently and significantly reduced bloating, abdominal pain and discomfort in irritable bowel syndrome (IBS) outpatients of Colinox® group (CG) compare to placebo group (PG) (Urgesi *et al*., 2014). Heo *et al*. (2014) also evaluated the therapeutic efficacy of Zhengchangsheng®, probiotic *B. licheniformis* capsules, in a randomized, double-blind, parallel-group clinical trial and found improvement in diarrhoeal symptoms and duration, such as rates of recovery for faeces formation, and frequency of stooling and quantity. Despite the pronounced beneficial health functions of probiotic microorganisms, their safety still needs to be assessed and ascertained, as per guidelines before applications, in particular the bacilli group. This is so because the use of *Bacillus* species as probiotics has raised a number of safety issues and concerns, most especially the *B. cereus* group and some species in the *B. subtilis* phylogeny that were reported to produce enterotoxins and emetic toxins food-borne outbreaks, including possession of transferable genetic elements coding for

virulent and antibiotic resistance in several microbial food chain (Salkinoja-Salonen *et al*., 1999; From *et al*., 2005; Ouoba *et al*., 2008).

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER THREE

3.0 Materials and Methods

3.1 Description of samples collection

Twenty-six traditionally processed *iru* were obtained from different local producers and retail markets in South-West (Lagos, Abeokuta, Ibadan, Oyo and Ado-Ekiti) and North-Central (Ilorin and Kaduna) Nigeria (Figure 3.1 and Table 3.1). Sampling plan and design was according to a randomized sampling technique (Smith, 2001). The condiments were transported immediately into the laboratory with the aid of ice-packs, stored in the refrigerator at 4^{o}C and analyzed within 48 h of collection.

3.2 Bacterial community profiling of *iru* samples

3.2.1 Culture-independent molecular analysis of bacterial community structure in *iru* samples

3.2.1.1 DNA extraction of *iru* samples

Total bacterial community DNA were extracted from 16 *iru* samples according to Ercolini *et al*. (2003) (enzymatic method) and Rantsiou *et al*. (2005) (chemical method), because out of the 26 *iru* samples no appreciable DNA was obtained from 4 samples and 6 samples also got deteriorated during transportation from Nigeria to India.

In the first method 10 g each of the condiments was homogenized in 90 ml sterile 0.1 M potassium phosphate buffer saline [potassium phosphate, monobasic (KH_2PO_4, 529568, Calibiochem, San Diego, CA, USA), potassium phosphate, dibasic, trihydrate (K_2HPO_4, 529567, Calibiochem, San Diego, CA, USA), NaCl (S3014, Sigma-Aldrich)] in a sterile stomacher bag (BA6141, Seward, London, UK) using a lab-blender (Stomacher 400 circulator, Seward, London, UK) at 200 rpm for 1 min. The homogenate was allowed to stand for about 2 min and 1 ml of the supernatant transferred into a screw-capped centrifuge tube. This was centrifuged (5424, Eppendorf) at room temperature for 10 min at 18,000xg and the

supernatant discarded, after which 500 µl lysozyme (20 mg/ml) in TES buffer (50 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0; 8.7% sucrose) and 5 µl mutanolysin (5U/µl) were added to the pellets, and the mixture vortexed for 1 min and incubated at 37^{o}C for 1 h. After 1 h incubation 50 µl of proteinase K (10 mg/ml) was added, and the samples further incubated at 65^{o}C for 10 min.

The resulting suspension was purified once with equal volume of phenol (pH 8.0) (P4557, Sigma-Aldrich) and twice with equal volume of chloroform:isoamylalcohol mixture (24:1) (Merck, Germany) with centrifugation each time at 4^{o}C for 15 min at 15,000xg (5415 R, Eppendorf). The aqueous solution obtained thereafter was precipitated with equal volume of cold isopropanol (-20^{o}C) (S. D. fine-chem., Mumbai, India) by centrifugation at 4^{o}C for 15 min at 15,000xg. The supernatant was discarded and the pellets washed with 1 ml of 70% ethanol (at 4^{o}C) and the DNA precipitated by centrifugation at 4^{o}C for 15 min at 15,000xg. Finally, the pellets were vacuum dried at room temperature and resuspended in 50 µl TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). DNA obtained was spectrophotometrically quantified and purity checked by taking absorbance reading at 260 nm (NanoDrop^{TM} 1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA), and DNA with purity level of 1.8 to 2.2 absorbance ratio (A_{260}/A_{280}) was used as template for PCR-DGGE and also stored at -20^{o}C (MDF-U333, SANYO, Japan).

In the second method of DNA extraction, homogenates of fermented condiments were prepared as earlier described. They were allowed to stand for about 2 min and 1.7 ml of the supernatant transferred into a screw-capped centrifuge tube and 0.3 g glass beads, acid washed of 0.5 mm diameter (G8772, Sigma-Aldrich) added and centrifuged at 4^{o}C for 10 min at 14,000xg. The supernatant was discarded, and the resulting pellets treated with 1 ml of petroleum ether:hexane (1:1) (Merck, Germany) for 10 min at room temperature in order to extract lipids. This was centrifuged at 4^{o}C for 10 min at 14,000xg and the pellets resuspended

in 150 µl of proteinase K buffer [(50 mM Tris.Cl, pH 8.0; 10 mM EDTA, pH 8.0; 0.5% (w/v) SDS]. Twenty-five microlitres of proteinase K, 25 mg/ml (P2308, Sigma-Aldrich) was added and the suspension incubated overnight at 65^{o}C. Thereafter, 150 µl of 2X breaking buffer [4% Triton X-100 (v/v) (H5141, Promega, Madison, WI, USA), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris (pH 8.0), 2 mM EDTA (pH 8.0)] was added to the tubes after which, 300 µl of phenol:chloroform:isoamylalcohol (25:24:1) (pH 6.7) (77617, Sigma-Aldrich) was also added to dissolve the proteins.

The resulting mixture was vortexed three times at maximum speed for 10 min. The tubes were then centrifuged at 4^{o}C for 10 min at 12,000xg, and the aqueous phase collected and mixed with cold absolute ethanol (-20^{o}C) (10107, Analar, England) of twice the quantity of the aqueous solution. The DNA was precipitated twice by centrifuging at 4^{o}C for 10 min at 14,000xg, and the pellets washed with 1 ml of cold 70% ethanol (at 4^{o}C). The pellets were vacuum dried at room temperature and 50 µl sterile TE buffer (10 mM Tris, pH 8.0; 0.5 mM EDTA, pH 8.0) added. The DNA obtained was spectrophotometrically quantified and purity checked as previously described.

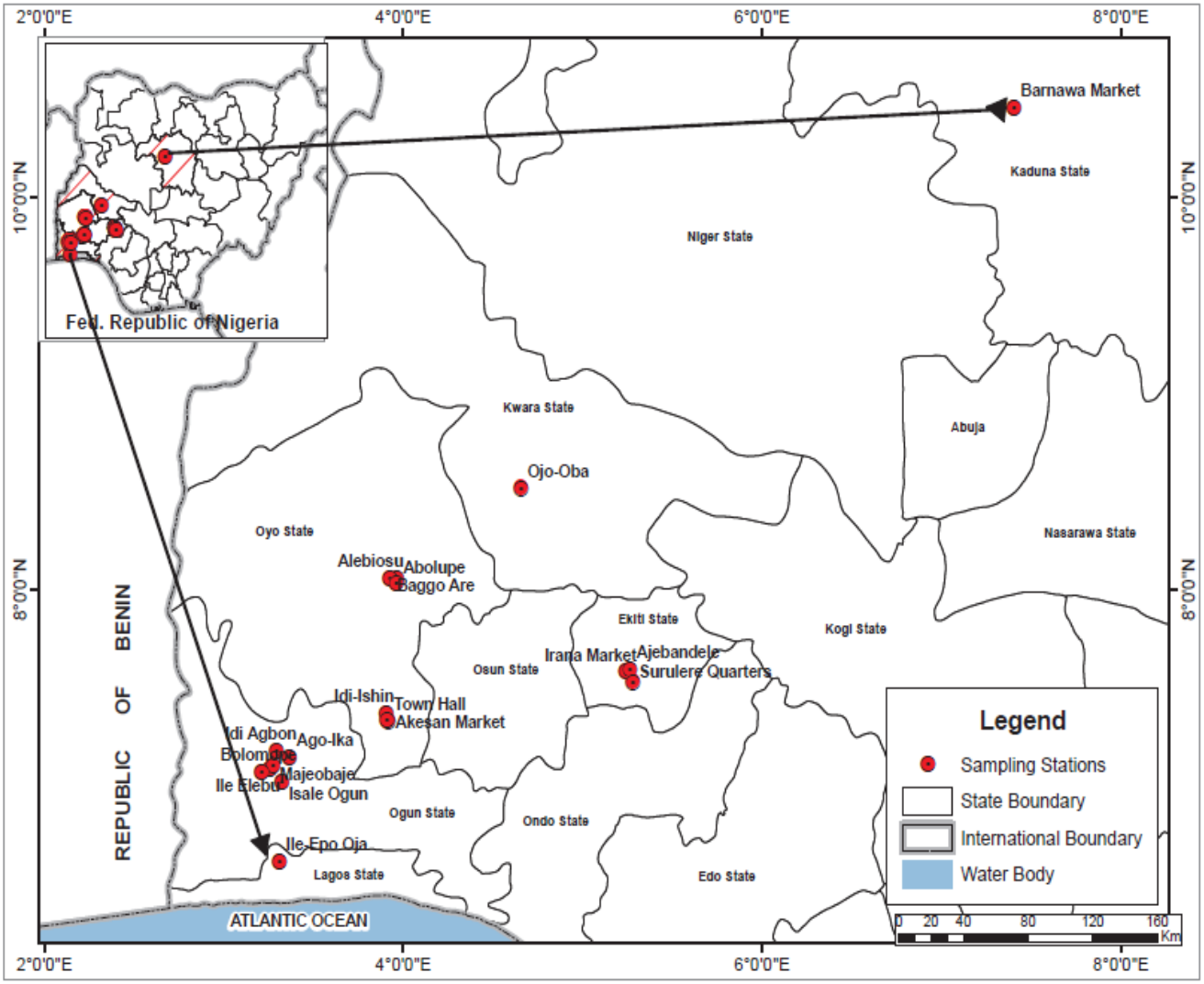


Figure 3.1. Map showing sampling locations of *iru* obtained from different manufacturers and retail markets in South-West and North-Central Nigeria.

Table 3.1: Coordinate descriptions of *iru* sampled at different locations in Nigeria.

Locations	GPS Coordinates	Towns/Cities
Abolupe	N8° 1' 42.391" E3° 57' 31.19"	Oyo
Baggo Are	N8° 3' 27.076" E3° 55' 18.491"	Oyo
Alebiosu	N8° 3' 38.873" E3° 57' 37.087"	Oyo
Town Hall	N7° 19' 51.586" E3° 54' 23.8"	Oyo
Akesan Market	N7° 19' 45.847" E3° 54' 50.195"	Oyo
Isale Ogun	N7° 8' 17.678" E3° 17' 8.948"	Abeokuta
Ile Elebu	N7° 5' 5.093" E3° 15' 16.848"	Abeokuta
Ago-Ika	N7° 8' 28.885" E3° 21' 48.895"	Abeokuta
Majeobaje	N7° 6' 19.825" E3° 16' 8.587"	Abeokuta
Idi Agbon	N7° 10' 41.398" E3° 17' 21.883"	Abeokuta
Bolomope	N7° 4' 3.292" E3° 12' 27.259"	Abeokuta
Barnawa Market	N10° 27' 33.97" E7° 23' 56.562"	Kaduna
Ajebandele	N7° 35' 33.792" E5° 15' 31.507"	Ado-Ekiti
Surulere Quarters	N7° 34' 45.793" E5° 16' 57.457"	Ado-Ekiti
Irana Market	N7° 34' 52.489" E5° 14' 6.673"	Ado-Ekiti
Idi-Ishin	N7° 22' 14.113" E3° 54' 9.162"	Ibadan
Ojo-Oba	N8° 31' 4.328" E4° 39' 4.892"	Ilorin
Ile-Epo Oja	N6° 36' 35.014" E3° 18' 21.229"	Lagos

3.2.1.2 PCR-DGGE amplification of variable V3 region of 16S rRNA gene

DNA templates from the various condiments analysed were subjected to PCR amplification using the set of primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') (Sigma-Aldrich), targeting the highly variable V3 region of the 16S rRNA gene. A 40-base-pair G+C-rich sequence (GC-clamp) (5'-**CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG**-3') was attached to the 5'-end of the forward primer as previously described (Sheffield *et al*., 1989; Muyzer *et al*., 1993), for optimum resolution of DGGE fragments.

Amplifications were carried out in a 25 µl master mixture containing 1.0 µl template DNA, 2.5 µl 10X PCR reaction buffer with 15 mM $MgCl_2$ (P2192, Sigma-Aldrich), 0.625 µl of 0.01 mM each of both forward and reverse primers at a final concentration of 0.25 µM (Sigma-Aldrich), 0.2 µl each dNTPs (Sigma-Aldrich) at a final concentration of 200 µM, 0.25 µl of 5 U/µl *Taq* DNA polymerase (D1806, Sigma-Aldrich) at a final concentration of 1.25 U and 19.8 µl sterile deionized water (Milli Q, Millipore, Bangalore, India). The 25 µl reaction mixture was short spun by centrifuge (5424, Eppendorf), after which amplification was performed in a thermal cycler (C1000, BIO-RAD, USA).

Template DNA was first denatured at 94^{o}C for 5 min. A touchdown PCR amplification (to minimize non-specific DNA bands) was performed as described by Ampe *et al*. (1999) with modification in the final extension time of 72^{o}C for 45 min. The initial annealing temperature was 65^{o}C and this was decreased by 1^{o}C every 2 cycles for 20 cycles; finally, 30 cycles were performed at 55^{o}C. The extension step for each cycle was 72^{o}C for 3 min, while the final extension was 72^{o}C for 45 min. Five microlitres PCR product was analysed on 2% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V

for about 1 h, and subsequently checked for non-specific amplifications. DNA fragments of about 240 bp obtained by comparing with PCR 100 bp low ladder (P1473, Sigma-Aldrich) was used for DGGE analysis.

3.2.1.3 DGGE analysis of bacterial community of *iru* samples

DGGE was performed using a DCODE^{TM} Universal Mutation Detection System (BIO-RAD, USA) following manufacturer's instructions. PCR products after analysis by agarose gel were subjected to denaturing gradient gel electrophoresis. Ten microlitres PCR-DGGE product mixed with equal volume of 2X gel loading dye [containing at a final concentration: 0.05% bromophenol blue (B6768, Sigma-Aldrich) (w/v); 0.05% xylene cyanol (w/v) and 70% (v/v) glycerol] was separated in a parallel denaturing gradients polyacrylamide gel [8% (w/v) acrylamide (V3111, Promega, Madison, WI, USA) -bisacrylamide (V3143, Promega, Madison, WI, USA)] at 37.5:1, containing 1X Tris-acetate-EDTA (TAE) buffer [40 mM Tris base, 20 mM acetic acid glacial (Merck, Germany), 1 mM EDTA (pH 8.0)] with a 25-55% urea (V3171, Promega, Madison, WI, USA) -formamide (344206, Calibiochem, San Diego, CA, USA) denaturing gradients [100% denaturant corresponds to 7 M urea and 40% (v/v) formamide] increasing in the direction of the electrophoretic run.

The electrophoresis was performed in a TAE buffer with an initial run of 20V for 10 min at a constant temperature of 60^{o}C, and finally at 140V for 5 h at 60^{o}C. A DGGE marker designed by mixing equal amount of 16S rRNA gene amplicons from 7 bacterial species (*B. subtilis* U170B, *B. amyloliquefaciens* U184B, *B. cereus* U175, *B. licheniformis* U126, *B. pumilus* U213A and *B. formosus* U185B all obtained during culture-dependent analysis, including *S. aureus* NB54 isolated from *ngari*, a fermented fish product of Manipur, India) was used as a reference ladder. After electrophoresis, gels were immediately stained for 20 min in 250 ml 1X TAE buffer containing 5 µg/ml ethidium bromide with gentle shaking at 2 rpm in a platform gel rocker (LI-GR-100, GeNei, Bangalore, India). After destaining for another 20

min in 1X TAE buffer, the gels were visualized under UV light and documented (Gene Snap, PerkinElmer, USA).

3.2.1.4 PCR-DGGE gel images analysis

The DGGE gel images obtained were converted to densitometric parameters using the software TL120 v2006 (Phoretix 1D Advanced Software, NonLinear Dynamics, Newcastle, UK). These data were further analysed to investigate similarities and differences in the DGGE gel lanes and bacterial composition of the different *iru* samples by constructing non-metric multidimensional scaling (non-metric MDS) scatter plots as described elsewhere (Boon *et al*., 2002; McOrist *et al*., 2008) using the PAST software (PAleontological STatistics, http://folk.uio.no/ohammer/past/). The Shannon-Weaver index of general diversity, *H*, and richness, *R*, were determined based on the number and relative intensity of the bands in each DGGE gel lane, where band intensity corresponded to peak height in the densitometric curves. The importance probability *Pi* and *H* were calculated using PAST (Shannon and Weaver, 1964).

3.2.1.5 Excision of DGGE bands and sequencing of amplicons

DGGE major bands were excised from the polyacrylamide gels using surface-sterilized microscope cover glass and the DNA eluted in 50 µl sterile deionized water (Milli Q, Millipore, Bangalore, India), and incubated overnight at 4^{o}C to allow diffusion of DNA fragments as described by Cocolin *et al*. (2001b). One microlitre of each eluate was used as DNA template for PCR reamplification using the reagents and cycling conditions as described above. PCR products originating from the excised bands were profiled and checked for quality in agarose gel; presence and position of the bands of interest in DGGE and comparison with parent DGGE profiles. Elution and reamplification were carried out at least twice or until a single band that co-migrate at the same position with the parent DGGE band is obtained. Sequencing was carried out using the corresponding primers as described earlier

without GC-clamp by a commercial facility (GeNei and MWG, Bangalore, India), and sequences of major bands from the DGGE gel were compared with those of NCBI GenBank database to determine closest known bacterial identities (Altschul *et al*., 1997).

3.3 Genomic characterization and sub-typing of autochthonous *Bacillus* species in *iru*

3.3.1 Culture-dependent microbiological analysis of bacterial species in *iru* samples

3.3.1.1 Isolation of microorganisms

Bacilli enrichment and enumeration were conducted, 10 g each of the condiments was aseptically weighed and homogenized in 90 ml sterile Ringer's solution using a surface sterilized mortar and pestle. This was heat treated in water bath (Memmert, Buchschlag-Frankfurt, Germany) for 5 min at 85^{o}C following the method of Guo *et al*. (2006) with slight modifications. The essence of this was to kill off the vegetative cells and ensure isolation of bacterial spore-formers. Six-fold serial dilutions were carried out on the homogenates, and 100 µl of appropriate decimal dilutions were plated in triplicates on nutrient agar (Scharlau Chemie S.A., Barcelona, Spain) using spread plate technique (Harrigan and McCance, 1976), and then incubated at 37^{o}C for 18-24 h. After incubation, the colonies that appeared on nutrient agar were counted and expressed as colony forming unit per gram (i.e. CFU/g).

The predominant representative colonies were selected based on colonial morphology and other features, and sub-cultured onto new nutrient agar plates and purified by repeated streaking to obtain pure cultures. A total of 280 bacterial strains obtained were grown and maintained in 5 ml sterile nutrient broth (Scharlau Chemie S.A., Barcelona, Spain), and subsequently preserved in 50% (v/v) glycerol (356352, Calibiochem, San Diego, CA, USA) at -20^{o}C (MDF-U333, SANYO, Japan). Reference *Bacillus* strains, *B. subtilis* MTCC 2451, *B. amyloliquefaciens* MTCC 1270, *B. licheniformis* MTCC 429, *B. cereus* MTCC 430 and *B. circulans* MTCC 490 were also obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Others include *B. subtilis*

MTCC 5480 isolated from *hawaijar*, fermented soybean of Manipur, northeast India and *B. subtilis* MTCC 1747 isolated from *kinema*, fermented soybean of Sikkim, northeast India. They were used to study the molecular and genetic diversity among *Bacillus* strains isolated from *iru*.

3.3.1.2 Determination of Hydrogen ion concentration (pH) of *iru* samples

During isolation, the pH of *iru* samples was determined using a digital pH meter (Model HI 255, Hanna Instruments, Bedfordshire, UK).

3.3.1.3 Phenotypic characterization of bacterial species isolated from *iru* samples

3.3.1.3.1 Cultural/colonial/macroscopic characteristics

The colony of each pure bacterial culture was examined for shape, elevation, edge, optical characteristics, consistency, surface and pigmentation according to Fawole and Oso (2002), using stereo zoom microscope (SZ-ST, Olympus, Japan).

3.3.1.3.2 Gram's staining

The Gram staining technique is the most important and frequently used staining method in bacteriology. It is a differential staining procedure that helps to differentiate bacteria into two main groups, possibly showing major evolutionary relationship (Willey *et al*., 2009). Gram staining was carried out by preparing a smear of each bacterial isolate from 18-24 h culture made on clean grease free slide, air dried and heat fixed by flaming over a spirit lamp. The smear was then flooded with Gram's crystal violet for 30-60 s. This dye was drained quickly after this time out, washed with Gram's iodine and left for 60 s. The iodine solution was drained off and the slide washed gently under the tap. Decolourization was followed by washing slide with 70% alcohol until no more crystal violet runs from it, and this last only for 10-30 s. After this, slide was rinsed under gentle-running tap water and counterstained with safranin for 30-60 s. The slide was finally drained, washed with water, blot dried and examined under the microscope with oil-immersion objective lens (Olutiola *et al*., 2000).

3.3.1.3.3 Endospore staining

This was carried out by preparing a heat-fixed smear of the test organism (18 h), which was flooded with 5% Malachite green. The stained slide was gently heated over a beaker of boiling water for about 10 min until steam rises and bubbles, while drying was avoided by occasional addition of more stain. The stain was washed off under the tap and the smear counterstained with 0.5% safranin for 30 s, again washed and rinsed under the tap and blot dried. Finally, slide was examined under the microscope with oil-immersion objective lens. While the vegetative portion of the bacterium stains pink to red, the spores stain green (Fawole and Oso, 2002).

3.3.1.3.4 Phase contrast microscopy

Thin smear of 18 h broth culture was made on a clean glass slide and allowed to dry. This was observed under the phase contrast microscope (BX61, Olympus, Japan), using oil-immersion objective lens, to view the bacterial endospores and the different forms.

3.3.1.3.5 Catalase test

Catalase test detects and demonstrates the presence or absence of catalase enzyme in each bacterial isolate. The test was carried out according to Harrigan and McCance (1976); 1 ml or few drops of 3% hydrogen peroxide (H_2O_2) solution was placed on a clean slide, and pure agar culture of each isolate of 18-24 h was emulsified with it, and with the aid of a sterile wire loop observing for immediate effervescence caused by the evolution (liberation) of oxygen gas. While bubbles indicate a catalase positive reaction, its absence or delayed bubbles indicate a negative reaction (Seeley *et al*., 1990).

3.3.1.3.6 Starch hydrolysis

This test was carried out by preparing starch agar, which contains per 100 ml, 2 g soluble starch (Spectrochem, Mumbai, India), 1 g peptone bacteriological (RM 001, HiMedia, India), 1 g beef extract powder (Merck, Mumbai, India), 0.3 g NaCl (Merck, Mumbai, India) and 1.8

g agar powder bacteriological (RM 026, HiMedia, India). The test isolates of 18 h growth were inoculated onto sterile starch agar Petri dishes by single streaking across the plates once using a sterile wire loop while uninoculated plates served as control. The plates were incubated for 24 h at 37^{o}C and then flooded with 0.1% iodine solution (containing 0.1 g potassium iodide, RM 252, HiMedia, India and 0.1 g crystal iodine, Loba Chemie, Mumbai, India) for 10-15 min and thereafter observed for clearing zone. Unhydrolyzed starch forms a blue or blue-black colour with the iodine on the plates as well as the control plates; hydrolyzed starch appears as a clear zone in addition to the presence of a bluish purple colouration, which results from α-amylase activity and of course confirms its presence. Reddish brown zones around the colony indicate partial hydrolysis of starch to dextrans, which results from α-amylase activity also (Olutiola *et al*., 2000).

3.3.1.3.7 Casein hydrolysis

Casein agar per 100 ml containing 2 g casein (C7078, Sigma-Aldrich), 0.5 g peptone bacteriological (RM 001, HiMedia, India) and 1.8 g agar powder bacteriological (RM 026, HiMedia, India) was used to determine the bacterial isolates' ability to produce protease required for hydrolysis of casein. Again, isolates were transferred onto sterile casein agar Petri dishes by single streaking across the plates once using a sterile wire loop while uninoculated plate served as control. After 24 h incubation at 37^{o}C, plates were observed and clear zones around the colonies on the culture medium i.e. transparency along the line of streaking indicates evidence of casein hydrolysis while opacity indicates non-hydrolysis of casein (Harrigan and McCance, 1976).

3.3.1.3.8 Lipid hydrolysis

The lipolytic activity of the bacterial isolates was determined using tributyrin oil agar, which contains per 100 ml, 1 ml tributyrin oil (FD081, HiMedia, India), 0.5 g peptone bacteriological (RM 001, HiMedia, India), 0.3 g beef extract powder (Merck, Mumbai, India)

and 1.8 g agar powder bacteriological (RM 026, HiMedia, India). The bacterial isolates were transferred onto sterile tributyrin oil agar Petri dishes by single streaking and allowed for 24 h incubation at 37^{o}C. After this, plates were observed for clear zone around the colonies, which indicates isolates ability to produce lipase.

3.3.1.3.9 Anaerobic growth

The ability of the bacterial isolates to grow under anaerobic condition was investigated using an anaerobic jar containing Anaerocult®C (Merck, Darmstadt, Germany) for the generation of an oxygen-depleted and CO_2-enriched atmosphere.

3.3.1.3.10 API 50 CHB carbohydrates fermentation profiles and identification system of *Bacillus* strains

In this test a single, pure bacterial colony (18 h) was suspended in 500 µl of 0.85% sterile normal saline of a turbidity equivalent to 2 McFarland standard. This was transferred into 10 ml API 50 CHB ampule/medium and mixed thoroughly. With the aid of a sterile pipette 200 µl cell suspensions was added to each of the 50 microtubes of the API CHB strips and incubated at 37^{o}C for 18-24 h following manufacturer's instructions (bioMérieux, Lyon, France). A positive result that corresponds to acidification is revealed by the phenol red indicator contained in the medium, which changes to yellow; for esculin test, a change in colour from red to black is indicative of a positive result. If a positive result becomes negative at the second reading, only the positive result will be considered (this is only caused by an alkalinisation due to production of ammonia from peptone).

3.3.2 Culture-dependent molecular assessment of bacterial species in *iru* samples

3.3.2.1 Bacterial genomic DNA extraction, quantification and purity determination

The extraction of bacterial genomic DNA follows the preparation of cell-free DNA lysate of pure bacterial isolates by lysozyme-heat lysis method as previously described (Zhang *et al*., 2002) with some modifications. A single colony of bacterial culture from nutrient HiVeg agar

(MV 561, HiMedia, India) or nutrient agar (VM035150 904, Merck, Darmstadt, Germany) was inoculated into 5 ml Luria-Bertani broth (VM941285 805, Merck, Darmstadt, Germany), and incubated overnight at 37^{o}C using an orbital shaking incubator (Remi Instruments, Mumbai, India) at 200 rpm. An optical density (O.D.) of 3 of the bacterial culture volume at 660 nm absorbance was measured (UV1700, Pharmaspec, Shimadzu, Japan).

Appropriate broth culture of each bacterial isolate was centrifuged by pelleting the cells at 8,000×g for 5 min (Microfuge-18, Beckman-Coulter, USA). The cell pellets was washed twice using the same centrifuge, with 1 ml sterile 0.5 M NaCl (K35855004 611, Merck, Darmstadt, Germany) and 1 ml sterile deionized water (Milli Q, Millipore, Bangalore, India), and finally re-suspended in 500 µl of 1X TE buffer (10 mM Tris.Cl, pH 8.0, H5121, Promega, Madison, WI, USA; 1 mM EDTA, pH 8.0, H5032, Promega, Madison, WI, USA) with 10 µl lysozyme (1 mg/ml) (M00069591, Novagen, USA). The cell suspension was incubated at 37°C in water bath (Memmert, Buchschlag-Frankfurt, Germany) for 30 min followed by heating at 95°C for 20 min in a serological water bath (Narang, New Delhi, India) and centrifuged at 10,000×g for 10 min at 4°C (5415 R, Eppendorf).

The supernatant was carefully pipetted into fresh centrifuge tube and the DNA quantity and purity determined by taking absorbance reading at 260 nm (NanoDrop^{TM} 1000 Spectrophotometer, Thermo Scientific, Waltham, MA, USA). The cell free lysate with absorbance ratio of 260 nm to that at 280 nm of approximately 1.8 indicates that the DNA sample is free of protein contamination, which absorbs strongly at 280 nm (Wilson and Walker, 2010). This was used for PCR analysis, and also stored at -20°C until required (MDF-U333, SANYO, Japan). Subsequently, for PCR analysis, DNA dilution was carried out based on the original concentration.

3.3.2.2 PCR amplification of 16S rRNA gene

The 16S rRNA gene ca. 1500 bp of the bacterial isolates was amplified with the universal primers pair fD1 and rD1 (Table 3.2). Each 25 µl PCR reaction mixture contained 2.0 µl of 50 ng DNA in the cell free DNA lysate, 2.5 µl of 10X PCR reaction buffer with 15 mM $MgCl_2$ (P2192, Sigma-Aldrich), 1.0 µl of 25 mM $MgCl_2$ (M8787, Sigma-Aldrich) at a final concentration of 1.0 mM, 0.2 µl of 0.1 mM each of forward and reverse primers (Sigma-Aldrich) at a final concentration of 0.8 µM, 0.2 µl each dNTPs (Sigma-Aldrich) at a final concentration of 200 µM, 0.5 µl of 3 U/µl *Taq* DNA polymerase (D6677, Sigma-Aldrich) and made up to 25 µl with sterile deionized water (Milli Q, Millipore, Bangalore, India).

The 25 µl PCR master mixture was short spun by centrifuge (Microfuge-18, Beckman-Coulter, USA), after which amplification was performed in a master cycler (5333, Eppendorf) and the cycling program was started with an initial denaturation at 94°C for 10 min followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 30 s. The PCR was ended with a final extension at 72°C for 7 min and the amplified products stored at -20°C (MDF-U333, SANYO, Japan) for further studies. Control reaction mixtures were also included in each experiment, positive control containing already amplified DNA sample and negative control lacking DNA template but instead contained sterile deionized water (Milli Q, Millipore, Bangalore, India).

The amplified 16S rRNA gene fragment of about 1500 bp was analyzed by applying 5 µl mixture of the PCR product mixed with 1 µl of 6X loading buffer (M00051831, Novagen, USA) in 0.8% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for 1.5 h. A 1 kb DNA ladder (G571A, Promega, Madison, WI, USA) was used as a size standard in each run; photographs of amplified products taken under UV

light using the gel documentation system (Gel DocEQ, BIO-RAD, USA), and scanning was carried out with Quantity One software (BIO-RAD 1-D Analysis Software version 4.6).

3.3.2.3 Amplified ribosomal DNA restriction analysis (ARDRA)

The amplified 16S rRNA gene of about 1500 bp intact band without non-specific amplification was digested separately each with *Hae*III (R617G, Promega, Madison, WI, USA), *Cfo*I (R624A, Promega, Madison, WI, USA), *Hinf*I (R620B, Promega, Madison, WI, USA), *Dde*I (R629B, Promega, Madison, WI, USA), *Taq*I (R415A, Promega, Madison, WI, USA) and *Rsa*I (R437A, Promega, Madison, WI, USA). An *in silico* analysis using Gene Runner (version 3.05), Clustal-X (version 8.1) and Bioedit (version 5.0.9) software were used to select the restriction enzymes based on their theoretical digestion of 16S rRNA gene sequences of type strains/genome data sourced from NCBI GenBank (release 183) and RDP database (release 10).

Briefly, 10 µl reaction mixture containing 5.0 µl of the 16S rRNA gene PCR amplified products, 1.0 µl of 10X buffer appropriate for each enzyme (1X final concentration) (Promega, Madison, WI, USA), 0.2 µl of 10 mg/ml bovine serum albumin (BSA) acetylated (0.2 mg/ml final concentration) (R396E, Promega, Madison, WI, USA), 2 U each of the restriction enzymes and 3.6 µl sterile deionized water (Milli Q, Millipore, Bangalore, India) was prepared. The reaction mixture was incubated at 37^{o}C overnight (10-16 h). The whole volume, 10 µl was mixed with 2 µl of 6X loading buffer (M00051831, Novagen, USA), and the restriction digested products analysed in 2% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for about 3 h. A 1 kb DNA ladder (G571A, Promega, Madison, WI, USA) or 100 bp DNA ladder (G210A, Promega,

Madison, WI, USA) was used as a size standard marker in each run, and the size of the DNA fragments measured using Quantity One software version 4.6 (BIO-RAD, USA).

3.3.2.4 16S-23S rRNA gene internal transcribed spacer (ITS) PCR amplification

The amplification of the 16S-23S rRNA gene internal transcribed spacer (ITS-PCR) was carried out in a 25 µl reaction volume containing 2.0 µl cell free DNA lysate of 50 ng, 2.5 µl of 10X PCR reaction buffer with 15 mM $MgCl_2$ (P2192, Sigma-Aldrich), 1.0 µl of 25 mM $MgCl_2$ (M8787, Sigma-Aldrich), 1.25 µl each of 10 pmol forward and reverse primers (Sigma-Aldrich) (Table 3.2) at a final concentration of 0.5 µM, 0.2 µl each dNTPs (Sigma-Aldrich) at a final concentration of 200 µM, 0.5 µl of 3 U/µl *Taq* DNA polymerase (D6677, Sigma-Aldrich) and made up to 25 µl with sterile deionized water (Milli Q, Millipore, Bangalore, India). The 25 µl PCR master mixture was short spun by centrifuge (Microfuge-18, Beckman-Coulter, USA), after which amplification was performed in a master cycler (5333, Eppendorf).

The PCR cycling program started with an initial denaturation at 94^{o}C for 5 min followed by 30 cycles of final denaturation at 94^{o}C for 30 s, annealing at 60^{o}C for 30 s and extension at 72^{o}C for 1 min, with a final extension at 72^{o}C for 7 min. The ITS-PCR products were analysed on 1.5% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for about 4 h. A 1 kb DNA ladder (G571A, Promega, Madison, WI, USA) was used as a size standard in each run. Photographs of amplified products were taken under UV light using the gel documentation system (Gel DocEQ, BIO-RAD, USA), and scanning carried out with Quantity One software (BIO-RAD 1-D Analysis Software version 4.6).

3.3.2.5 16S-23S rRNA internal transcribed spacer-restriction fragment length polymorphism (ITS-PCR-RFLP)

The amplified 16S-23S rRNA internal transcribed spacer (ITS) fragments were digested singly with *Cfo*I (R624A, Promega, Madison, WI, USA). Ten microlitres reaction mixture containing 2.0 µl of the ITS-PCR amplified product, 1.0 µl of 10X buffer (1X final concentration) (Promega, Madison, WI, USA), 0.2 µl of 10 mg/ml bovine serum albumin (BSA) acetylated (0.2 mg/ml final concentration) (R396E, Promega, Madison, WI, USA), 2 U of *Cfo*I and 6.6 µl sterile deionized water (Milli Q, Millipore, Bangalore, India) was incubated at 37^{o}C overnight (10-16 h). The whole volume, 10 µl was mixed with 2 µl of 6X loading buffer (M00051831, Novagen, USA), and the restriction digested products analysed in 2% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for about 3 h. A 1 kb DNA ladder (G571A, Promega, Madison, WI, USA) or 100 bp DNA ladder (G210A, Promega, Madison, WI, USA) was used as a size standard marker in each run.

3.3.2.6 Randomly amplified polymorphic DNA PCR (RAPD-PCR)

Six primers, which include OPA 17, OPA 18, OPA 20, OPI 04, OPL 14 and M13, were used for the RAPD-PCR analysis (Table 3.2). RAPD-PCR was carried out in a 12.5 µl master mixture, which contains 1.0 µl DNA template cell free lysate of 25 ng, 1.25 µl of 10X PCR reaction buffer with 15 mM $MgCl_2$ (P2192, Sigma-Aldrich), 1.0 µl of 10 pmol each of the primers, 0.1 µl each dNTPs (Sigma-Aldrich) at a final concentration of 200 µM, 0.2 µl of 3 U/µl *Taq* DNA polymerase (D6677, Sigma-Aldrich) and made up to 12.5 µl with sterile deionized water (Milli Q, Millipore, Bangalore, India). The 12.5 µl reaction mixture was

short spun by centrifuge (5424, Eppendorf), after which amplification was performed in a thermal cycler (BIO-RAD, iCycler, USA).

The first step of the PCR cycling was initial denaturation at 94^{o}C for 5 min followed by 35 cycles of final denaturation at 94^{o}C for 1 min, annealing at 35^{o}C for 1 min and extension at 72^{o}C for 2 min. The PCR was ended with a final extension temperature of 70^{o}C for 7 min. RAPD-PCR products were then analysed on 1.5% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for about 2.5 h. A 1 kb DNA ladder (G571A, Promega, Madison, WI, USA) was used as a size standard in each run and thereafter, photographs of amplified products were taken under UV light using the gel documentation system (Gene Snap, PerkinElmer, USA).

RAPD-PCR using M13 primer was carried out in a master mixture that contained 1.0 µl of 25 ng DNA cell free lysate, 1.25 µl of 10X *Taq* buffer A with 15 mM $MgCl_2$ (105876, Genei, Bangalore, India), 1.0 µl of 10 pmol M13, 0.1 µl each dNTPs (Genei, Bangalore, India) at a final concentration of 200 µM, 0.12 µl of 5 U/µl *Taq* DNA polymerase (116634, Genei, Bangalore, India) and made up to 12.5 µl with sterile deionized water (Milli Q, Millipore, Bangalore, India). Amplification of the short spun 12.5 µl reaction volume was performed in a master cycler (5333, Eppendorf) and the thermocycle programme consisted of one cycle of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 36°C for 1 min and 72°C for 1.5 min, and then one cycle of 72°C for 5 min. The PCR products (2 µl) were analysed as described previously. Banding patterns of ARDRA, ITS-PCR, ITS-PCR-RFLP and RAPD-PCR polymorphisms were scored manually and grouped using NTSYSpc. 2.20e (Rohlf, 1998) for the generation of clusters in a dendrogram based on Jaccard similarity coefficient (S_J) and unweighted pair group method using arithmetic averages (UPGMA).

3.3.2.7 Multilocus sequence analysis (MLSA)

Five housekeeping genes were used for differentiation of *B. cereus* phylogeny (Table 3.2). PCR amplification of 25 µl reaction volume, which contained 2.0 µl DNA template cell free lysate of 50 ng, 2.5 µl of 10X PCR reaction buffer with 15 mM $MgCl_2$ (P2192, Sigma-Aldrich), 0.625 µl of 0.01 mM each of both forward and reverse primers at a final concentration of 0.25 µM (Sigma-Aldrich), 0.2 µl each dNTPs (Sigma-Aldrich) at a final concentration of 200 µM, 0.25 µl of 5 U/µl *Taq* DNA polymerase (D1806, Sigma-Aldrich) at a final concentration of 1.25 U and 18.8 µl sterile deionized water (Milli Q, Millipore, Bangalore, India) was performed in a master cycler (5333, Eppendorf).

The PCR conditions included an initial denaturation at 94°C for 5 min followed by 36 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 30 s. The PCR was ended with a final extension at 72°C for 7 min and the amplified product stored at -20°C (MDF-U333, SANYO, Japan). Control reaction mixtures were also included in each experiment, positive control containing already amplified sample and negative control lacking DNA template but instead contained sterile deionized water (Milli Q, Millipore, Bangalore, India).

The amplified products were analyzed by applying 2 µl of the PCR product in 1.5% agarose (V3121, Promega, Madison, WI, USA) gel containing 5 µl ethidium bromide of 0.5 µg/ml (E1510, Sigma-Aldrich), using 0.5X TBE buffer (45 mM Tris-borate; 1 mM EDTA, pH 8.0) in BIO-RAD Sub-cell GT and accessories (BIO-RAD, Berkeley, CA, USA) and run at 80V for about 2 h. A PCR 100 bp low ladder (P1473, Sigma-Aldrich) was used as a size standard in each run and photographs of amplified products taken under UV light using the gel documentation system (Gene Snap, PerkinElmer, USA).

Table 3.2: List of PCR primers for *Bacillus* species characterization and identification using culture-dependent molecular techniques.

Primer names	Primer sequences	Target regions	References
fD1	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA	Weisburg *et al.*(1991); Escalante *et al.* (2001)
rD1	5′-AAGGAGGTGATCCAGCCGCA-3′		
16Sf-R2	5′-CGCGGGATCCTTGTACACACCGCCCGTC-3′	16S-23S rRNA	Lechner *et al.* (1998)
23Sr-R10	5′-GGCCGTCGACCCTTTCCCTCACGGTACTG-3′		
OPA 17	5′-GACCGCTTGT-3′	ra	This study
OPA 18	5′-AGGTGACCGT-3′	ra	Pulido *et al.* (2005)
OPA 20	5′-GTTGCGTCC-3′	ra	This study
OPI 04	5′-CCGCCTAGTC-3′	ra	This study
OPL 14	5′-GTGACAGGCT-3′	ra	This study
M13	5'-GAGGGTGGCGGTTCT-3'	ra	This study
gyrB-BC-F	5'-TGAATTGACACGCCGCAAGAGC-3'	DNA gyrase subunit B	This study
gyrB-BC-R	5'-AACGCACTTCTGGGTCCATTGTCG-3'		
glpF-BC-F	5'-GCGTTTGTGCTGGTGTAAGT-3'	glycerol uptake facilitator protein	This study
glpF-BC-R	5'-CTGCAATCGGAAGGAAGAAG-3'		
gmk-BC-F	5'-ATTTAAGTGAGGAAGGGTAGG-3'	guanylate kinase putative	This study
gmk-BC-R	5'-GCAATGTTCACCAACCACAA-3'		
rpoA-BC-F	5'-CCTGGTGCCGCTGTTACTGCTATC-3'	RNA polymerase subunit alpha	This study
rpoA-BC-R	5'-GCCCGATGCTTCCATCCGTCCATAC-3'		
pheS-BC-F	5'-AGCAGAAGAGCGTCCACGTATGGG-3'	phenylalanyl-tRNA synthetase subunit alpha	This study
pheS-BC-R	5'-CGCAACCTTTGCCGTGACACATC-3'		

[ra] random amplification

3.3.2.8 Phylogenetic construction of 16S rRNA and housekeeping genes sequences

The 16S rRNA genes of representative bacterial strains within the formed clusters, including *gyrB, glpF* and *gmk* housekeeping amplicon genes were sequenced using ABI 3100 DNA sequencer (Applied Biosystems, Life Technologies, NY, USA) with the same primers used for amplification as carried out by service providers (GeNei and MWG, Bangalore, India). The electropherogram sequence data obtained were validated using Chromas LITE (version 2.01) and Gene Runner (version 3.05) software. In determining the closest known relatives of the sequences, searches were performed using the GenBank database BLAST programme and compared with those deposited in GenBank (Altschul *et al*., 1997). These sequences were eventually submitted to GenBank NCBI for accession numbers. Pairwise and multiple alignments of these sequences including those of related bacterial species were carried out using ClustalW 2.0.12 (Thompson *et al*., 1994), according to the Kimura two-parameter model (Kimura, 1980). Phylogenetic and molecular evolutionary analyses were conducted using MEGA5 (Tamura *et al*., 2011). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985).

3.4 Probiotic examinations of *Bacillus* strains

Two *B. subtilis* strains, *B. subtilis* U170B and *B. subtilis* U146A (accession numbers: JN255720 and JN255713 respectively) were selected for probiotic investigations due to better functional properties such as production of hydrolytic enzymes, especially protease, faster growth rate (lower generation time) and adaptation to the fermentation substrate (*P. biglobosa*). *B. clausii* UBBC-07 (MTCC 5472), a probiotic reference strain kindly provided by Unique Biotechnology Limited, Hyderabad, India, was also included.

3.4.1 Resistance of *Bacillus* strains to acidic pH and bile salts tolerance

Bacilli vegetative cells and spores resistance to simulated gastric juice (SGJ) of the stomach and simulated intestinal fluid (SIF) of the small intestine were assayed as described by Duc *et*

al. (2004a) with some modifications. *Bacillus* spores were previously induced and prepared in Difco Sporulating Medium (DSM) using modified nutrient exhaustion method of Nicholson and Setlow (1990). Both vegetative cells and spores were suspended in 0.85% NaCl, pH 2 containing 1 mg/ml pepsin (porcine gastric mucosa P7000, Sigma-Aldrich, UK), and isotonic buffer [Bott and Wilson salts: K_2HPO_4-1.24%; KH_2PO_4-0.76%; tri-Sodium citrate-0.1%; $(NH_4)_2SO_4$-0.6%, pH 6.7] containing 0.3% (w/v) bile salts concentration (CR 008, HiMedia, India) and 1 mg/ml pancreatin (porcine pancreas P7545, Sigma-Aldrich, USA) for acid resistance and bile salts tolerance respectively. Aliquots were immediately taken and after 1, 2 and 3 h incubation at 37^{o}C with agitation. Appropriate serial dilutions of suspensions were pour-plated for CFU/ml on nutrient and DSM agar to determine initial cells and spores counts and subsequent populations. The experiments were conducted in two replicate for both acid and bile.

3.4.2 Antimicrobial activity of *Bacillus* strains against food-borne pathogens

The ability of *Bacillus* strains to produce antimicrobial substances against closely related species of organisms or genera, including food-borne pathogens was determined using two methods. The first is the agar spot-on-lawn test as described by Schillinger and Lücke (1989) and the colony overlay assay method by Pugsley (1985) with some modifications. The second is the agar well diffusion assay, which involves the use of cell-free supernatant as earlier described (Wang *et al*., 2010).

For the first method, overnight bacilli cultures in brain heart infusion (BHI) broth (M 210, HiMedia, India) were centrifuged at 8,000 rpm for 5 min. Cell pellets washed in 0.5 M NaCl (Merck, Germany) were resuspended in peptone saline, corresponding to 10^8 CFU/ml. Five microlitres of the cell suspension was spotted onto the surface of dried BHI agar (M 211 HiMedia, Mumbai, India), aerated and allowed to dry, followed by incubation at 37^{o}C for 18 h. Duplicate spotting was carried out for results validation. The seeded agar plates were

overlaid with 7 ml soft BHI agar (0.7%) containing 100 µl broth culture of the indicator organisms; allowed to solidify and re-incubated.

The presence of zones of growth inhibition around the bacterial spots at any of the times examined (7, 14, 18 h post-inoculation) indicates a positive result. The tested indicator bacterial strains studied include *L. sakei* DSM 20017, *L. casei* DSM 20011, *S. aureus* subsp. *aureus* ATCC 11632, *E. coli* ATCC 11229, *Listeria monocytogenes* ATCC 19118, *E. faecium* ATCC 35667, *B. cereus* MTCC 430, and in addition, *B. cereus* MBU 1011, *S. aureus* MBU 1023, *E. coli* MBU 1035 and *S. enterica* serovar Typhimurium MBU 1047 provided by the culture collection, Molecular Biology Unit (MBU), National Dairy Research Institute (NDRI), Karnal, India.

In the second method, already prepared BHI agar plates were overlaid with soft BHI agar (0.7%), 7 ml containing 100 µl of the indicator organisms. Dried plates with bored holes of 5 mm diameter were filled with 30 µl cell-free culture supernatant (pH 7.4) of producer strains obtained by centrifugation (8,000 rpm for 7 min) and sterilization through filtration [(0.22 µm), Millipore, Tullagreen, Carrigtwohill, Ireland], and thereafter incubated.

3.4.3 Bacterial cell surface hydrophobicity (BCSH)

Cell surface hydrophobicity (CSH) of the *Bacillus* strains was quantitatively determined by applying bacterial adhesion to solvents (BATS) assay (Bellon-Fontaine *et al*., 1996), based on their relative tendency to adhere to non-polar/apolar materials/solvents such as *n*-hexadecane, xylene and toluene, including chloroform, a monopolar acidic solvent (electron donor), compare to water (Doyle and Rosenberg, 1990).

A single colony of pure bacterial agar plate was inoculated into nutrient broth (M002, HiMedia, India) overnight at 37^{o}C in incubator shaker (innova 4230, New Brunswick, NJ, USA) at 180 rpm. The broth culture at mid-log growth phase (16 h) was centrifuged (Sigma 6K 15, Sartorius, Germany) at 8,000 rpm room temperature for 7 min to make cell pellets.

The pellets were washed twice with phosphate-urea-magnesium (PUM) buffer containing 22.2 g K_2HPO_4. $3H_2O$ (RM 168, HiMedia, India); 7.26 g KH_2PO_4 (RM1188, HiMedia, India); 1.8 g urea (300191, Stratagene, CA, USA); 0.2 g $MgSO_4$. 7 H_2O (M2773, Sigma-Aldrich, Japan) per 1000 ml distilled water; pH 7.1 and resuspended in appropriate volume of PUM buffer with optical density (O.D.) of 0.8±0.02 at 600 nm (Elisa Reader, BioTek, VT, USA), usually containing approximately 10^8-10^9 colony forming unit per ml (CFU/ml).

Aliquots of 3.6 ml bacterial cell suspension was transferred into moisture-free, dried sterile test-tubes followed by addition of 400 µl *n*-hexadecane (RM 2238, HiMedia, India), toluene (34866, Sigma-Aldrich, Germany) and chloroform (67-66-3, Thermo Fisher Scientific, India). The tubes were incubated at room temperature for 10 min for temperature equilibrium and vortexed for 15 s. Thereafter, they were incubated at 37^{o}C undisturbed for 1 h to enable phase separation and partitioning of the hydrocarbons and aqueous phases. The lower aqueous phase was carefully collected and O.D. determined at 600 nm. Percentage hydrophobicity was calculated as described by Rosenberg *et al*. (1983).

$$\% \text{ hydrophobicity} = ODi - OD_t/OD_i \times 100$$

where OD_i = initial O.D. of cell suspension at 600 nm and OD_t = O.D. of aqueous phase at time t.

3.4.4 Autoaggregation assay

Autoaggregation assay was carried out as described by Del Re *et al*. (2000) with slight modifications. Bacterial cells were pelleted, washed twice and resuspended in sterile phosphate-buffered saline [PBS, pH 7.4, NaCl-8g (RM853, HiMedia, India), KH_2PO_4-0.34g, K_2HPO_4-1.21g per liter]. The $O.D._{600\ nm}$ was adjusted to a bacterial population of about 10^9 CFU/ml, and 4 ml aliquots cell suspension transferred into moisture-free, dried sterile test-tubes, which were vortexed for 15 s and incubated undisturbed for 30 min, 60 min, 90 min,

120 min, 180 min and 240 min at 37^{o}C, where a tube represent a time interval each. After incubation, the upper fraction was carefully collected and O.D. measured at 600 nm.

% autoaggregation = ODi - OD_t/OD_i x100

where OD_i = initial O.D. of cell suspension at 600 nm and OD_t = O.D. of aqueous phase at time t.

3.4.5 Coaggregation assay

Coaggregation assay was performed according to Handley *et al*. (1987) with modifications. *Bacillus* strains and *S. enterica* subsp. *enterica* serovar Typhimurium LT2 [obtained from Culture Collection, Molecular Biology Unit (MBU), NDRI, Karnal, India] were cultured in nutrient and BHI broths respectively. Cell pellets of both organisms were washed and resuspended in sterile PBS (pH 7.4), with O.D. adjusted to ca. 10^9 CFU/ml. Two millilitres aliquots cell suspension each of *Bacillus* and *Salmonella* were mixed together in a sterile test-tube and vortexed for 10 s. Also, 4 ml aliquots of both cultures as individual control were made and all suspensions incubated undisturbed for 30 min, 60 min, 90 min, 120 min, 180 min and 240 min at 37^{o}C. The upper fraction was carefully collected and $O.D._{600\ nm}$ determined. Kinetics of coaggregation was calculated as follows:

% Coaggregation = [(ODx+ODy)/2-(ODxy)/(ODx+ODy)/2] X 100

where ODx and ODy are the optical densities of *Bacillus* and *Salmonella* control suspension respectively and ODxy represents optical density of mixed cultures after time t, at 600 nm.

3.4.6 Cell culture studies

3.4.6.1 HT-29 cell lines propagation and maintenance

HT-29 cell lines (NCI-PBCF-HTB38), originated from human colon adenocarcinoma epithelial was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were routinely cultured and maintained in 25 cm^2 tissue culture flask (Falcon, Beckton Dickinson, Sparks, MD, USA) and 24-well tissue culture plates (Falcon 24-

well, Beckton Dickinson, Sparks, MD, USA) containing Dulbecco's modified Eagle's minimal essential medium (4.5 g/l glucose; DMEM-high glucose, L-glutamine and sodium pyruvate) (DMEM, D7777-1L, Sigma-Aldrich, USA), supplemented with 10% (v/v) heat-inactivated (56^{o}C for 30 min) foetal bovine serum (FBS, 10270, Gibco, Invitrogen, MA, USA), 1% (v/v) non-essential amino acids (SH3023801, HyClone, Thermo Scientific, MA, USA), antibiotics [100 U/ml penicillin G (PENNA, Sigma-Aldrich, China) and 100 μg/ml streptomycin sulphate (S1277, Sigma-Aldrich, USA)], 2.5 g/l sodium bicarbonate ($NaHCO_3$; 1944142, SRL, Mumbai, India), 1% (w/v) HEPES (H-0891, Sigma-Aldrich, USA) at 37^{o}C in an incubator of atmospheric conditions of 95% (v/v) humidified air, 5% (v/v) CO_2 and 19.8% O_2 (NuAire, Plymouth, MN, USA).

For maintenance and routine cell line analyses, HT-29 was passaged/splitted after attaining 80% confluent. Spent DMEM was carefully drained and cells recovered washed once with 1 ml sterile PBS/DMEM. This was followed by addition of 1 ml of 0.25% trypsin-EDTA solution (SH30042.02, HyClone, Thermo Scientific, USA) for cell detachment. Thereafter, 2 ml DMEM was added to the flask to deactivate trypsin, and the whole content centrifuged (8,000 rpm for 4 min) to pellet the cells. They were later resuspended in 5 ml DMEM, dispensed into different tissue culture flasks and plates meant for passaging and incubated until confluent is formed.

3.4.6.2 *In vitro* adhesion assay of *Bacillus* strains

Adhesion properties of *Bacillus* strains were examined using propagated HT-29 differentiated and undifferentiated cells. Spent medium was completely removed 18 h prior to challenge study and cells fed with fresh basal DMEM devoid of FBS and antibiotics [for differentiated cells HT-29 were treated with forskolin (F3917, Sigma-Aldrich, USA)]. *Bacillus* cultures at mid-log phase grown in BHI broth at 37^{o}C (bacteria grown in BHI do not form spores) were centrifuged at 7,000 rpm for 6 min, washed twice and resuspended in sterile PBS (pH 7.4).

Five microlitres of 2 μM 6-carboxyfluorescein diacetate [(CFDA), C5041, Sigma-Aldrich, USA] was added and incubated for 30 min at 37^{o}C in the dark for fluorescent-tagging and staining of bacterial cells. After incubation, cells were washed 4 times with sterile PBS (pH 7.4) at 7,000 rpm for 6 min, and later resuspended in basal DMEM of O.D. corresponding to ca. 10^8 CFU/ml. Just prior to assays, HT-29 cell line monolayers, in 24-well tissue culture plates, were washed, each well twice using 1 ml sterile PBS (pH 7.4) and inoculated with 1 ml of 10^8 CFU bacterial cells seeded with 10^6 cell line monolayers, which makes a bacterial (prokaryotic) to cell (eukaryotic) ratio of 100:1, i.e. a multiplicity of infection (MOI) of 100:1 bacteria per epithelial cells.

Assay was performed in two replicate and plates incubated for 2 h at 37^{o}C under humidified atmosphere. After incubation and infection, supernatants were drained and discarded, and wells were gently washed 3 times each with 1 ml sterile PBS (pH 7.4) to remove non-adherent bacterial cells. HT-29 cell monolayers were lysed with 1 ml of 0.05% Triton-X 100 (T8787, Sigma-Aldrich, USA) or 1 ml of sterile Milli Q water containing 0.1% bovine serum albumin (BSA, MB083, HiMedia, India) for 3-5 min and then aspirated to collect the adherent bacterial cells.

Three independent techniques were used to assess the adhesion potential or otherwise percent adhesion of the bacterial cells to HT-29, namely, microscopic counting (dark field), fluorescence (Elisa Reader, BioTek, VT, USA) and viable count. For enumeration using microscope, HT-29 cells cultured in glass cover slips were used for counting the number of adhering bacterial cells. For the second method, fluorescence of diluted bacterial suspension, i.e. initial bacterial suspension (10^8 CFU/ml) and those from the control wells were measured at 490 nm filters for excitation and 525 nm filters for emission. The readings obtained were plotted against corresponding bacterial population of the diluents to have a standard curve. For viable counts, adherent bacterial cell suspension recovered by lysis of HT-29 were

serially diluted in 0.85% saline and pour-plated on BHI agar, incubated at 37^{o}C for 18-24 h and cells counted (B_1 CFU/ml). Bacterial cells initially added to each of the 24-well plates were also serially diluted and counted as (B_0 CFU/ml). Percentage adhesion was then calculated as:

$$\% \text{ adhesion} = (B_1/B_0) \times 100$$

3.4.6.3 Bacilli inhibition of *S. enterica* serovar Typhimurium MBU 1047 adherence to HT-29 cell lines

The ability of *Bacillus* strains to inhibit *S. enterica* serovar Typhimurium MBU 1047 adhesion to HT-29 cells was assayed following previously described methods with modifications (Yu *et al*., 2011). First, HT-29 cell monolayers at 80% confluent (approximately 10^6 cells) were seeded in 24-well tissue culture plates and medium replaced with fresh basal DMEM 18 h prior to experiment. Bacterial cell suspensions were prepared as earlier described (but not tagged with CFDA), with MOI of 100:1 (*Bacillus* strains to epithelial cells) and 10:1 (*S. enterica* serovar Typhimurium MBU 1047 to epithelial cells).

In the exclusion assay, HT-29 cells were washed twice with 1 ml sterile PBS, pH 7.4 and thereafter inoculated with 500 µl cell suspensions of bacilli (10^8 CFU/well) in DMEM and incubated at 37^{o}C for 2 h in 5% CO_2 (v/v) and 95% humidified air (v/v). After incubation, medium was discarded and non-adherent cells of *Bacillus* strains removed by washing cell lines three times with 1 ml sterile PBS (pH 7.4), each well. Immediately after this, HT-29 were again inoculated with 150 µl cell suspensions of *S. enterica* serovar Typhimurium (10^7 CFU/well) in DMEM and further incubated for 2 h at 37^{o}C in 5% CO_2 (v/v) and 95% humidified air (v/v).

In the competition assay, HT-29 cell monolayers were infected with *Bacillus* strains (10^8 CFU/well) and *S. enterica* serovar Typhimurium MBU 1047 (10^7 CFU/well) simultaneously, and also incubated under conditions stated earlier. For displacement assay, *S. enterica* serovar

Typhimurium MBU 1047 at 10^7 CFU/well were added first to HT-29 cells and incubated at 37^{o}C for 2 h in 5% CO_2 (v/v) and 95% humidified air (v/v). Medium was discarded after incubation and non-adhering cells of *S. enterica* serovar Typhimurium MBU 1047 removed by washing cells three times with 1 ml sterile PBS (pH 7.4). Following this, HT-29 cell lines were further challenged with *Bacillus* strains (10^8 CFU/well) and cultured plates subsequently incubated for another 2 h at 37^{o}C in 5% CO_2 (v/v) and 95% humidified air (v/v). HT-29 wells containing *S. enterica* serovar Typhimurium MBU 1047 alone without *Bacillus* strains served as controls.

At the end of all assays, supernatants were drained and discarded, while non-adhering bacterial cells detached by gentle washing of cell lines three times with sterile PBS (pH 7.4). Adhering cell suspensions of *S. enterica* serovar Typhimurium MBU 1047 were obtained by lysis of HT-29 cell monolayers with Milli Q water containing 0.1% bovine serum albumin (BSA, MB083, HiMedia, India). Serial dilution of cell suspensions in 0.85% saline was carried out, spread-plated on xylose, lysine, deoxycholate (XLD) agar (MH031, HiMedia, India) and incubated overnight at 37^{o}C to obtain viable counts of *S. enterica* serovar Typhimurium MBU 1047. In all the experiments, two biological replicates were conducted for each bacterial strain per assay. The ability of *Bacillus* strains to exclude, compete and displace *S. enterica* serovar Typhimurium MBU 1047 was determined by comparing adhesion of *S. enterica* serovar Typhimurium MBU 1047 to cell lines in the presence of *Bacillus* ($S.T._{test}$) to that of *S. enterica* serovar Typhimurium MBU 1047 alone, i.e. in the absence of *Bacillus* ($S.T._{control}$). Percentage of *S. enterica* serovar Typhimurium MBU 1047 prevented from adhering to HT-29 cells = $(S.T._{control}-S.T._{test})/(S.T._{control}) \times 100$

3.4.7 Safety assessment of *Bacillus* strains

3.4.7.1 Haemolysis on blood agar

Bacillus strains (*B. subtilis* U170B, *B. subtilis* U146A, *B. clausii* UBBC-07, *B. cereus* U175 and a reference haemolytic *B. cereus* MBU 1011) grown in nutrient broth (M002 HiMedia, India) for 18 h at 37^{o}C were streaked on BHI agar supplemented with 5% sheep blood and incubated at 37^{o}C for 18-24 h. A β-haemolytic activity is indicated by the presence of clear zone around the streaked area (positive), α-haemolysis is associated with partial clearance zone and greenish colouration around the streaked region, while (γ-haemolysis) is without clearance zone, which is considered negative (De Vuyst *et al*., 2003).

3.4.7.2 Antibiotic susceptibility testing

The susceptibility of *Bacillus* strains to antibiotics was determined by modification of the standard disk and agar overlay diffusion methods of Clinical and Laboratory Standards Institute (2009), formerly National Committee for Clinical Laboratory Standards (NCCLS). Antibiotic disks analysed include: penicillin G (P, 10 units/disk), ampicillin (AMP, 10 µg/disk) (β-lactams); erythromycin (E, 15 µg/disk), vancomycin (VA, 30 µg/disk) (Gram+ve spectrum); nalidixic acid (NA, 30 µg/disk) (Gram-ve spectrum); chloramphenicol (C, 30 µg/disk), rifampicin (RIF, 5 µg/disk), tetracycline (TE, 30 µg/disk) (broad spectrum); kanamycin (K, 30 µg/disk), gentamicin (GEN, 10 µg/disk), streptomycin (S, 10 µg/disk) (aminoglycosides), others are amoxyclav (AMC, 30 µg/disk), clindamycin (CD, 2 µg/disk), ciprofloxacin (CIP, 5 µg/disk), methicillin (MET, 5 µg/disk), trimethoprim (TR, 5 µg/disk) and norfloxacin (NX, 10 µg/disk).

In brief, already prepared BHI agar plates were overlaid with soft BHI agar (0.7%), 7 ml at 50^{o}C containing 100 µl of bacterial isolates at log phase growth. After solidification, the antibiotic disks (HiMedia, India) were aseptically placed onto the agar surface and plates subsequently incubated at 37^{o}C for 18-24 h to allow for bacterial-antibiotic interaction.

Diameters of zones of inhibition in two replicate, including that of disks (in mm) were measured and results expressed in terms of resistance (R) and susceptibility (S) in accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS).

3.4.7.3 Mucin degradation by Petri-dish assay

The minimal anaerobic culture medium (medium B) according to Ruseler-van Embden *et al*. (1995) was used for mucin degradation analysis with slight modifications, which contained (g/l): tryptone (RM 014, HiMedia, India) 7.5; peptone, bacteriological (RM 001, HiMedia, India) 7.5; yeast extract powder (RM 027, HiMedia, India) 3.0; beef extract powder (RM 002, HiMedia, India) 5.0; NaCl (RM853 HiMedia, India) 5.0; $K_2HPO_4.3H_2O$ (RM 168, HiMedia, India) 3.0; KH_2PO_4 (RM 1188, HiMedia, India) 0.5; $MgSO_4.7H_2O$ (RM683, HiMedia, India) 0.5. Mucin from porcine stomach, type III (M1778, Sigma-Aldrich, USA) and agarose (014011, SRL, Mumbai, India) were incorporated at concentrations of 0.5% (w/v) and 1.5% (w/v) respectively, and with or without 3% dextrose (RM077, HiMedia, India).

Overnight grown *Bacillus* cultures (4 µl), including positive (faecal flora) and negative (heat killed faecal flora) samples were spotted onto the plates containing medium B, mucin, agarose and with or without 3% dextrose. After about 18 h incubation, plates were stained with 0.1% amido black 10B (Loba Chemie, Mumbai, India) in 3.5 M acetic acid glacial (21057, Qualigens, Thermo Fisher Scientific, Mumbai, Inida) for 30 min and thereafter destained with 1.2 M acetic acid glacial until the mucin lysis zone (discoloured halo) around the colony of positive control culture appeared. The mucin degradation activity was defined as the size of the mucin lysis zone.

3.5 Statistical analysis

Data were analyzed using GraphPad Prism 5 software and expressed as means ± standard error of mean (SEM). Statistical tests of significance were performed using Multifactor ANOVA, and where appropriate, the post-hoc mean comparison based on Duncan Multiple Range Test (DMRT) was used (Statgraphics® Centurion XVI).

CHAPTER FOUR

RESULTS

CHAPTER FOUR

4.0 Results

4.1 Analysis of bacterial communities and diversity of *iru* using PCR-DGGE

The two genomic DNA extraction protocols- enzymatic and chemical were efficient in terms of quantity and purity, producing DNA templates suitable for PCR amplification (Figure 4.1). The results of the PCR-DGGE gel fingerprints of the V3 variable region of amplified 16S rRNA gene showed identical DGGE patterns and high degree of similarity in DNA fragments with the two DNA extraction methods used (Figure 4.2). Similarities and variations in DGGE gel patterns of the various fermented condiments were established based on combined analysis of Dice similarity coefficient and nMDS.

The results showed that the bacterial composition of the sixteen *iru* samples clustered into five groups- a, b, c, d and e (Figure 4.2). Groups: (a) consists of 7 samples (5 from South-West and 2 from North-Central Nigeria); (b) has only 1 sample from South-West Nigeria; (c) comprise 5 samples (all from South-West Nigeria); (d) contains 2 samples from South-West Nigeria; (e) also has only 1 sample from South-West Nigeria. Noticeable similarity was found in the bacterial community structure of some samples of *iru* obtained from the two geographical zones under study (South-West and North-Central Nigeria). Apart from the two *iru* samples from Lagos South-West Nigeria that clustered into the same group 'a', variation was observed among samples within the same geographical location especially South-West *iru* samples.

PCR-DGGE gel profiles were also used to assess the bacterial species richness, biodiversity and dominance indexes of *iru* samples obtained from different geographical locations in Nigeria. Bacterial diversity index (*H*) calculated on the basis of number and intensity of bands on a gel track was highest for *iru* samples from Ilorin (*H* 2.92) with the lowest being Abeokuta (2.19<*H*<2.80). The species richness index (*R*) determined was also highest

for DGGE profiles of *iru* samples from Ilorin (*R*▭19), exhibiting the highest number of bands/bacterial species than those of Lagos (16<*R*<18); Ado-Ekiti (*R*◊16); Ibadan (11<*R*<14); Oyo (*R*▽10) and Abeokuta (9<*R*<17) (Table 4.1).

4.2 Identification of major bacterial PCR-DGGE bands

DNA sequencing was carried out on the major DGGE bacterial bands. The closest known identities, percentage similarities and frequency of occurrence of each band in the fermented condiments are as shown in Table 4.2. *B. subtilis* occurred most frequently (band 10; Figure 4.3), and was found to be present in 94% of the *iru* samples, which confirmed its consistency, dominance and viability in the fermented condiments. *B. licheniformis* and *Brevibacillus parabrevis* were also found to be present in at least one *iru* sample (Table 4.2). Other bands identified included close relatives of *Tetragenococcus halophilus*, *Salinicoccus jeotgali*, *Ureibacillus thermosphaericus*, *Brevibacterium* sp., including food-borne pathogens and contaminants such as *Staphylococcus vitulinus*, *S. saprophyticus*, *B. thuringiensis*, *Morganella morganii* and uncultured bacteria clones.

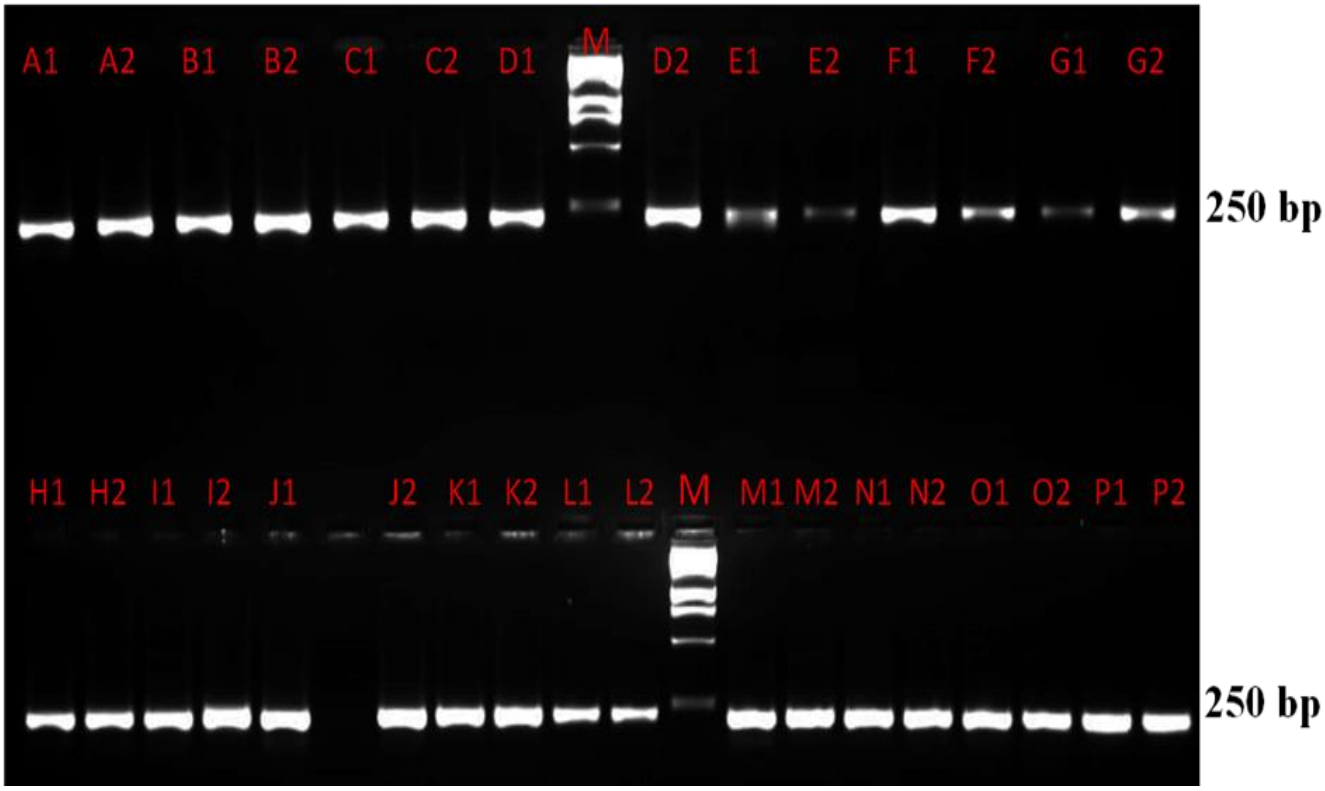


Figure 4.1. PCR amplification of variable V3 (~ 240 bp) region of bacterial 16S rRNA gene in different *iru* samples, based on two genomic DNA extraction protocols- enzymatic (1) and chemical (2). Lanes: A1 & A2 (*iru* Abeokuta 1); B1 & B2 (*iru* Abeokuta 2); C1 & C2 (*iru* Abeokuta 3); D1 & D2 (*iru* Abeokuta 4); E1 & E2 (*iru* Abeokuta 5); F1 & F2 (*iru* Abeokuta 6); G1 & G2 (*iru* Abeokuta 7); H1 & H2 (*iru* Abeokuta 8); I1 & I2 (*iru* Lagos 1); J1 & J2 (*iru* Lagos 2); K1 & K2 (*iru* Ilorin 1); L1 & L2 (*iru* Ilorin 2); M1 & M2 (*iru* Ibadan 1); N1 & N2 (*iru* Ibadan 2); O1 & O2 (*iru* Oyo); P1 & P2 (*iru* Ado-Ekiti); M: 1 kb DNA ladder.

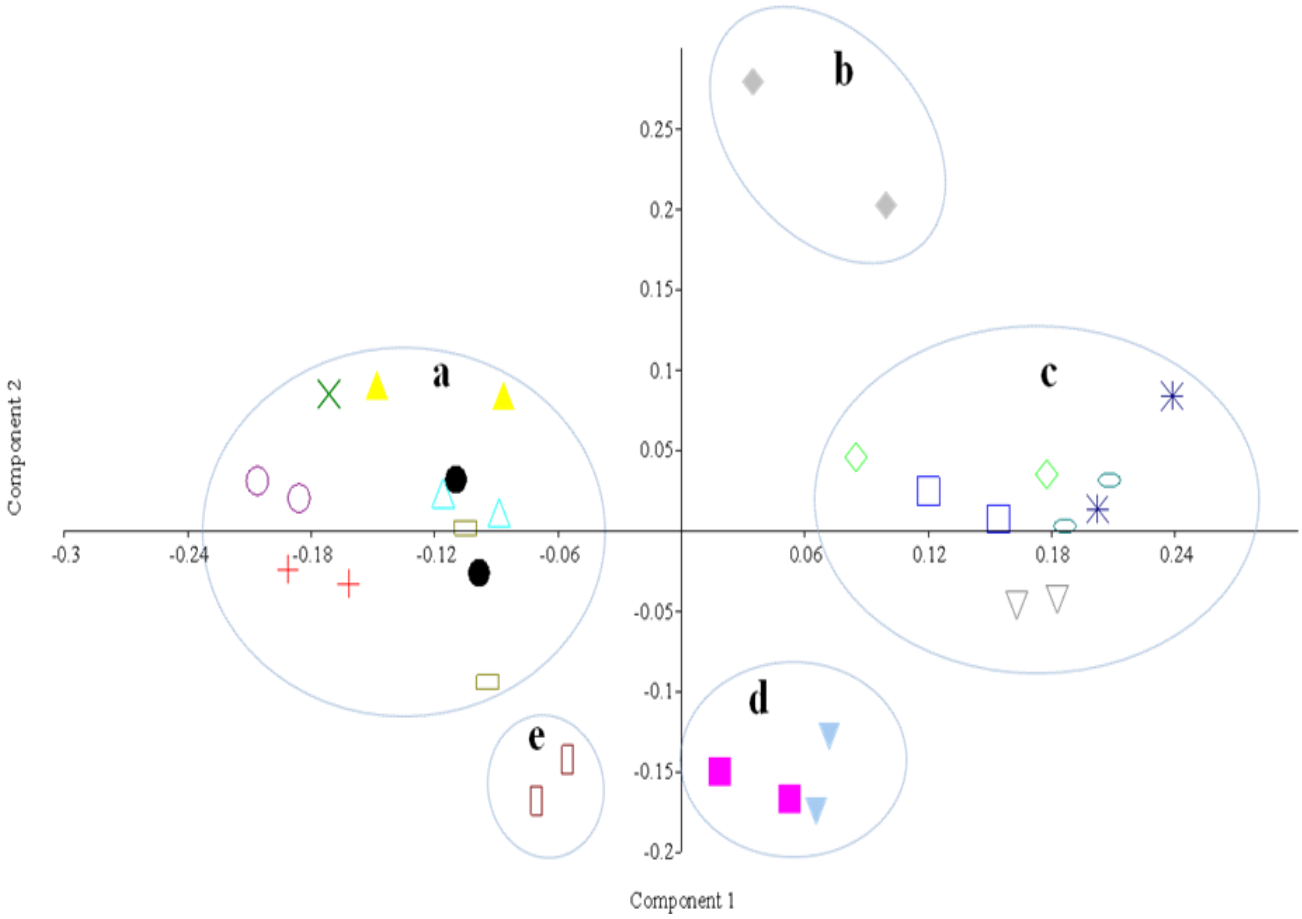


Figure 4.2. Principal component of non-metric multidimensional scaling analysis of DGGE data. Group (a): *iru* Abeokuta 2 South-West Nigeria (+); *iru* Abeokuta 3 South-West Nigeria (○); *iru* Lagos 1 South-West Nigeria (▲); *iru* Lagos 2 South-West Nigeria (●); *iru* Ilorin 1 North-Central Nigeria (△); *iru* Ilorin 2 North-Central Nigeria (▭); *iru* Ibadan 1 South-West Nigeria (≈). Group (b): *iru* Abeokuta 4 South-West Nigeria (◆). Group (c): *iru* Abeokuta 1 South-West Nigeria (□); *iru* Abeokuta 5 South-West Nigeria (⬭); *iru* Abeokuta 8 South-West Nigeria (☆); *iru* Oyo South-West Nigeria (▽); *iru* Ado-Ekiti South-West Nigeria (◇). Group (d): *iru* Abeokuta 6 South-West Nigeria (■); *iru* Ibadan 2 South-West Nigeria (▼). Group (e): *iru* Abeokuta 7 South-West Nigeria (▯).

Table 4.1: Bacterial species richness estimates (*R*) and Shannon's index of diversity (*H*) of DGGE profiles of *iru* samples obtained from various geographical locations in Nigeria.

Products/locations	*R*	*H*
iru Abeokuta 1	12	2.44
iru Abeokuta 2	13	2.57
iru Abeokuta 3	17	2.80
iru Abeokuta 4	12	2.49
iru Abeokuta 5	11	2.34
iru Abeokuta 6	11	2.35
iru Abeokuta 7	15	2.70
iru Abeokuta 8	9	2.19
iru Lagos 1	18	2.88
iru Lagos 2	16	2.74
iru Ilorin 1	19	2.92
iru Ilorin 2	19	2.92
iru Ibadan 1	11	2.40
iru Ibadan 2	14	2.60
iru Oyo	10	2.25
iru Ado-Ekiti	16	2.77
Mean *iru* Abeokuta (±STDEV)	12.50±2.35	2.49±0.19
Mean *iru* Lagos (±STDEV)	17.00±1.00	2.81±0.05
Mean *iru* Ilorin (±STDEV)	19.00±0.00	2.92±0.00
Mean *iru* Ibadan (±STDEV)	12.50±1.50	2.50±0.10

Table 4.2: Identities of major bacterial bands excised from PCR-DGGE gels of *iru* samples.

Bands	Sources/locations[ϕ]	16S rRNA gene closest known relative	% Similarity	Accession no.[†]
1	Oyo (1), Abeokuta (4), Ado-Ekiti (1), Lagos (1), Ibadan (1), Ilorin (2)	*S. vitulinus*	98	KF318035
2	Ado-Ekiti (1), Ibadan (2), Abeokuta (4), Lagos (2), Ilorin (2)	*M. morganii*	98	KC250021
3	Abeokuta (2), Ibadan (1), Lagos (1)	*B. thuringiensis*	99	KF583683
4	Abeokuta (2), Ibadan (1), Ilorin (1)	*B. licheniformis*	98	KF242348
5	Oyo (1), Abeokuta (1), Lagos (1), Ilorin (1)	*S. saprophyticus*	99	JF920014
6	Oyo (1), Abeokuta (7), Ado-Ekiti (1), Lagos (2), Ilorin (2)	Uncultured bacterium clone	97	JF154559
7	Abeokuta (3), Ibadan (1), Lagos (1)	*T. halophilus*	95	EU689055
8	Oyo (2), Ibadan (2), Abeokuta (1), Lagos (1)	*U. thermosphaericus*	100	AB210915
9	Ado-Ekiti (1), Ibadan (1), Abeokuta (1), Lagos (1)	*Bacillus* sp.	98	AB375760
10	Oyo (1), Abeokuta (7), Ado-Ekiti (1), Ibadan (2), Lagos (2), Ilorin (2)	*B. subtilis*	99	KF258841
11	Abeokuta (1)	*B. parabrevis*	97	KF377324
12	Ibadan (1), Abeokuta (1)	*S. jeotgali*	99	NR_043877
13	Ado-Ekiti (1), Lagos (1)	*Brevibacterium* sp.	97	DQ153941

[ϕ]Values in parenthesis represent number of *iru* samples. [†]Accession no. of closest relative organisms of nucleotide sequences found in GenBank database; sequences < 200 bp are not assigned accession no.

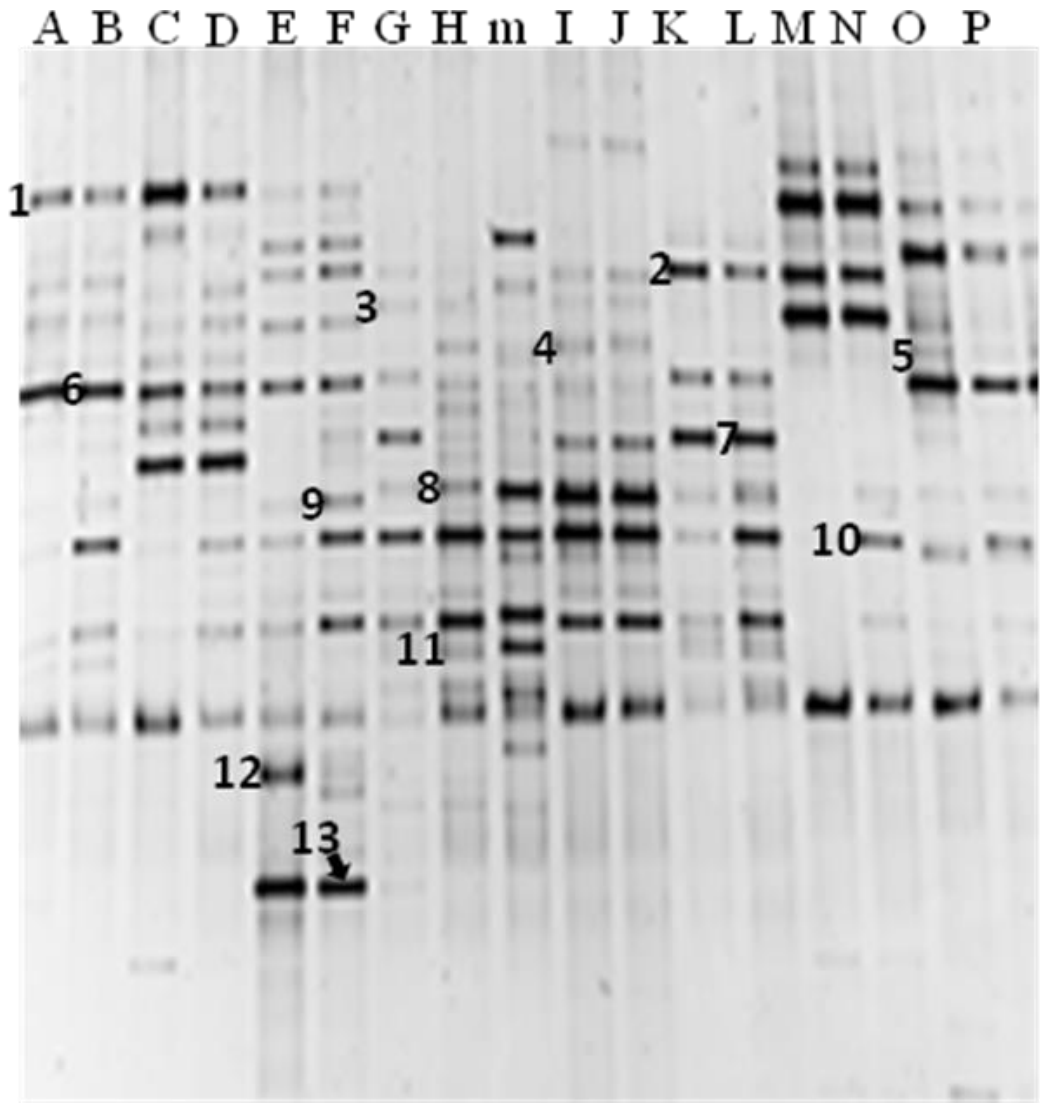


Figure 4.3. DGGE profiles of PCR-amplified 16S rRNA gene fragments of sixteen (16) *iru* samples (alphabets A-P) showing major bacterial amplicons. 'm' is DGGE reference DNA ladder. Bands 1: *S. vitulinus*; 2: *M. morganii*; 3: *B. thuringiensis*; 4: *B. licheniformis*; 5: *S. saprophyticus*; 6: Uncultured bacterium clone; 7: *T. halophilus*; 8: *U. thermosphaericus*; 9: *Bacillus* sp.; 10: *B. subtilis*; 11: *B. parabrevis*; 12: *S. jeotgali*; 13: *Brevibacterium* sp.

4.3 Bacterial counts and pH of *iru* samples

Bacterial population counts on nutrient agar and determination of pH as presented in Figures 4.4 and 4.5 respectively, are expressed as means of three replicate values. Multifactor ANOVA tests were performed to analyse differences in plate counts and pH values of the different *iru* samples, and where appropriate, the post-hoc mean comparison based on Duncan Multiple Range Test (DMRT) was used (Statgraphics® Centurion XVI).

The total viable counts of *iru* ranged from 5.23±0.77 Log_{10} CFU/g (OYO 2) to 7.17±0.19 Log_{10} CFU/g (ADK 1), signifying an average population density of 10^6. In general, there were similarities and/or variations in the population densities and pH of *iru* sampled at different production sites within the same town and between towns. *Iru* samples from Oyo differ significantly at $p<0.05$. Five out of ten *iru* sampled in Abeokuta showed no statistical difference in their counts; similarly ABK 2 and ABK 6. Meanwhile, plate counts from ABK 1, ABK 3 and ABK 9 were significantly different from others at $p<0.05$. The two *iru* samples each obtained from Kaduna and Ibadan differ statistically at significant level of $p<0.05$; likewise two out of three samples from Ado-Ekiti. Lagos and Ilorin samples each showed no significant difference in their counts at $p<0.05$.

The pH values of *iru* ranging from 6.00±0.23 (ABK 3) to 8.40±0.74 (OYO 2 & KAD 1) were recorded. No significant deviation in the pH of *iru* OYO 1 and OYO 5; OYO 3 and OYO4, while OYO 2 differ statistically at $p<0.05$. Most of the *iru* produced in Abeokuta differ significantly in pH at $p<0.05$. The two *iru* samples each from Kaduna, Lagos and Ibadan also differ significantly in pH values at $p<0.05$, as well as two out of three samples from Ado-Ekiti. Ilorin samples were statistically similar in pH values at $p<0.05$. Population counts of some *iru* samples were averagely in close range with their corresponding pH values, while others with low counts have high pH values (e.g. OYO 2).

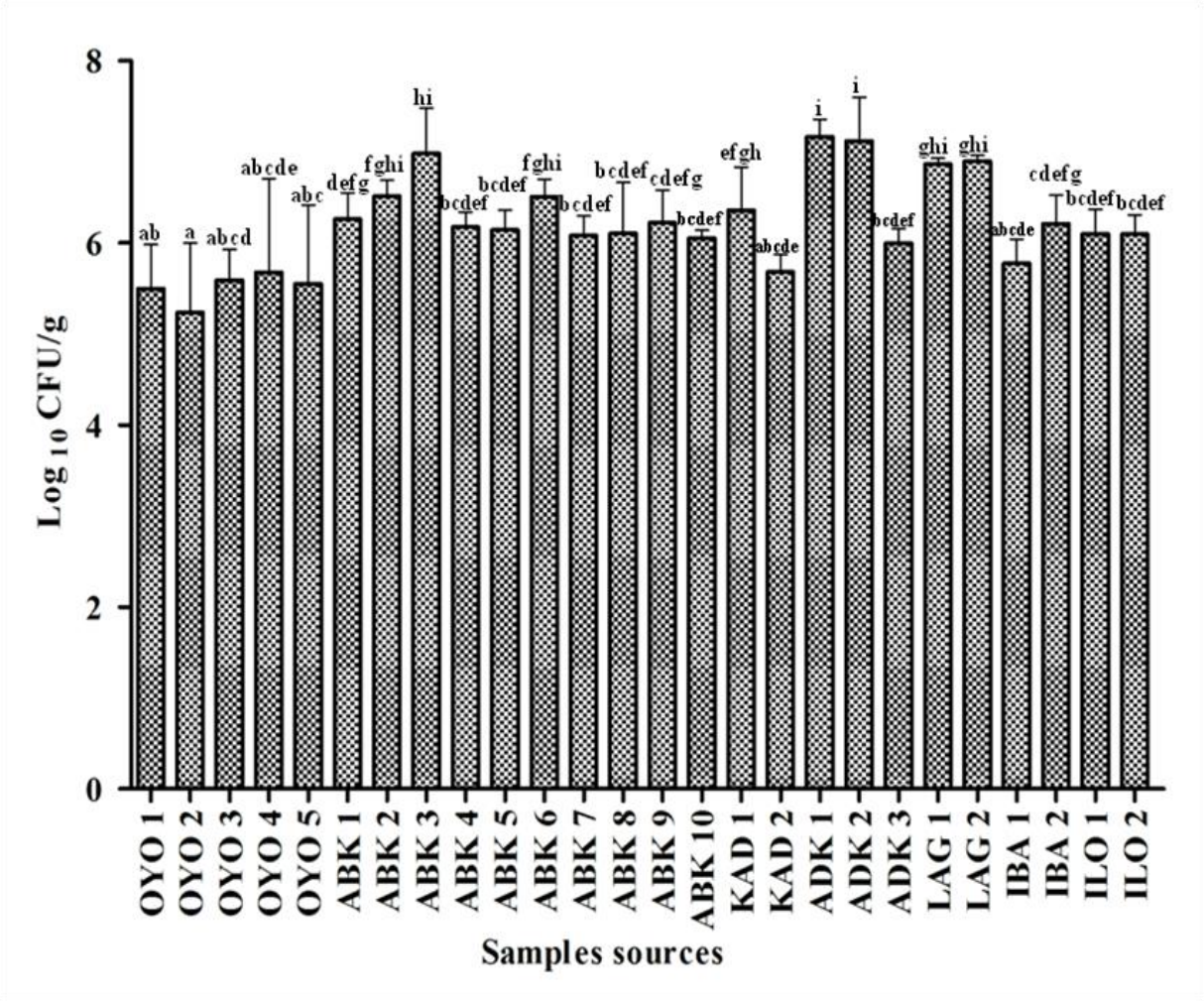


Figure 4.4. Population counts of bacterial colonies in *iru* samples obtained from Oyo (OYO), Abeokuta (ABK), Kaduna (KAD), Ado-Ekiti (ADK), Lagos (LAG), Ibadan (IBA) and Ilorin (ILO). Error bars represent standard error of mean (SEM) of three replicate values. Bars with different alphabets differ significantly at $p<0.05$; Duncan test.

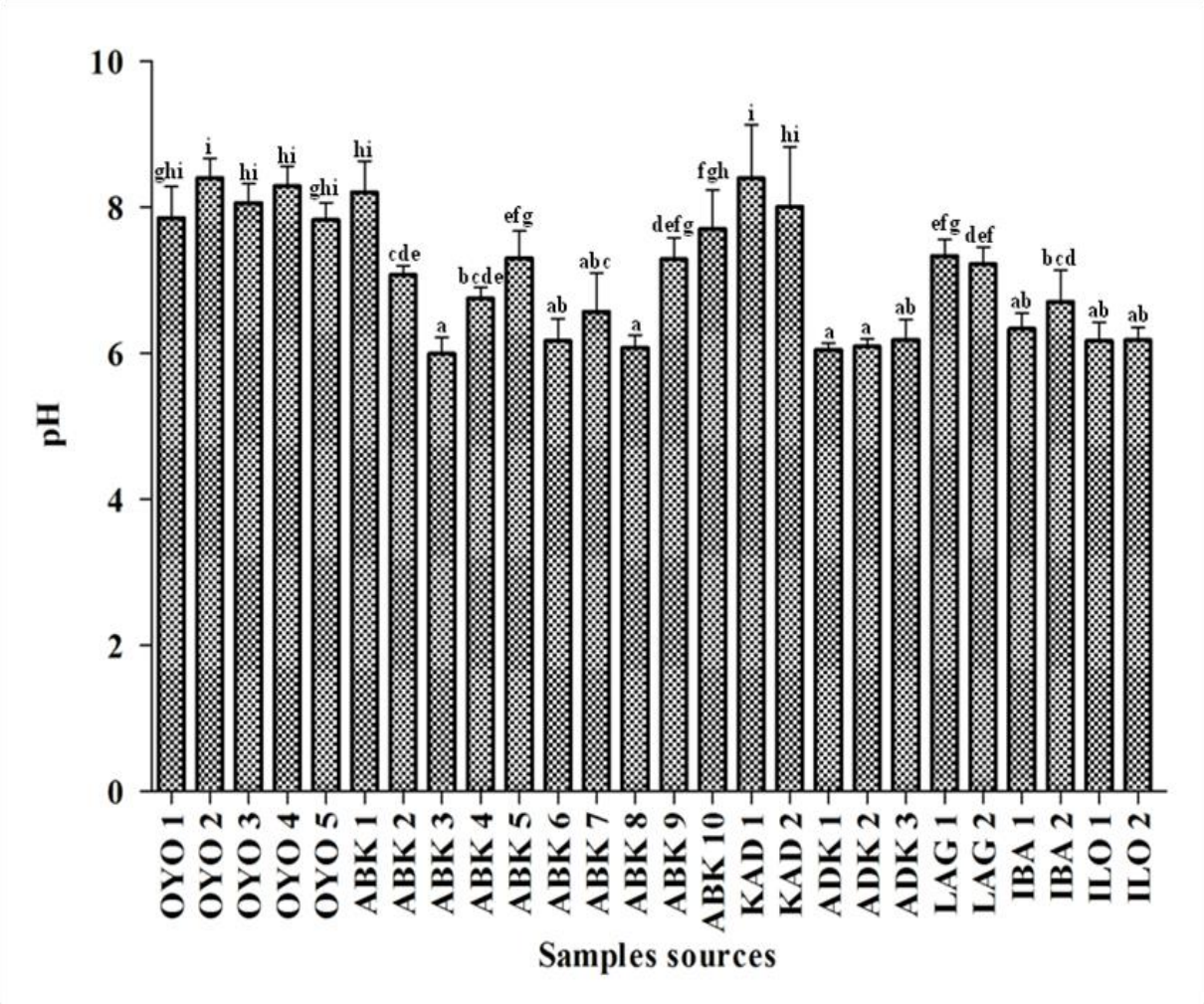


Figure 4.5. pH values of *iru* samples obtained from Oyo (OYO), Abeokuta (ABK), Kaduna (KAD), Ado-Ekiti (ADK), Lagos (LAG), Ibadan (IBA) and Ilorin (ILO). Error bars represent standard error of mean (SEM) of three replicate values. Bars with different alphabets differ significantly at $p<0.05$; Duncan test.

4.4 Phenotypic characterization and identification of bacterial species isolated from *iru*

Two hundred and eighty (280) bacterial colonies, representing the predominant isolates in *iru* samples were phenotyped and found to be Gram-positive rods, catalase-positive, aerobic and facultative anaerobic endospore-formers (Figure 4.6), and presumptively identified as *Bacillus* species.

On the basis of hydrolytic enzymes production (amylase and protease), two *Bacillus* strains were found to possess higher activities compare to others; and further identified with API 50 CHB kit (Table 4.3). They both fermented glycerol, D-ribose, D-fructose, D-maltose, D-raffinose, starch and glycogen, while *B. subtilis*/*B. amyloliquefaciens* U146A only fermented L-arabinose and D-saccharose. *B. subtilis*/*B. amyloliquefaciens* U170B fermented most other sugars, which includes, D-glucose, D-mannose, inositol, D-mannitol, methyl-αD-glucopyranoside, esculin ferric citrate, salicin, D-turanose, D-celiobiose, D-melibiose and inulin. However, none utilized erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, methyl-αD-mannopyranoside, amygdalin, arbutin, D-lactose, D-melezitose, xylitol etc. (Table 4.3).

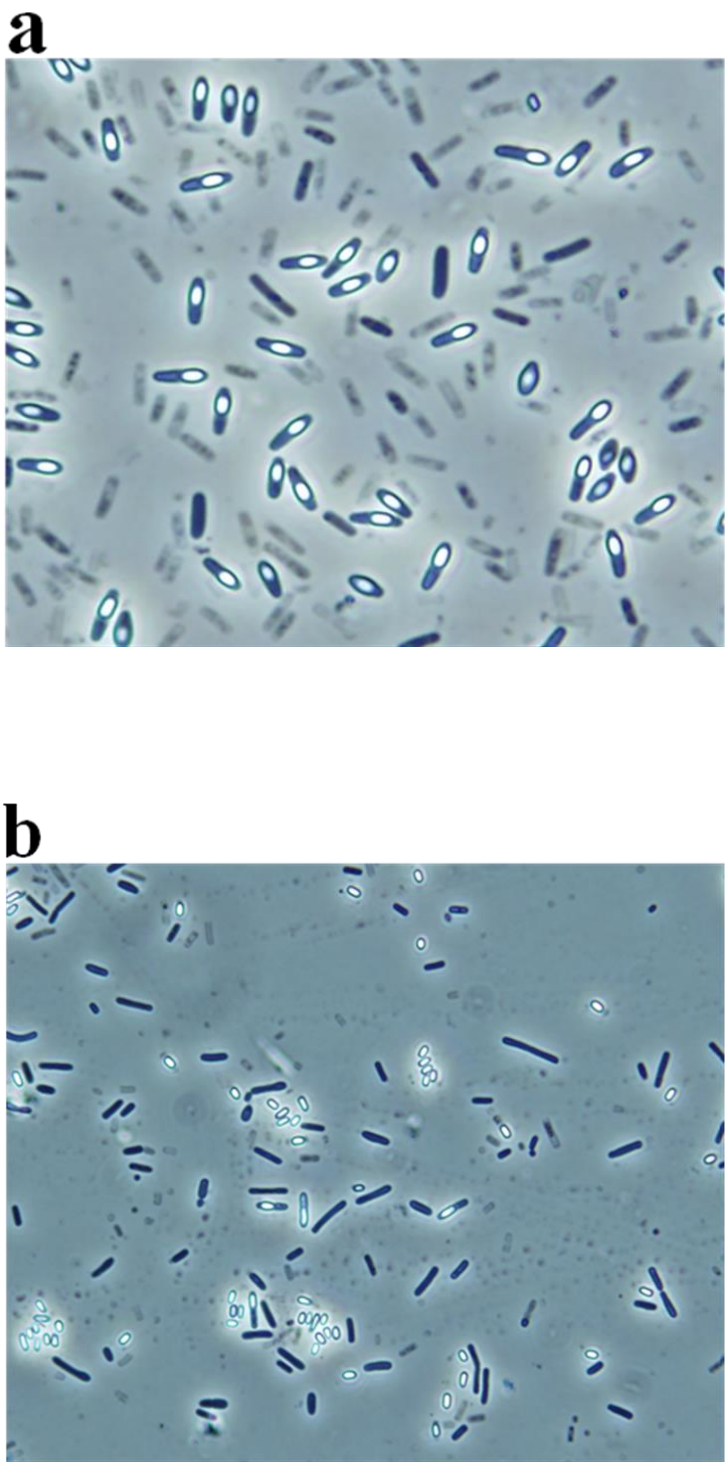


Figure 4.6. Phase contrast microscope of *Bacillus* species endospores [a, U146A (mostly terminal and few central endospores); b, U170B (central endospores)]. Magnification 100X.

Table 4.3: Phenotypic features and API 50 CHB identification of selected *Bacillus* strains in *iru* samples.

Bacillus strains	U146A	U170B	Probable identities
Gram's reaction	+	+	
Cell morphology	rods in chains	rods in chains	
Catalase	+	+	
Endospore	central; terminal	terminal; lateral	
Glycerol	+	+	
Erythritol	-	-	
D-arabinose	-	-	
L-arabinose	+	-	
D-ribose	+	+	
D-xylose	-	-	
L-xylose	-	-	
D-adonitol	-	-	
Methyl-βD-xylopyranoside	-	-	
D-galactose	-	-	
D-glucose	-	+	
D-fructose	+	+	
D-mannose	-	+	
L-sorbose	-	-	
L-rhamnose	-	-	
Dulcitol	-	-	
Inositol	a	+	
D-mannitol	-	+	
D-sorbitol	a	-	
Methyl-αD-mannopyranoside	-	-	
Methyl-αD-glucopyranoside	-	+	
N-acetylglycosamine	-	a	
Amygdalin	-	-	
Arbutin	-	-	
Esculin ferric citrate	-	+	
Salicin	-	+	
D-celiobiose	-	+	
D-maltose	+	+	
D-lactose	-	-	
D-melibiose	a	+	
D-saccharose	+	-	
D-trehalose	-	+	
Inulin	-	+	
D-melezitose	-	-	
D-raffinose	+	+	
Starch	+	+	
Glycogen	+	+	
Xylitol	-	-	
Gentiobiose	-	-	
D-turanose	-	+	
D-lyxose	-	-	
D-tagatose	-	-	
D-fucose	-	-	
L-fucose	-	-	
D-arabitol	-	-	
L-arabitol	-	-	
Potassium gluconate	p	-	
Potassium 2-ketogluconate	-	-	
Potassium 5-ketogluconate	-	-	
	B. subtilis/B. amyloliquefaciens	*B. subtilis/B. amyloliquefaciens*	

[a]assimilation of carbohydrate without acid production; [p]pink colour formation due to alkalination

4.5 *Bacillus* genomic characterization and strains sub-typing using polyphasic approaches

Amplified 16S rRNA gene ca. 1500 bp of 280 bacilli from *iru* samples and reference strains were digested with *Hae*III, *Cfo*I, *Hinf*I, *Dde*I, *Taq*I and *Rsa*I. Combined analysis of ARDRA grouped the *Bacillus* species into six groups (Table 4.4). All the restriction endonucleases distinctly differentiated the strains into two phylotypes of *B. subtilis* and *B. cereus* (Figure 4.7). The restriction digested products of *Rsa*I clearly differentiated *B. subtilis* from *B. amyloliquefaciens*; *B. subtilis* from *B. pumilus*; *B. amyloliquefaciens* from *B. licheniformis*; *B. pumilus* from *B. licheniformis* (Figure 4.8). However, it failed to distinguish *B. amyloliquefaciens* from *B. pumilus* and also *B. subtilis* from *B. licheniformis*, as they were observed to possess similar polymorphisms. The difficulty in differentiating *B. subtilis* from *B. licheniformis* was overcome with the *Cfo*I digestion (Figure 4.9). These results altogether evidently indicate that ARDRA can be successfully used for differentiation of *Bacillus* species, particularly *B. subtilis* phylogeny, when appropriate restriction enzymes are employed.

PCR amplification of the 16S-23S rRNA gene internal transcribed spacer (ITS-PCR) could not bring about any strain differentiation; rather it maintained the interspecies differentiation generated by ARDRA, except for *B. licheniformis* strains (Table 4.4; Figure 4.10). However, ITS-PCR and restriction analysis with *Cfo*I (ITS-PCR-RFLP) showed intraspecies variation among the *B. subtilis* group (*B. subtilis*, *B. pumilus* and *B. amyloliquefaciens*) (Table 4.4) aside from differentiating *B. pumilus* from *B. amyloliquefaciens* (Figure 4.11).

Dendrogram constructed based on combined analysis of the gel fingerprints of ARDRA, ITS-PCR and ITS-PCR-RFLP is shown in Figure 4.12. Two major clusters at 35% similarity level were obtained. Cluster 1, identified as *B. subtilis* phylogeny consisting of 3 main sub-clusters and 14 strains, all clustered together at 64%, and cluster 2, identified as *B. cereus* phylogeny

consisting of 2 strains clustered at 45%. Sub-clusters of *B. subtilis* phylogeny showed a high degree of strain diversity, with *B. subtilis* and *B. amyloliquefaciens* confirmed as dominant species. *B. subtilis* U104 from *iru* phylogenetically differ from reference strains *B. subtilis* MTCC 2451, MTCC 5480 and MTCC 1747 from *hawaijar* and *kinema* respectively. However, *B. amyloliquefaciens* U184B and the two *B. cereus* strains (U175 and U243) from *iru* clustered with reference strains, *B. amyloliquefaciens* MTCC 1270 and *B. cereus* MTCC 430 respectively.

The two dominant species in *B. subtilis* phylogeny found in *iru*, *B. subtilis* and *B. amyloliquefaciens*, were further subjected to genomic strain diversity using RAPD-PCR OPA 18 primer (Figures 4.13 & 4.14). The results in Figure 4.15 showed 60% similarity level, between two clusters I and II identified as *B. subtilis* and *B. amyloliquefaciens* strains. Also, at about 70% similarity level, two sub-groups each of *B. subtilis* (IA & IB) and *B. amyloliquefaciens* (IIA & IIB) strains identified were genetically distinct from the reference strains, *B. subtilis* MTCC 5480 and MTCC 1747 from *hawaijar* and *kinema* respectively, and *B. amyloliquefaciens* MTCC 1270. The dominant strains of *B. subtilis* and *B. amyloliquefaciens* were further studied using RAPD-PCR M13 primer. This gave better diversity profiles than OPA 18 primer (Figures 4.16 & 4.17). At 85% similarity level, higher strain diversity was found within *B. subtilis* (16 strains), and were genetically distinct from the reference strain *B. subtilis* MTCC 2451 (Figure 4.18). At 63% similarity level, higher strain diversity was also observed within *B. amyloliquefaciens* (12 strains), which were genetically different from reference strain *B. amyloliquefaciens* MTCC 1270 (Figure 4.19). Thus, polyphasic genomic techniques used in this study were useful in identification, strain differentiation and comprehensive understanding of the diversity of *Bacillus* strains.

Table 4.4: Genomic characterization and sub-typing of *Bacillus* species isolated from *iru*, traditional fermented *Parkia biglobosa* seeds.

ARDRA groups	16S rRNA-RFLP profiles (sizes in bp)						ITS-PCR sub-groups (sizes in bp)	ITS-PCR-RFLP sub-groups (sizes in bp)	No. of isolates	Representative isolates	16S rRNA gene sequencing		
											Closest known relatives	Similarity	NCBI acc. no.
	*Hae*III	*Cfo*I	*Dde*I	*Hinf* I	*Taq*I	*Rsa*I							
I.	*627 473	908	508	581	573	525	**IA.**	**IA1.**	93	U4E BA	*B. subtilis*	99%	JN255703
	318	451	465	355	458	477	1065, 945, 869,	502, 417, 339, 278, 260, 214,					
	156	248	237	317	349	429		143, 136					
			150	177	214	149		**IA2.**	26	U104	*B. subtilis*	98%	JN165753†
			122					506, 339, 280, 141					
II.	622 467	904	504	592	590	515	**IIA.**	**IIA1.**	1	U154B	*B. subtilis*	100%	JN255715
	314 151	445	381	362	412	470	1036, 845	485, 401, 322, 263, 247, 218,					
		243	280	324	361	417		138, 129					
			147	183	221	146		**IIA2.**	3	U191A	*B. subtilis*	97%	JN255725
			117					489, 329, 271, 231, 137					
								IIA3.	1	U233	*B. subtilis*	100%	JN255727
								494, 406, 330, 269, 255, 225,					
								127, 120					
								IIA4.	1	U122A	*B. subtilis*	99%	JN255710
								494, 329, 268, 223, 134					
								IIA5.	2	U213A	*B. pumilus*	99%	JN255726
								490, 339, 259, 149		U158	*B. pumilus*	100%	JN255717
III.	627 472	912	510	609	722	515	**IIIA.**	**IIIA1.**	62	U184B	*B. amyloliquefaciens*	99%	JN255722
	318 156	451	392	374	375	424	1057, 939, 863	504, 420, 343, 274, 259, 145,					
		247	286	335	232	147		137					
			236	190				**IIIA2.**	37	U79BA	*B. amyloliquefaciens*	98%	JN255707
			122					506, 424, 384, 369, 343, 274,					
								260, 147, 138					
IV.	622 475	414	518	581	570	498	**IVA.**	**IVA1.**	3	U186	*B. licheniformis*	98%	JN255724
	324 156	326	288	344	395	459	1024, 845	496, 336, 272, 229, 142					
		228	277	307	345	409	**IVB.**	**IVB1**.	1	U126	*B. licheniformis*	100%	JN255711
		172	237	166	211	142	1044, 926, 842,	489, 435, 414, 335, 271, 129					
			132				339						
			115										
V.	595 477	567	516	943	600	494	**VA.**	**VA1.**	49	U175	*B. cereus*	96%	JN255721
	322 161	417	287	312	481	411	1078, 827	506, 311, 168, 136		U243	*B. cereus*	92%	JN255728
		332	275	170	134	355							
		175	172			142							
			150										
			115										
VI.	627, 477,	412,	546	973	575	452	**VIA.**	**VIA1.**	1	U185B	*Brevibacillus formosus*	99%	JN255723
	228, 157	324,	441	304	389	411	1359, 1161,	461, 355, 294, 262, 225, 205,					
		218,	238	234	365	353	1103, 1020, 910	198, 193, 167, 157, 142, 133					
		172	167	167	196	131							
			120			107							

*DNA fragment sizes. †Accession no. of closest relative organism of nucleotide sequence found in GenBank database.

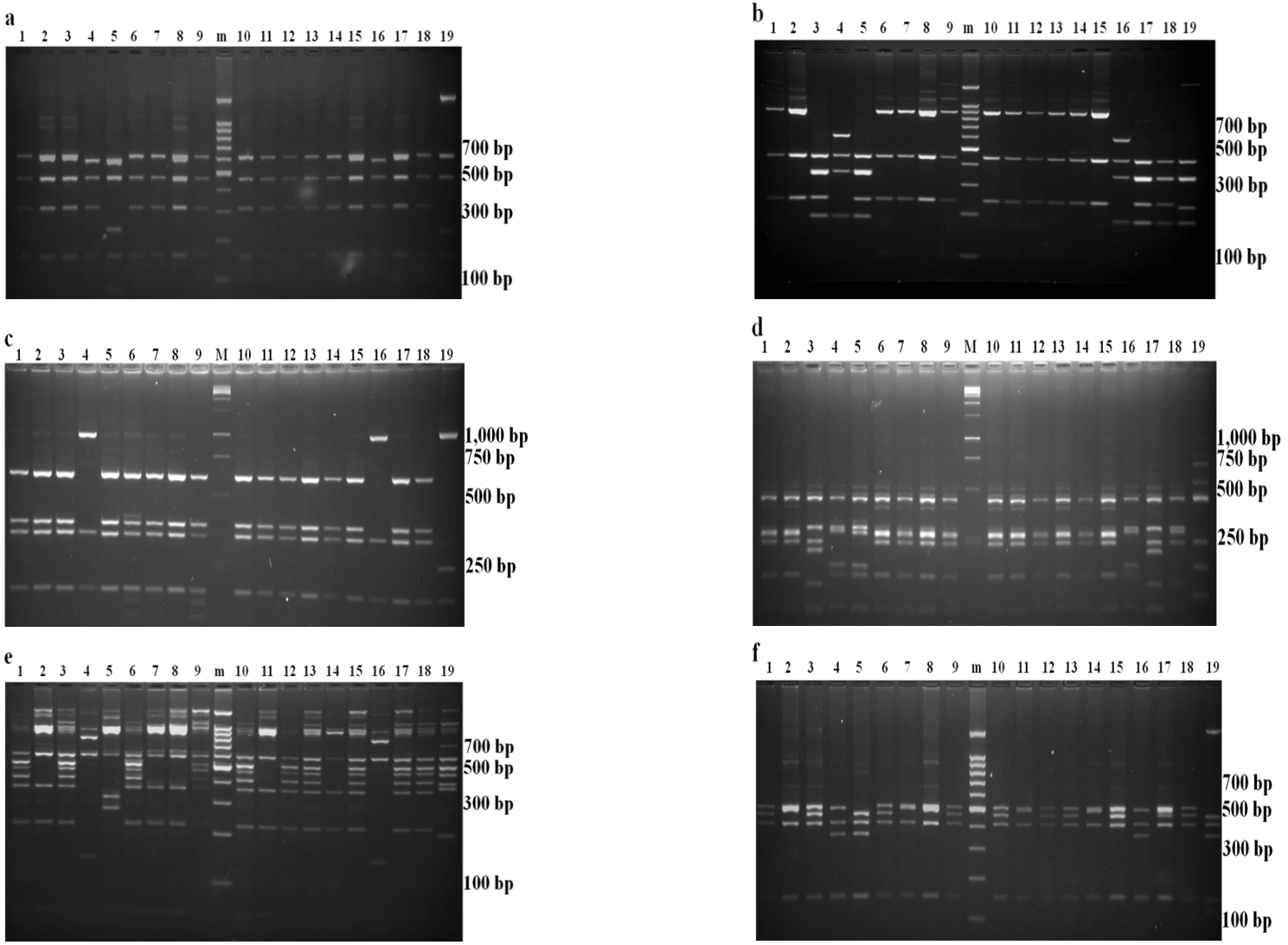


Figure 4.7. ARDRA gel profiles based on (a) *Hae*III (b) *Cfo*I (c) *Hinf*I (d) *Dde*I (e) *Taq*I (f) *Rsa*I showing differentiation of *Bacillus* species into phylogeny of *B. subtilis* and *B. cereus*. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 5: *B. circulans* MTCC 490; 6,7,8,9,10,11,12,13,14,15,17&18: *B. subtilis* phylotype; 4&16: *B. cereus* MTCC 430 and *B. cereus* U175; 19: *Brevibacillus formosus* U185B; m: 100 bp DNA ladder; M: 1 kb DNA ladder.

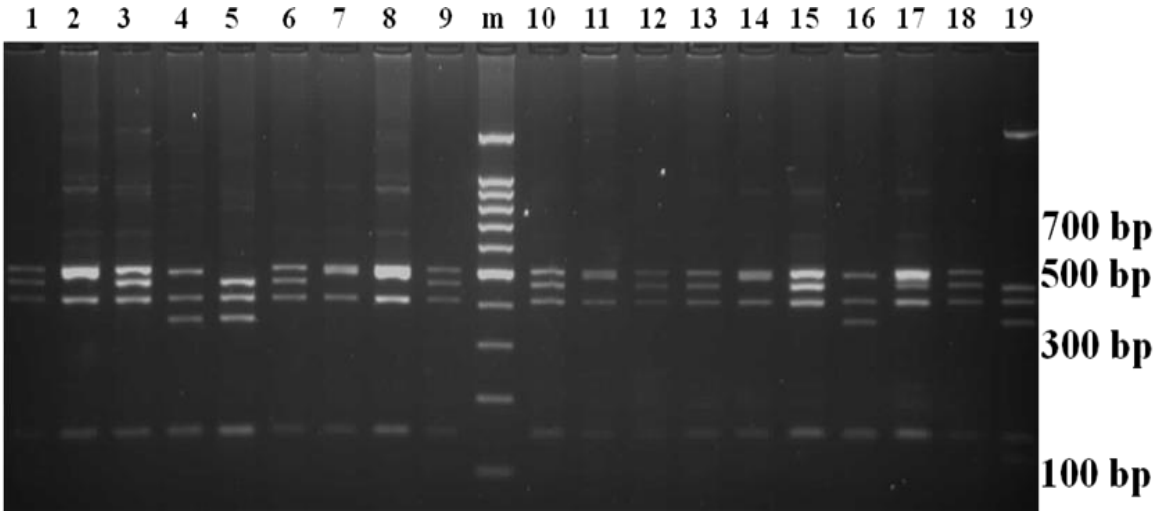


Figure 4.8. ARDRA gel profile based on *Rsa*I showing differentiation among *B. subtilis* phylogeny. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 4: *B. cereus* MTCC 430; 5: *B. circulans* MTCC 490; 6, 9, 10, 12, 13 & 15: *B. subtilis* strains; 7 & 8: *B. amyloliquefaciens* strains; 11 & 14: *B. pumilus* strains; 16: *B. cereus* U175; 17 & 18: *B. licheniformis* strains; 19: *Brevibacillus formosus* U185B; m: 100 bp DNA ladder.

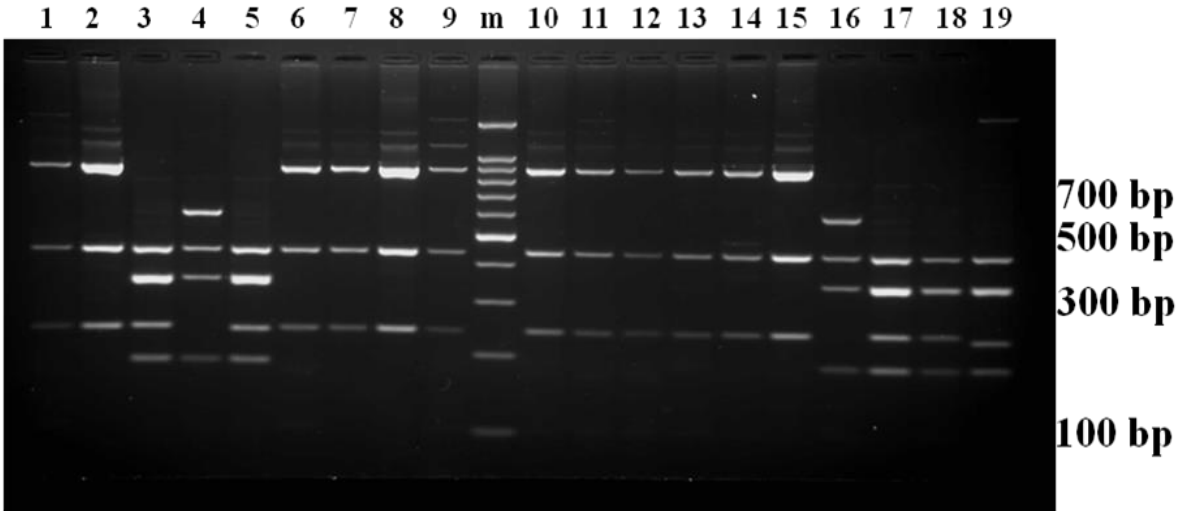


Figure 4.9. ARDRA gel profile based on *Cfo*I showing differentiation of *B. subtilis* and *B. licheniformis*. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 4: *B. cereus* MTCC 430; 5: *B. circulans* MTCC 490; 6, 9, 10, 12, 13 & 15: *B. subtilis* strains; 17 & 18: *B. licheniformis* strains; 19: *Brevibacillus formosus* U185B; m: 100 bp DNA ladder.

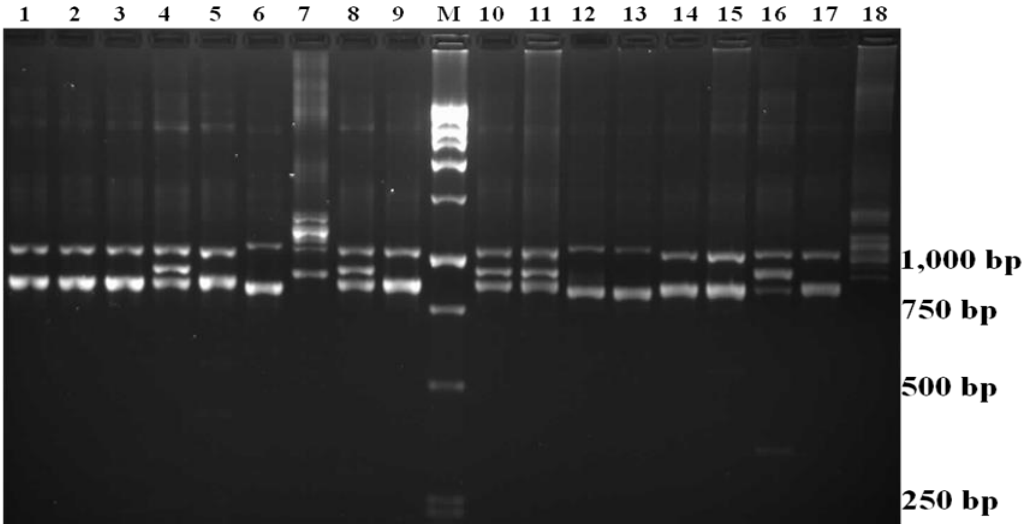


Figure 4.10. ITS-PCR gel profile of different *B. licheniformis* strains. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. subtilis* MTCC 5480; 3: *B. subtilis* MTCC 1747; 4: *B. amyloliquefaciens* MTCC 1270; 5: *B. licheniformis* MTCC 429; 6: *B. cereus* MTCC 430; 7: *B. circulans* MTCC 490; 9 & 14: *B. subtilis* strains; 8, 10 & 11: *B. amyloliquefaciens* strains; 12 &13: *B. cereus* strains; 15 & 16: *B. licheniformis* strains; 17: *B. pumilus* U158; 18: *Brevibacillus formosus* U185B; M: 1 kb DNA ladder.

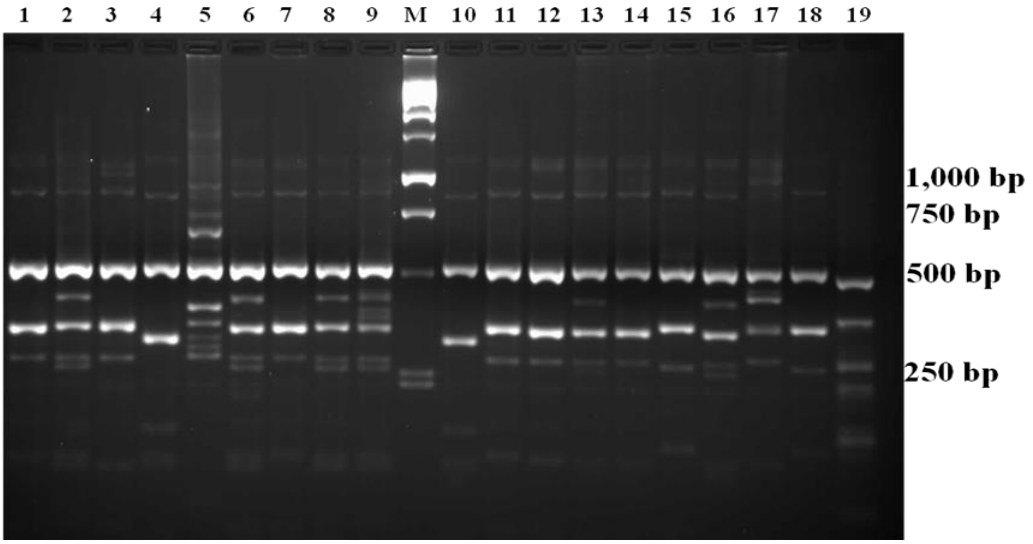


Figure 4.11. ITS-PCR-RFLP gel profile showing strain diversity within *B. subtilis*, *B. pumilus* and *B. amyloliquefaciens*, also differentiating *B. pumilus* from *B. amyloliquefaciens*. Lanes 1: *B. subtilis* MTCC 2451; 2: *B. amyloliquefaciens* MTCC 1270; 3: *B. licheniformis* MTCC 429; 4: *B. cereus* MTCC 430; 5: *B. circulans* MTCC 490; 6, 7, 12, 13, 14 &16: *B. subtilis* strains; 8 & 9: *B. amyloliquefaciens* strains; 10: *B. cereus* U175; 11 & 17: *B. licheniformis* strains; 15 & 18: *B. pumilus* strains; 19: *Brevibacillus formosus* U185B; M: 1 kb DNA ladder.

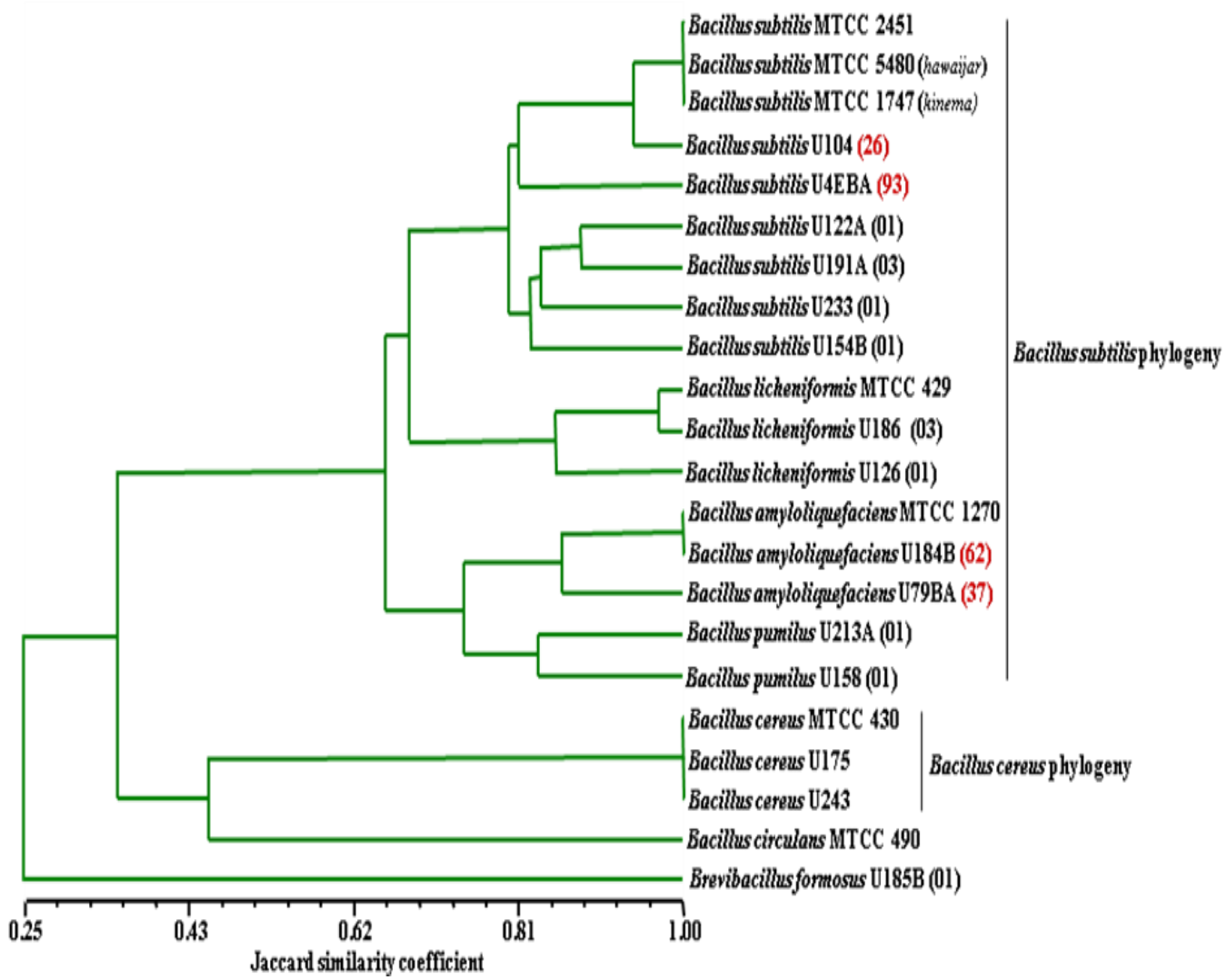


Figure 4.12. Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized combined ARDRA, ITS-PCR and ITS-PCR-RFLP fingerprints of *Bacillus* species isolated from *iru* and reference strains.

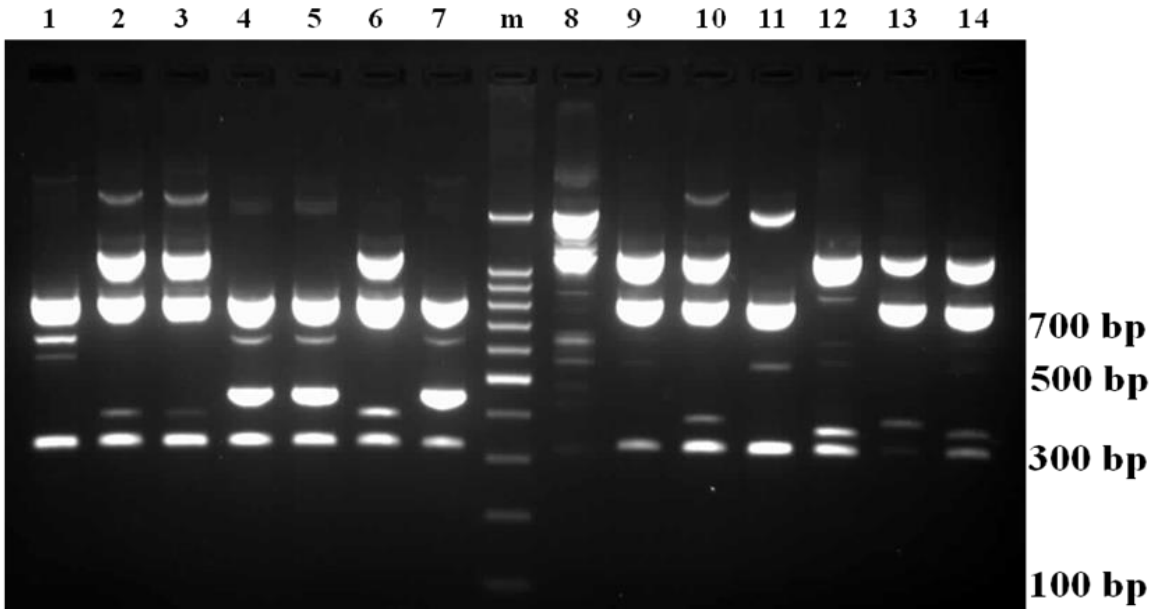


Figure 4.13. RAPD-PCR (OPA 18 primer) of *B. subtilis* strains from *iru*. Lanes 1: *B. subtilis* MTCC 2451; 2-14: *B. subtilis* strains; m: 100 bp DNA ladder.

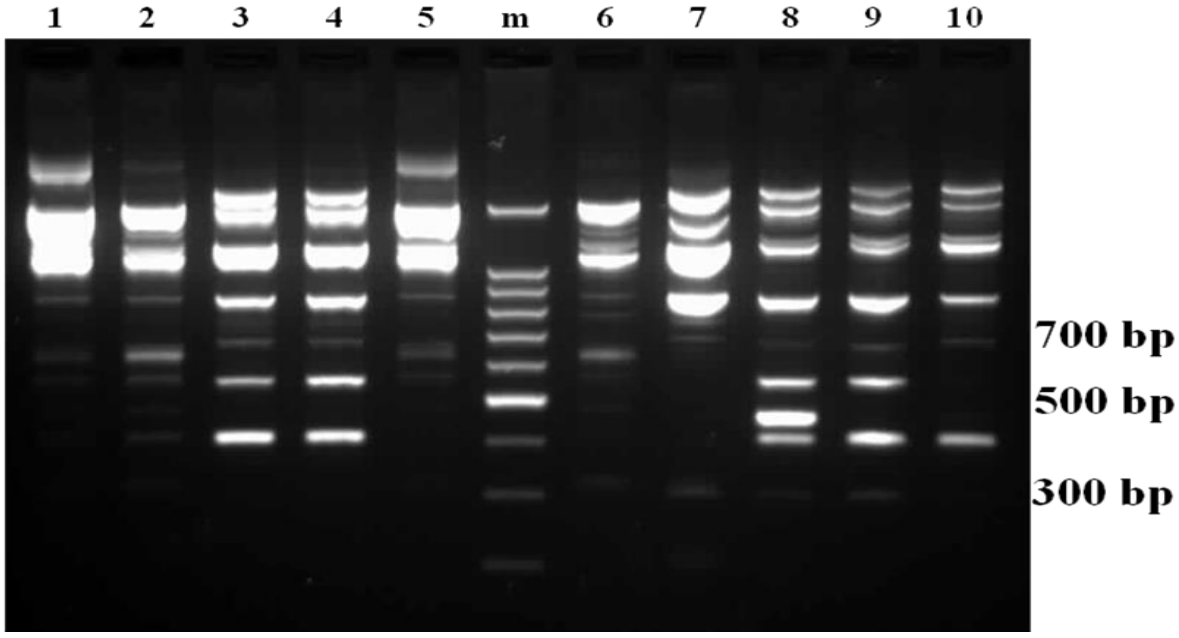


Figure 4.14. RAPD-PCR (OPA 18 primer) of *B. amyloliquefaciens* strains from *iru*. Lanes 1: *B. amyloliquefaciens* MTCC 1270; 2-10: *B. amyloliquefaciens* strains; m: 100 bp DNA ladder.

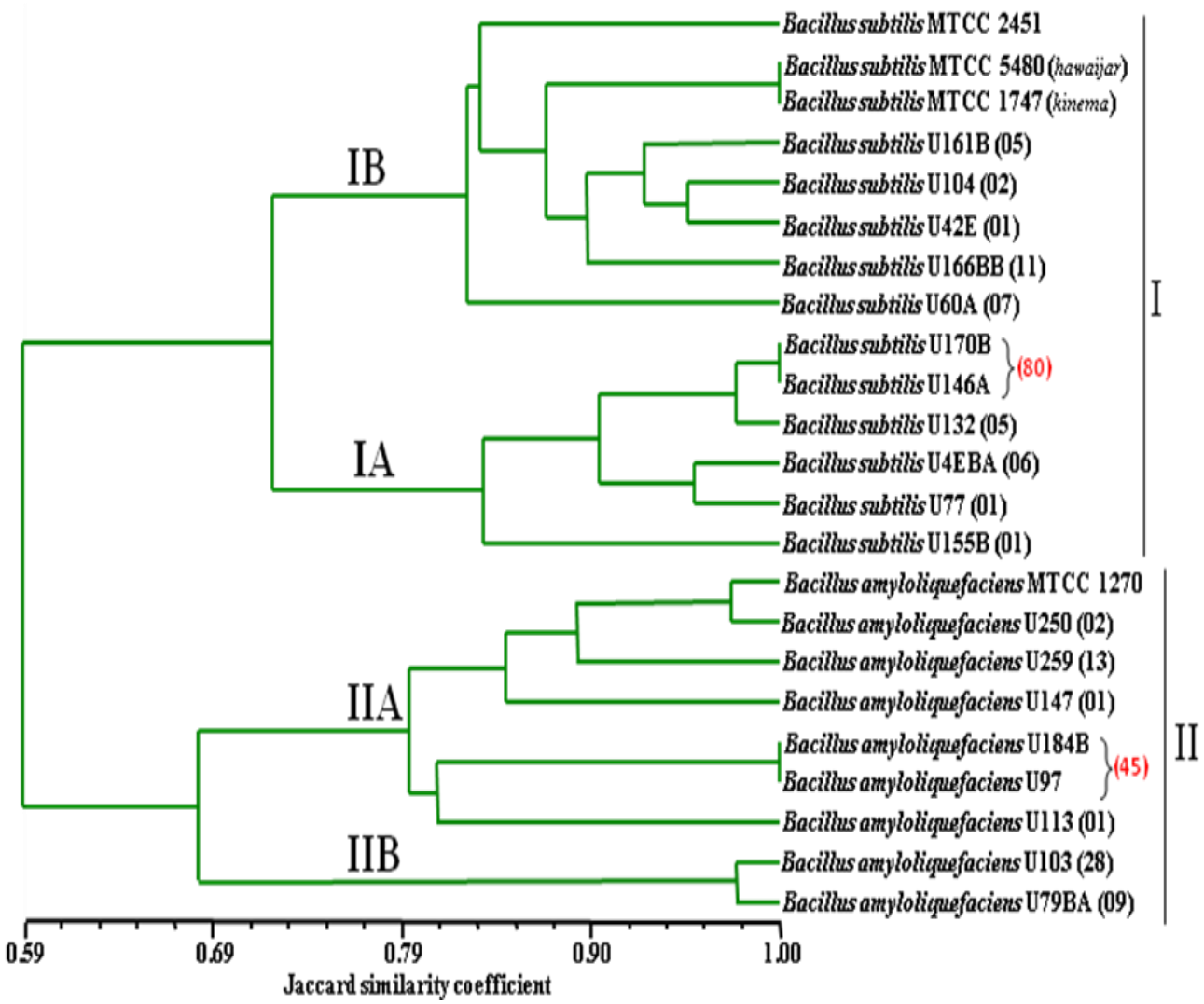


Figure 4.15. Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized OPA 18 RAPD-PCR fingerprints of dominant *B. subtilis* and *B. amyloliquefaciens* strains from *iru* and reference strains.

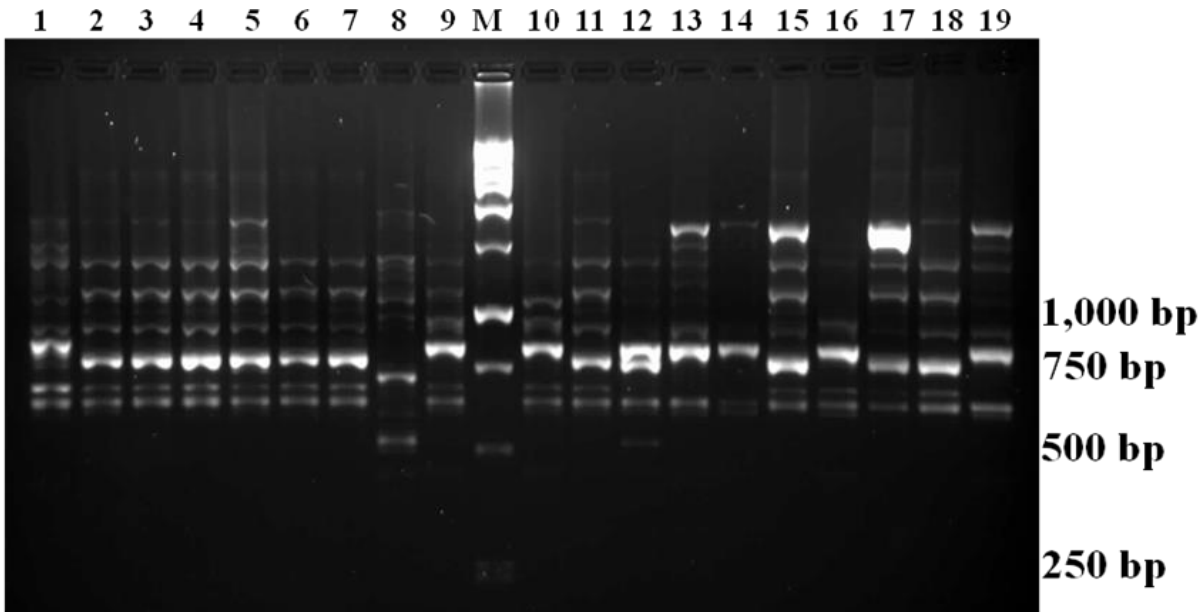


Figure 4.16. RAPD-PCR (M13 primer) of *B. subtilis* strains from *iru*. Lanes 1: *B. subtilis* MTCC 2451; 2-19: *B. subtilis* strains; M: 1 kb DNA ladder.

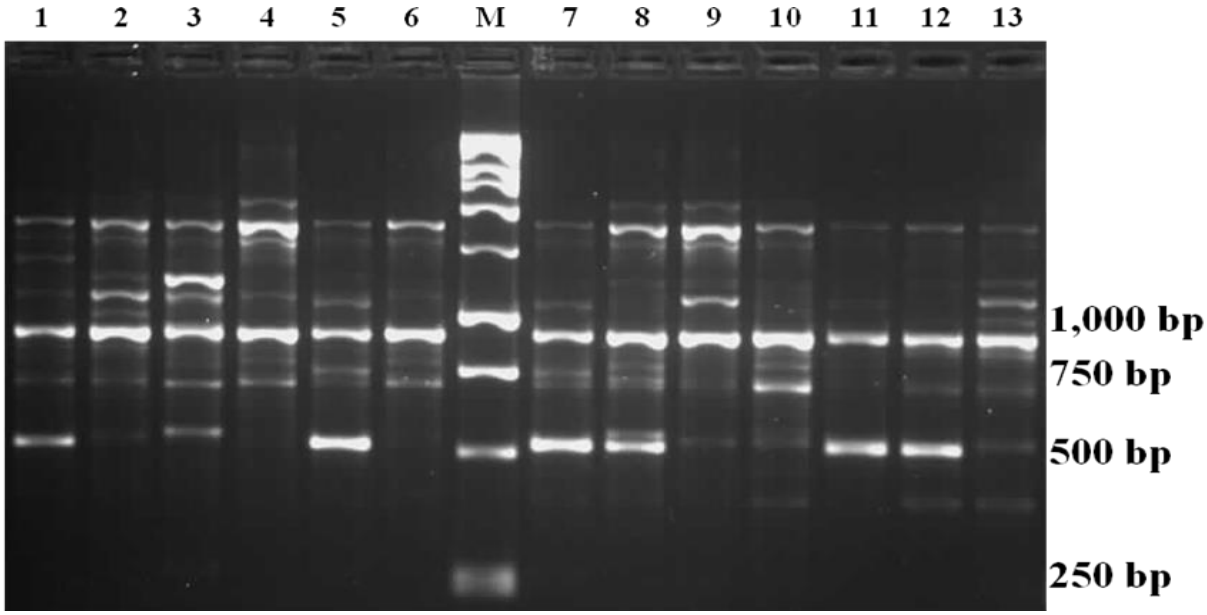


Figure 4.17. RAPD-PCR (M13 primer) of *B. amyloliquefaciens* strains from *iru*. Lanes 1: *B. amyloliquefaciens* MTCC 1270; 2-13: *B. amyloliquefaciens* strains; M: 1 kb DNA ladder.

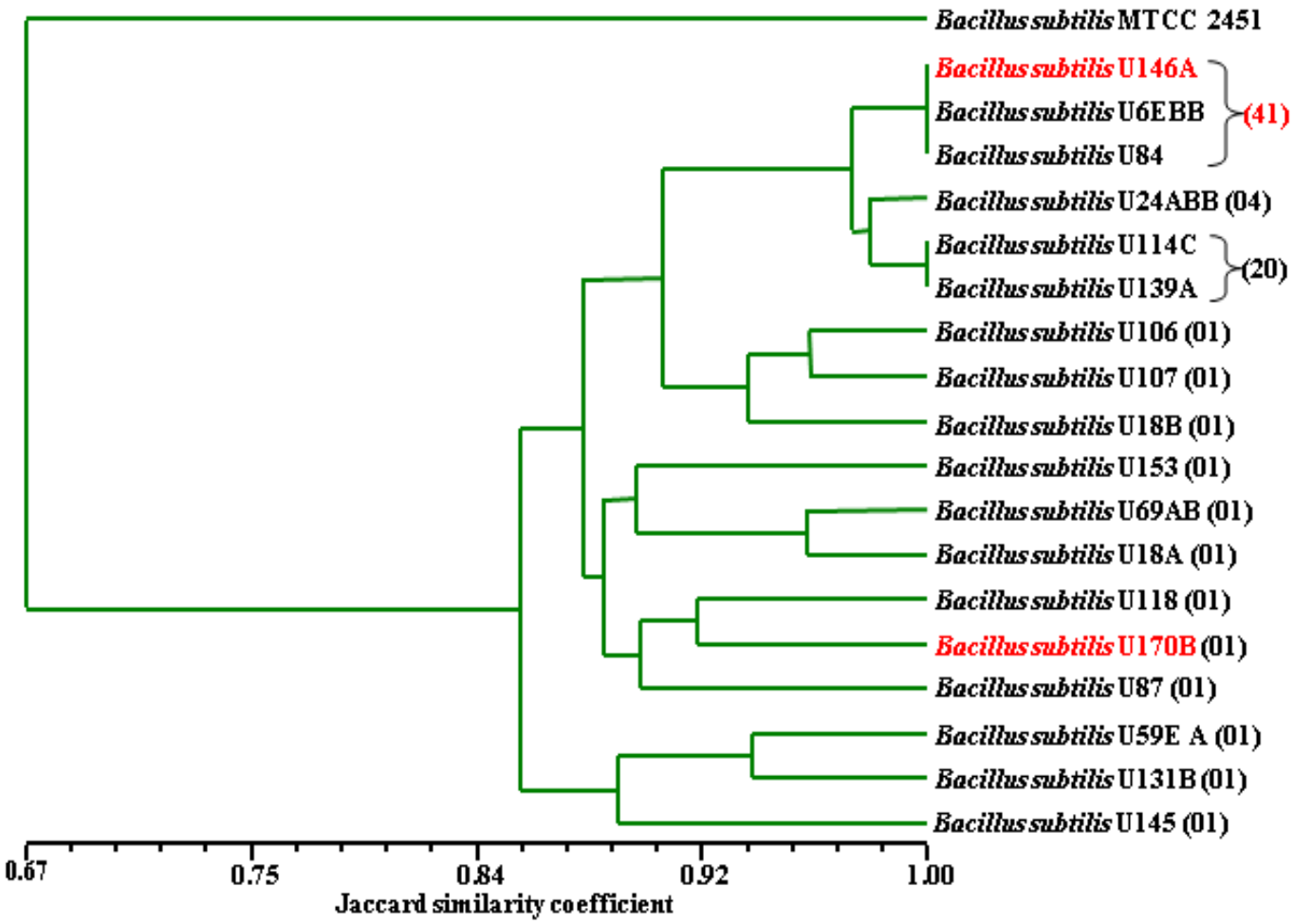


Figure 4.18. Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized M13 RAPD-PCR fingerprints of dominant *B. subtilis* strains.

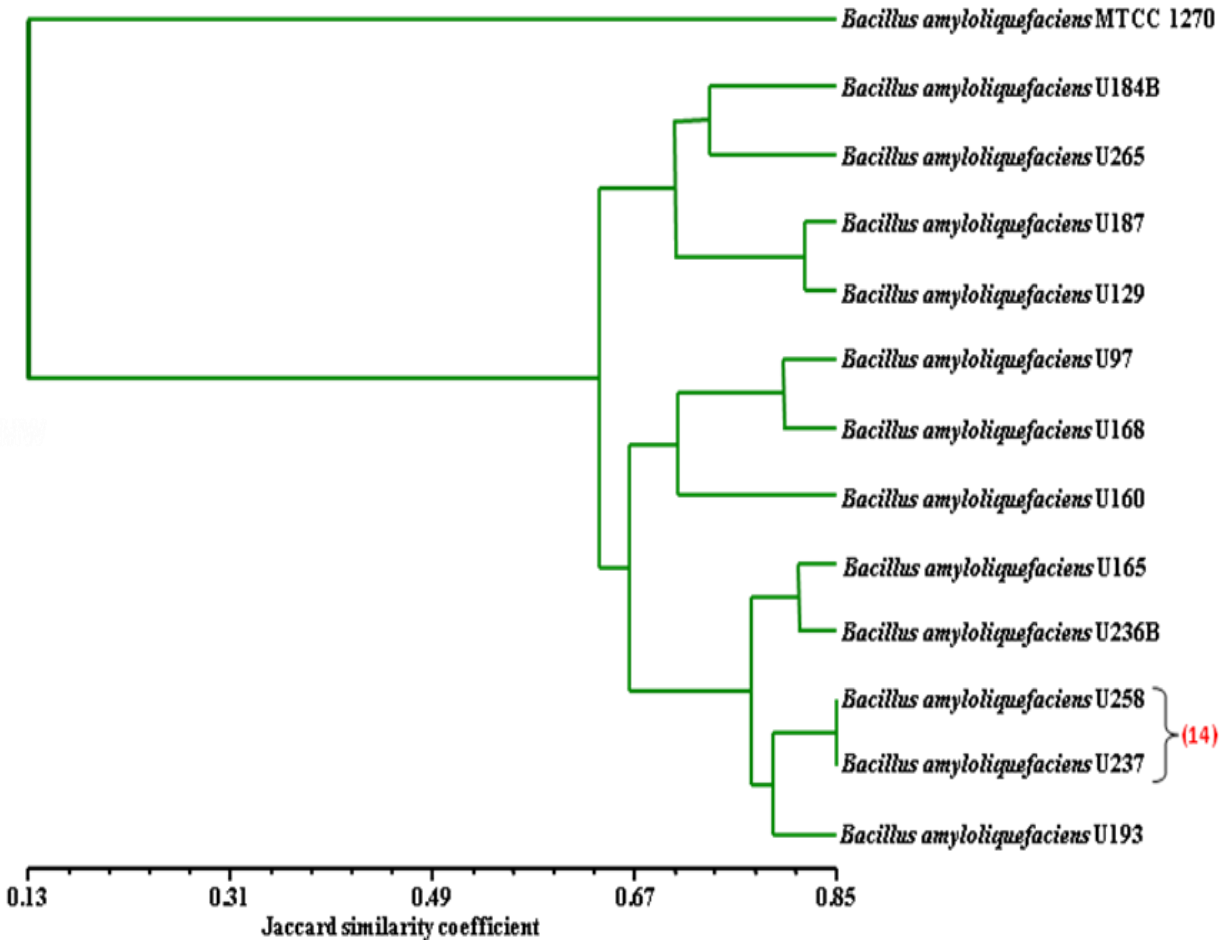


Figure 4.19. Dendrogram based on UPGMA clustering of Jaccard similarity coefficient (S_j) of normalized M13 RAPD-PCR fingerprints of dominant *B. amyloliquefaciens* strains.

4.6 Phylogenetic analysis

Nucleotide sequences of almost complete 16S rRNA gene of representative *Bacillus* strains based on polyphasic genomic sub-typing and dendrogram generated were compared with those of NCBI database (www.ncbi.nlm.nih.gov). The BLAST analysis first identified them as species closely related to *B. subtilis* (44.6%), *B. amyloliquefaciens* (35.4%), *B. cereus* (17.5%), *B. licheniformis* (1.4%), *B. pumilus* (0.7%) and *Brevibacillus formosus* (0.4%) in decreasing order of occurrence. Secondly, it revealed *Bacillus* species as the major group of bacteria associated with fermentation of *P. biglobosa* for *iru* production. Thirdly, the 16S rRNA gene sequences aided construction of a phylogenetic tree of *Bacillus* strains obtained from African fermented condiments (*iru* or *daddawa*); Asian fermented condiments (*kinema, hawaijar, thua nao, douche* and *cheonggukjang*) and other typed and referenced bacilli strains deposited in different culture collections.

The results of the phylogenetic relationship are as shown in Figure 4.20, with five major bacilli groups identified. Group I consists of *B. subtilis* and *B. amyloliquefaciens* strains from different condiments. The cluster analysis also showed the strains to be closest relative of *B. subtilis* DSM 10^T. However, the analysis could not differentiate *B. subtilis* from *B. amyloliquefaciens*. Group II is a sub-cluster of Group I that consists of species identified as *B. licheniformis*; the two strains clustered with *B. licheniformis* ATCC 14580^T. Group III consists of species closely related to *B. pumilus* ATCC 7061^T. Group IV comprises *B. cereus* that clustered with *B. cereus* ATCC 14579^T. Group V could be referred to an outcast; it comprises *Brevibacillus formosus* strain that clustered with the type strain *Brevibacillus formosus* DSM 9885^T. Finally, the nucleotide sequences were submitted to GenBank NCBI and received accession no. JN255703 to JN255730.

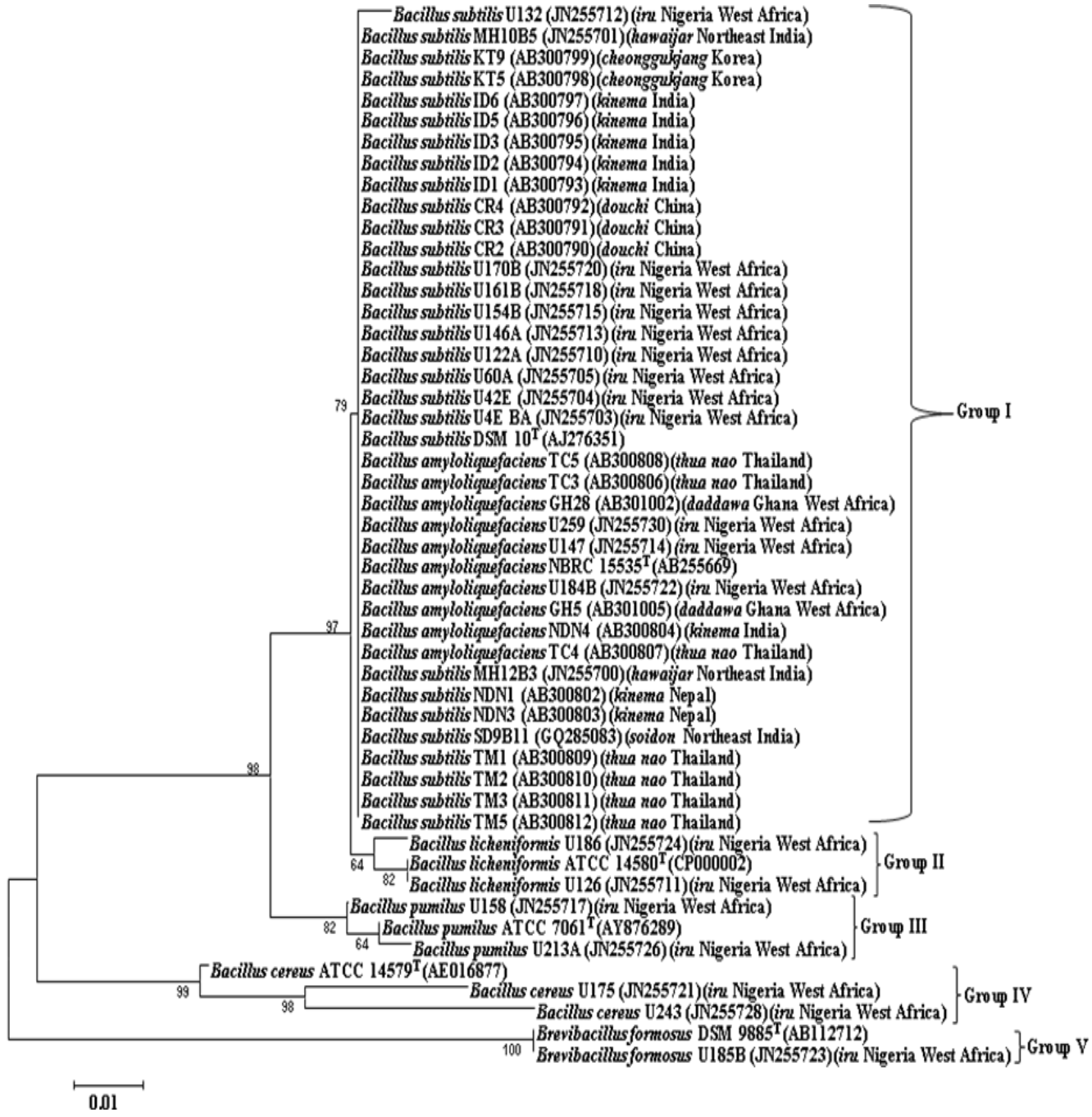


Figure 4.20. Phylogenetic tree showing multiple sequence alignment of 16S rRNA gene sequences of *Bacillus* species isolated in Africa from *iru* or *daddawa*; *hawaijar*, *cheonggukjang*, *kinema*, *douchi*, *thua nao* in Asia; referenced and typed strains. Pairwise phylogenetic distances were calculated based on 1400nt of 16S rRNA gene. Tree generated using MEGA5 (http://www.megasoftware.net).

4.7 MLSA for sub-typing *Bacillus cereus sensu lato*

The *B. cereus* phylogeny comprising *B. cereus sensu stricto, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. anthracis, B. thuringiensis* and *B. cytotoxicus* showed similar ARDRA, ITS-PCR and ITS-PCR-RFLP band patterns (Figures 4.7, 4.8, 4.9, 4.10 & 4.11). RAPD-PCR (M13 primer) amplification employed thereafter grouped the *B. cereus sensu lato* into four representative groups (Figure 4.21), which was followed by PCR amplification of *gyrB, glpF, gmk, rpoA* and *pheS* housekeeping genes for possible species discrimination (Figure 4.22). DNA sequences for *gyrB, glpF* and *gmk* (*rpoA* and *pheS* gave invalid sequence reads with no favourable identities) obtained were compared with those deposited in GenBank using the BLAST programme; this identified 96% of the *B. cereus* group as *B. cereus sensu stricto* and 4% as *B. thuringiensis*. *gyrB, glpF* and *gmk* sequences of *B. cereus* and *B. thuringiensis* strains from *iru*, including sequences of other strains retrieved from NCBI database were further analysed by constructing phylogenetic trees (Figure 4.23). This revealed phylogenetic and clonal relationship of common ancestral origin between *B. cereus* and *B. thuringiensis*; it also established *B. cereus sensu stricto* as the major member of the *B. cereus sensu lato* found in *iru*. Also, *B. cereus* and *B. thuringiensis* strains from the present study are totally diverged from the other strains (Figure 4.23).

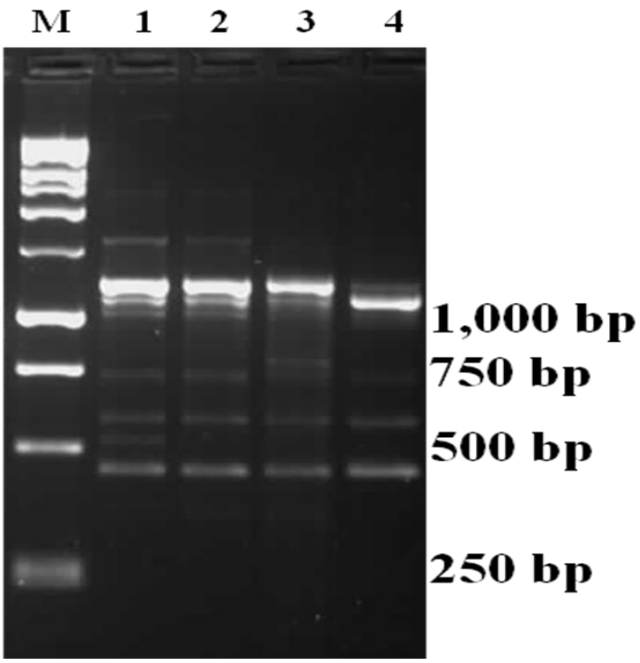


Figure 4.21. RAPD-PCR (M13 primer) of representative *B. cereus* strains from *iru*. Lanes 1: *B. cereus* U173A; 2: *B. cereus* U9E; 3: *B. cereus* U27; 4: *B. cereus* U240; M: 1 kb DNA ladder.

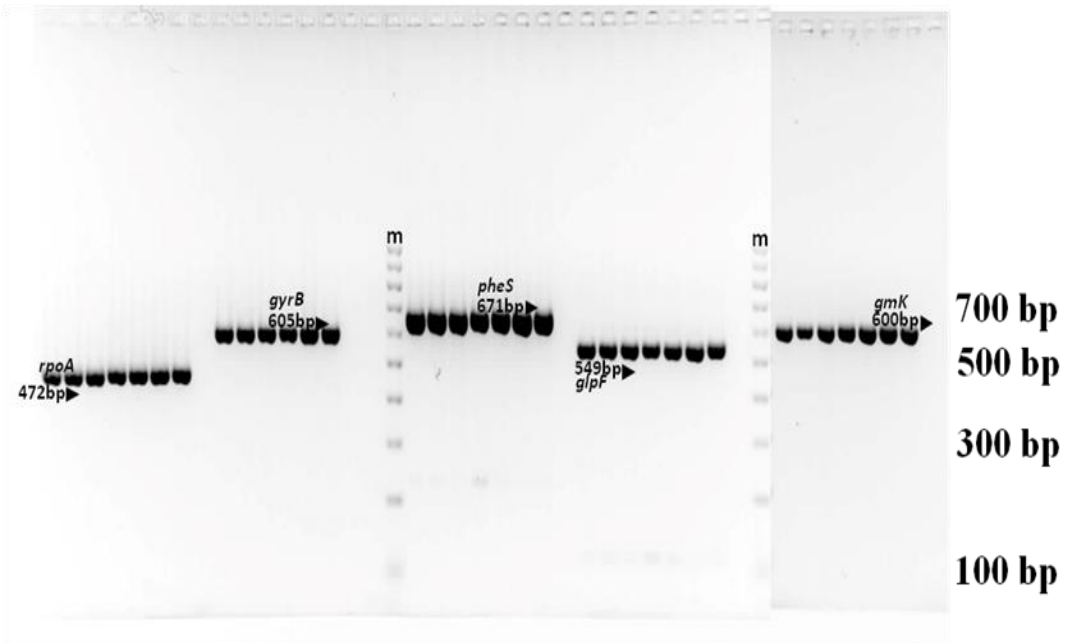
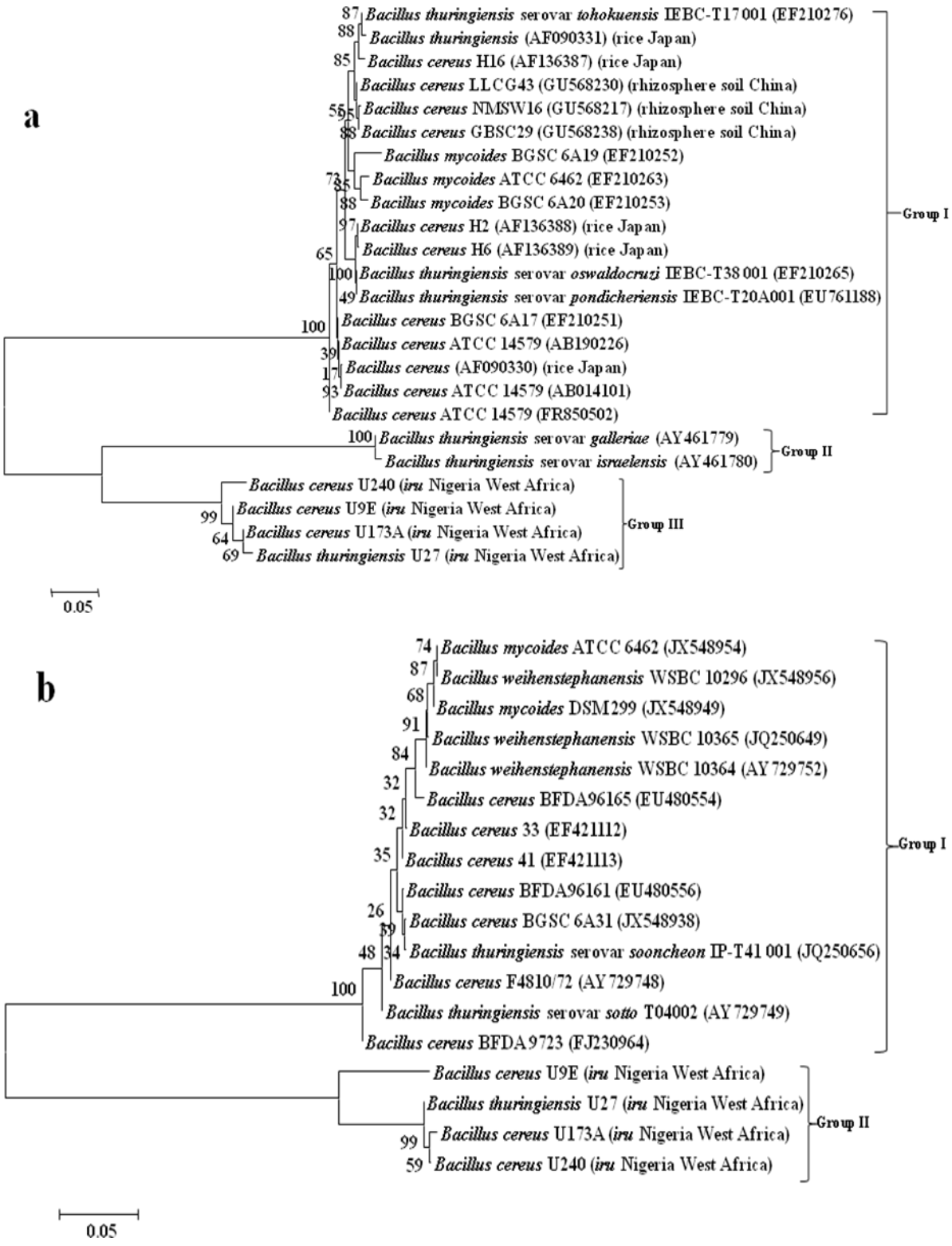


Figure 4.22. PCR amplicons of housekeeping genes (*rpoA*, *gyrB*, *pheS*, *glpF* and *gmk*) of *B. cereus* group from *iru* based on RAPD-PCR M13 grouping. m: 100 bp DNA ladder.


a
87 Bacillus thuringiensis serovar tohokuensis IEBC-T17001 (EF210276)
88 Bacillus thuringiensis (AF090331) (rice Japan)
85 Bacillus cereus H16 (AF136387) (rice Japan)
Bacillus cereus LLCG43 (GU568230) (rhizosphere soil China)
55 Bacillus cereus NMSW16 (GU568217) (rhizosphere soil China)
88 Bacillus cereus GBSC29 (GU568238) (rhizosphere soil China)
Bacillus mycoides BGSC 6A19 (EF210252)
73 Bacillus mycoides ATCC 6462 (EF210263)
88 Bacillus mycoides BGSC 6A20 (EF210253)
97 Bacillus cereus H2 (AF136388) (rice Japan)
65 Bacillus cereus H6 (AF136389) (rice Japan)
100 Bacillus thuringiensis serovar oswaldocruzi IEBC-T38001 (EF210265)
49 Bacillus thuringiensis serovar pondicheriensis IEBC-T20A001 (EU761188)
100 Bacillus cereus BGSC 6A17 (EF210251)
39 Bacillus cereus ATCC 14579 (AB190226)
17 Bacillus cereus (AF090330) (rice Japan)
93 Bacillus cereus ATCC 14579 (AB014101)
Bacillus cereus ATCC 14579 (FR850502)
Group I
100 Bacillus thuringiensis serovar galleriae (AY461779)
Bacillus thuringiensis serovar israelensis (AY461780)
Group II
Bacillus cereus U240 (iru Nigeria West Africa)
99 Bacillus cereus U9E (iru Nigeria West Africa)
64 Bacillus cereus U173A (iru Nigeria West Africa)
69 Bacillus thuringiensis U27 (iru Nigeria West Africa)
Group III
0.05
b
74 Bacillus mycoides ATCC 6462 (JX548954)
87 Bacillus weihenstephanensis WSBC 10296 (JX548956)
68 Bacillus mycoides DSM299 (JX548949)
91 Bacillus weihenstephanensis WSBC 10365 (JQ250649)
84 Bacillus weihenstephanensis WSBC 10364 (AY729752)
32 Bacillus cereus BFDA96165 (EU480554)
32 Bacillus cereus 33 (EF421112)
35 Bacillus cereus 41 (EF421113)
Bacillus cereus BFDA96161 (EU480556)
26 Bacillus cereus BGSC 6A31 (JX548938)
59
48 34 Bacillus thuringiensis serovar sooncheon IP-T41001 (JQ250656)
Bacillus cereus F4810/72 (AY729748)
100 Bacillus thuringiensis serovar sotto T04002 (AY729749)
Bacillus cereus BFDA9723 (FJ230964)
Group I
Bacillus cereus U9E (iru Nigeria West Africa)
Bacillus thuringiensis U27 (iru Nigeria West Africa)
99 Bacillus cereus U173A (iru Nigeria West Africa)
59 Bacillus cereus U240 (iru Nigeria West Africa)
Group II
0.05

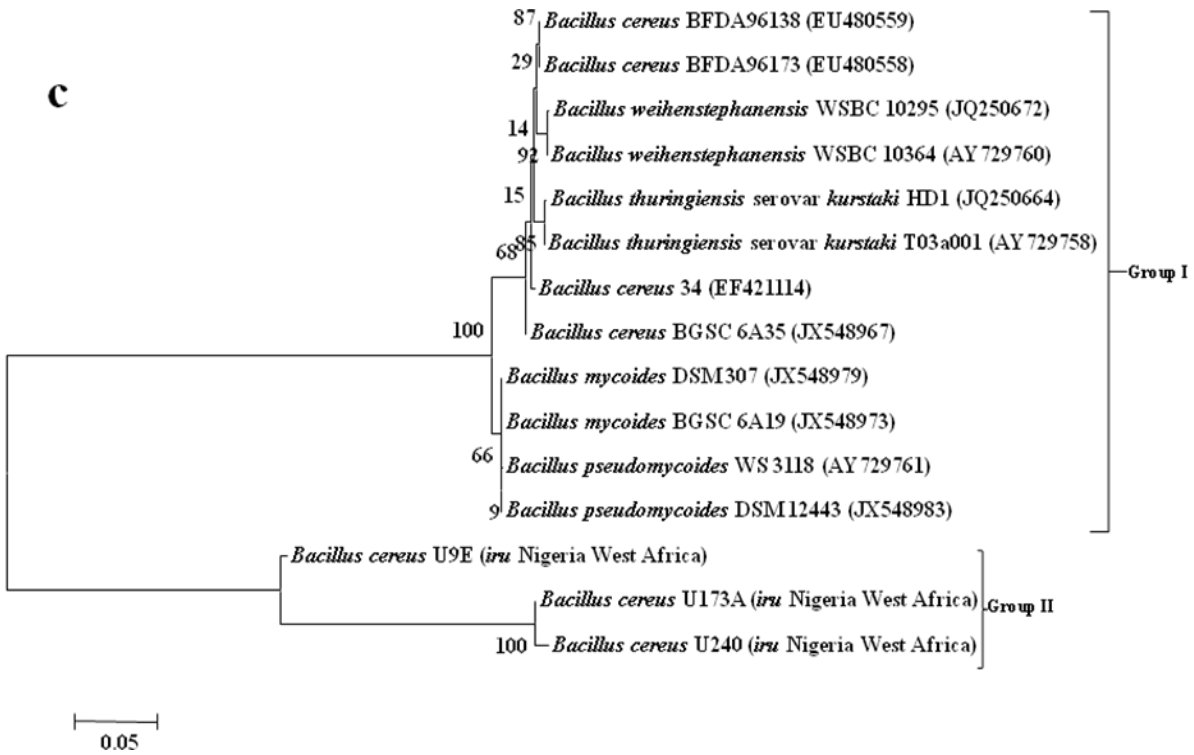


Figure 4.23. Phylogenetic relationship of pairwise and multiple alignments of (a) *gyrB* (b) *glpF* (c) *gmk* genes sequences of *B. cereus* phylotype obtained from *iru*, and other food and environmental samples, including referenced and typed strains. Trees generated using MEGA5 (http://www.megasoftware.net).

4.8 Probiotic functional properties of *Bacillus* strains

4.8.1 Acid resistance and bile salts tolerance

Autochthonous *B. subtilis* strains, *B. subtilis* U170B and *B. subtilis* U146A (accession numbers: JN255720 and JN255713 respectively) from *iru*, and a reference probiotic *B. clausii* UBB-07 were examined for their ability to withstand and survive physiological environment prevalent in the human GIT under simulated gastric acidic and bile intestinal conditions of the stomach and small intestine respectively. Vegetative cells of bacilli analysed generally showed resistance to acidic condition of pH 2 at 37^{o}C. *B. subtilis* U170B and *B. clausii* UBB-07 had just < 1 Log unit reduction after 3 h incubation, while *B. subtilis* U146A had 1 Log unit lower (Figure 4.24a). The survival rate (expressed in percentage) of the *Bacillus* strains based on initial viable colony counts and population counts after 3 h incubation at 37^{o}C were 33.45%, 12.44% and 9.53% for *B. subtilis* U170B, *B. clausii* UBB-07 and *B. subtilis* U146A respectively.

The three strains exhibited higher tolerance to bile salts concentration of 0.3% alkaline pH, typical of the duodenum conditions, compared to their resistance to acidic pH. In terms of log cycle loss, they all had < 1 Log unit reduction (Figure 4.24b). Cell viability of these strains after 3 h passage in simulated intestinal fluid was also higher compared to that of gastric juice. *B. subtilis* U170B demonstrated the highest survival rate (43.45%), followed by *B. subtilis* U146A (25%) and *B. clausii* UBB-07 (18.94%). In general, vegetative cells of *B. subtilis* U170B was more acid resistant and bile tolerant than the other two strains tested, with adequate survivability during the simulated gastrointestinal transit. However, only *B. clausii* UBB-07 spores survived the acidic and bile salts conditions; others were found to be highly sensitive as no single spore count was recorded. The reference strain thrived well in both low pH and bile salts with an average marginal decrease of 0.5 Log cycle losses (Figure 4.24c). Survival rate obtained were 42.27% and 20.80% for acid and bile salts respectively.

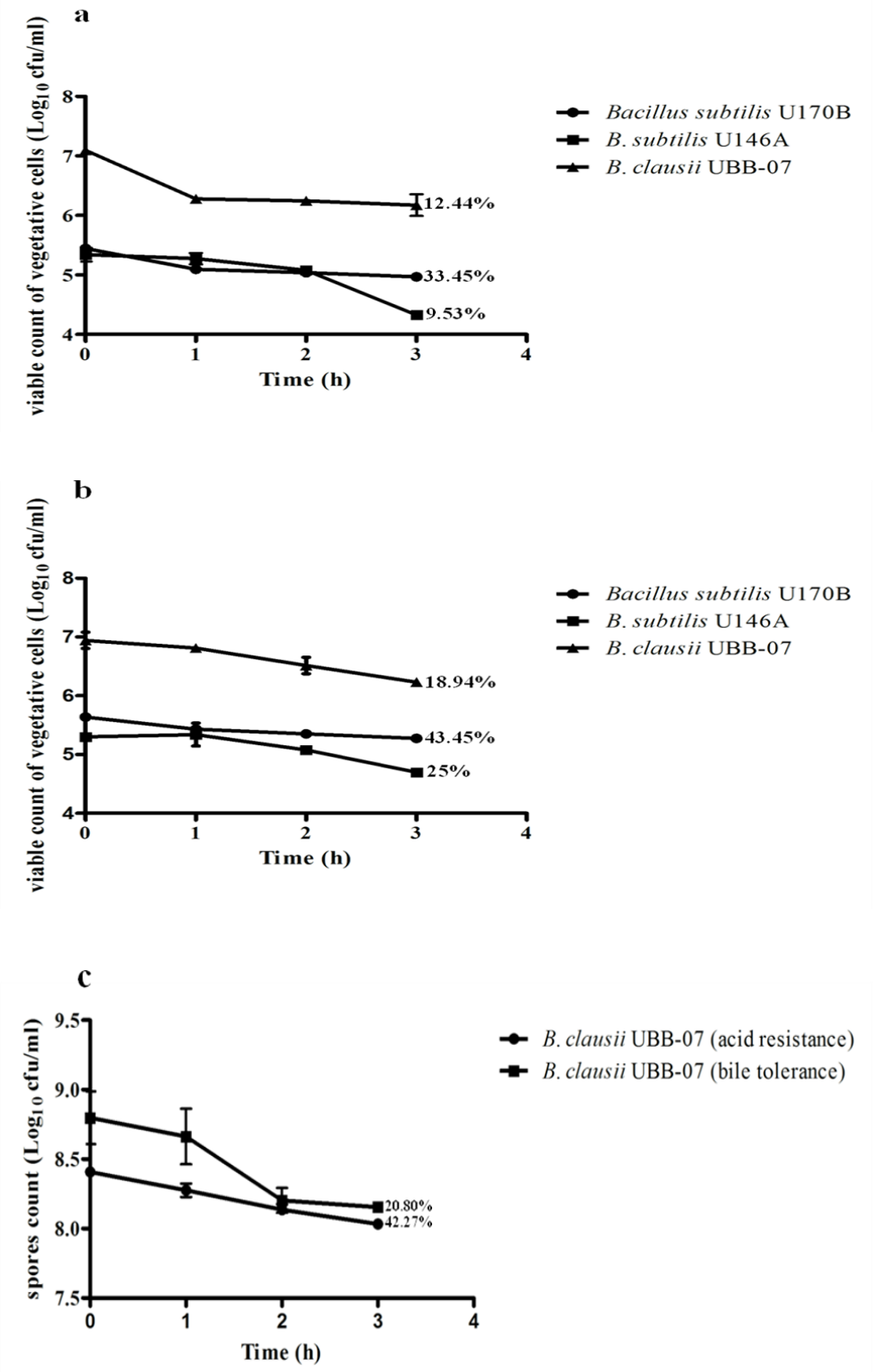


Figure 4.24. Growth kinetic and viability of *Bacillus* strains under simulated gastric juice and intestinal bile conditions. Acid resistance (a) and bile tolerance (b) of vegetative cells of *B. subtilis* strains from *iru* and *B. clausii* UBB-07 reference probiotic strain; (c) acid and bile tolerance of *B. clausii* UBB-07 spores. Error bars represent standard error of mean (SEM) of two replicate values.

4.8.2 *In vitro* antagonistic potentials of *Bacillus* strains against food-borne pathogens

The production of antibacterial compounds by *Bacillus* strains active towards eleven indicator strains, which included both Gram-positive and -negative bacteria were demonstrated using agar spot-on-lawn and well diffusion agar assays. The bacilli tested could not inhibit most of the indicator organisms (Table 4.5). However, *B. subtilis* U170B and *B. subtilis* U146A showed variable to weak inhibition zone against *B. cereus* MTCC 430 and *B. cereus* MBU 1011 with the agar spot-on-lawn method. No zone of inhibition was detected using the cell-free supernatant of these strains. *B. clausii* UBB-07 displayed strong antimicrobial activity against *B. cereus* MBU 1011 (Plate 1), though wider inhibitory zone was recorded for agar spot-on-lawn compared to cell-free culture supernatant.

4.8.3 Bacterial hydrophobicity

The hydrophobicity nature of the *Bacillus* strains were spectrophotometrically determined based on their ability to adsorb to *n*-hexadecane, toluene and chloroform at the aqueous-hydrocarbon interface. A subjective visual observation and scoring technique for assessing bacterial surface hydrophobicity showed that *B. subtilis* U146A formed strong and wider ring adsorption diameter with the various solvents used in comparison to other strains. Its adsorbed cells in the experimental test-tubes rose to the top with the hydrocarbon droplets, which resulted in reduced optical density and turbidity of the lower aqueous phase. The drop in turbidity corresponded with the disappearance of the bacterial cells from the lower aqueous phase and their appearance on the hydrocarbon droplets. Hydrophobicity percentage ranged from 18.31-44.96%, 16.14-44.11% and 20.32-61.73% for *n*-hexadecane, toluene and chloroform respectively. *B. subtilis* U146A had the highest percentage in all the solvents analysed, followed by *B. clausii* UBB-07 for toluene and chloroform, while the least affinity was observed in *B. subtilis* U170B also for toluene and chloroform. However, *B. subtilis* U146A adhesion value was not statistically different from others at $p<0.05$ for *n*-hexadecane,

except toluene and chloroform (Figure 4.25). When compared, hydrocarbons effect on hydrophobicity, no statistical difference exist in their values at $p<0.05$.

4.8.4 Autoaggregation

The cell autoaggregation properties of the *Bacillus* strains were analyzed over a period of 4 h based on their sedimentation characteristics. A phenotypic inspection of the cell aggregates showed that *B. subtilis* U146A formed quick and larger sediments, which precipitated into a clear solution. *B. clausii* UBB-07 sediments less quickly and produced constant turbidity, while *B. subtilis* U170B formed smaller aggregates, whose suspension showed both a precipitate and constant turbidity. The sedimentation rate of the cell suspension was also observed to increase with time at 37^{o}C. Percentage autoaggregation of the tested bacilli strains ranged from 21.94-46.50%, 12.54-68.14% and 7.76-19.97% for *B. subtilis* U170B, *B. subtilis* U146A and *B. clausii* UBB-07 respectively with respect to 4 h incubation (Figure 4.26a). It was evident that *B. subtilis* U146A possessed the maximum aggregation potential, followed by *B. subtilis* U170B, while the least was *B. clausii* UBB-07 after 4 h incubation at 37^{o}C.

4.8.5 Coaggregation

The coaggregation assay was used to substantiate the ability of *Bacillus* strains to closely interact with food-borne pathogens when co-cultured. The results obtained were expressed as the percentage reduction in the optical density of a mixed suspension of probiotic strain and pathogen compared with the individual suspensions. The three strains coaggregated with the pathogen (*S. enterica* subsp. *enterica* serovar Typhimurium LT2), but the percentages depended on each strain and time of co-incubation. These ranged from 5.00-33.80%, 26.98-56.71% and 3.40-23.43% for *B. subtilis* U170B, *B. subtilis* U146A and *B. clausii* UBB-07 respectively over 4 h incubation time (Figure 4.26b). *B. subtilis* U146A tended to coaggregate with the pathogen more than others, displaying 56.71% after 4 h at 37^{o}C.

Table 4.5: Antagonistic activities of *B. subtilis* strains isolated from *iru* and reference probiotic *B. clausii* UBBC-07 against eleven indicator bacteria strains.

	Test organisms		
Indicator strains	*B. subtilis* U170B	*B. subtilis* U146A	*B. clausii* UBBC-07
L. sakei DSM 20017	-	-	nd
L. casei DSM 20011	-	-	nd
S. aureus subsp. *aureus* ATCC 11632	-	-	nd
E. coli ATCC 11229	-	-	nd
L. monocytogenes ATCC 19118	-	-	nd
E. faecium ATCC 35667	-	-	nd
B. cereus MTCC 430	+ (2 mm)*	-	nd
B. cereus MBU 1011	+ (2 mm)*	+ (4 mm)*	+ (9 mm)†
S. aureus MBU 1023	-	-	-
E. coli MBU 1035	-	-	-
S. enterica serovar Typhimurium MBU 1047	-	-	-

DSM: Deutsche Sammlung von Mikroorganismen Gottingen, Germany; ATCC: American Type Culture Collection; MTCC: Microbial Type Culture Collection, Chandigarh, India. *Weak activity using agar spot-on-lawn method. †Strong inhibition with agar well diffusion assay. nd: not determined. Diameter of inhibition zone obtained after subtracting diameter of bored hole from the entire halo.

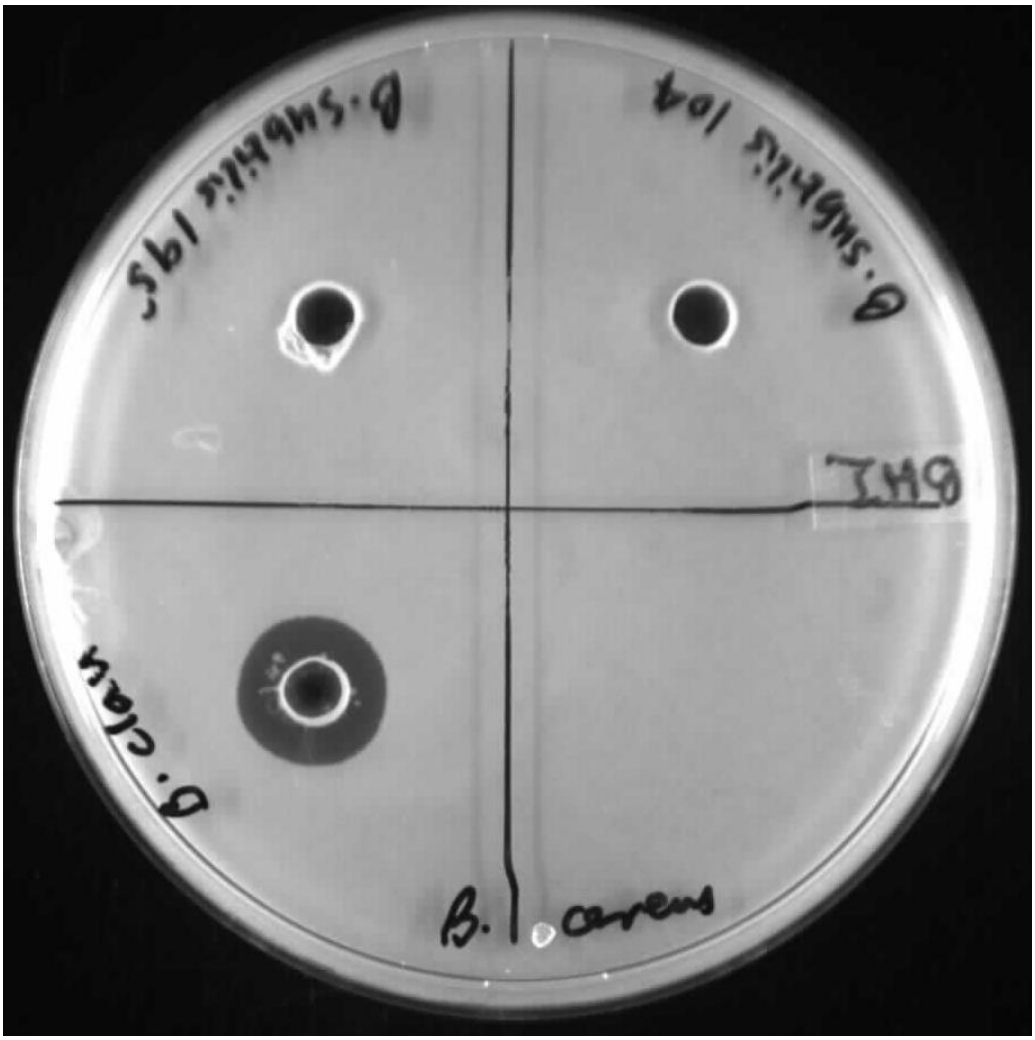


Plate 4.1. Petri dish containing cell-free extract of *B. clausii* UBBC-07 showing inhibitory activity against *B. cereus* MBU 1011 by agar well diffusion method on BHI agar.

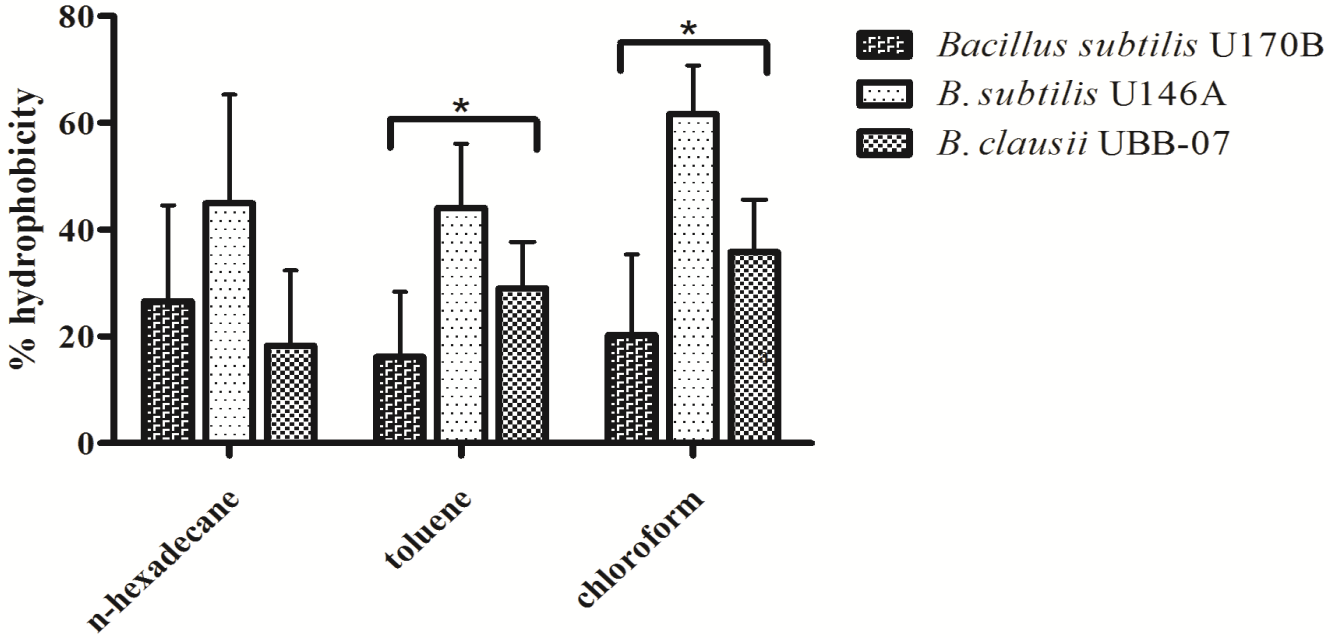


Figure 4.25. Cell surface adhesion of *B. subtilis* U170B and *B. subtilis* U146A from *iru* and reference probiotic *B. clausii* UBB-07 to *n*-hexadecane, toluene and chloroform. Error bars represent standard error of mean (SEM) of three replicate values. Asterik indicates statistical significance difference ($p<0.05$) in % hydrophobicity within the same group of hydrocarbon; Duncan test.

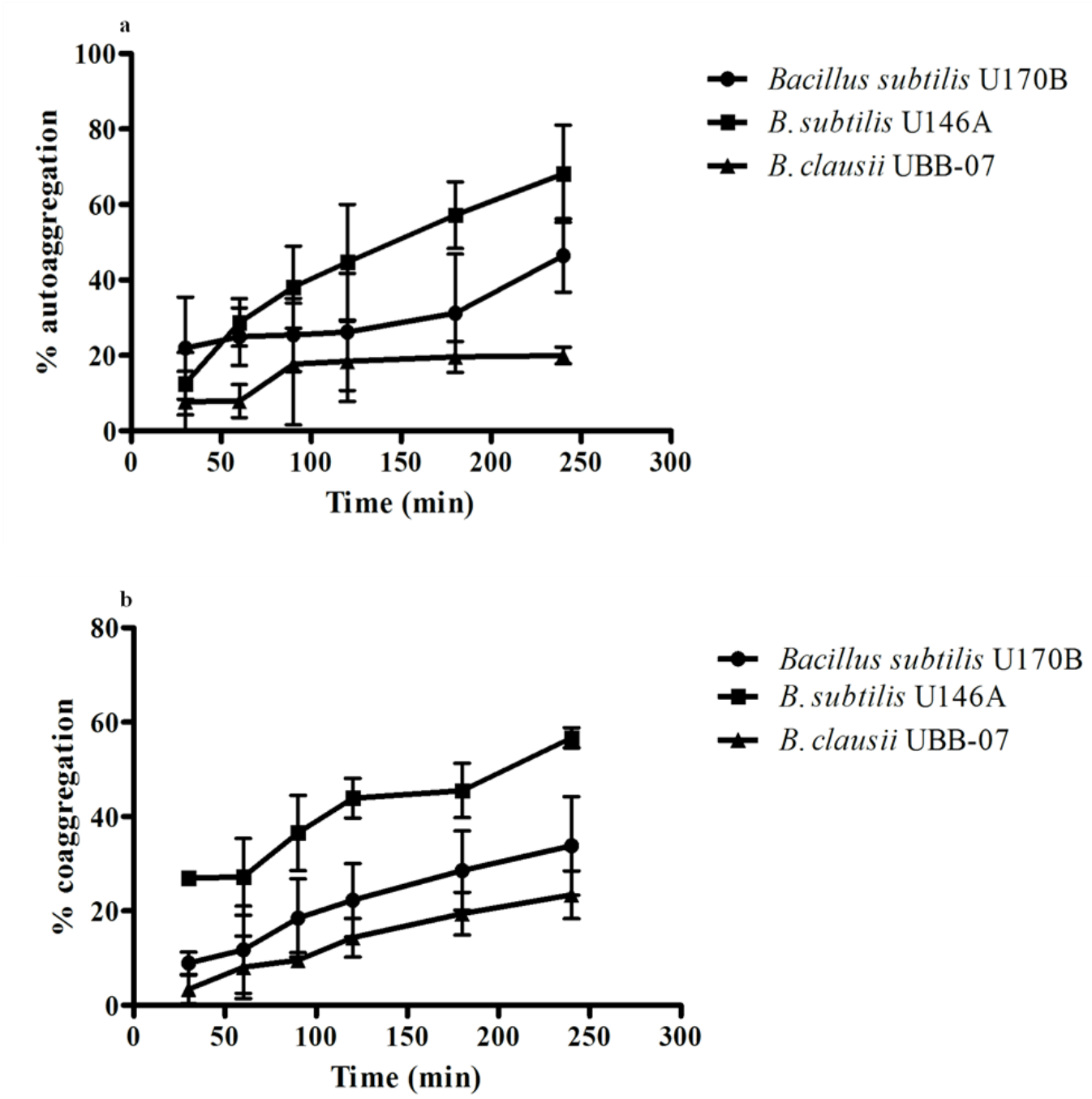


Figure 4.26. Time-course of (a) autoaggregation and (b) coaggregation characteristics of *B. subtilis* U170B and *B. subtilis* U146A from *iru* and reference probiotic *B. clausii* UBB-07 for a period of 4 h incubation at 37^{o}C. Error bars represent standard error of mean (SEM) of three replicate values.

4.8.6 Adhesion of *Bacillus* strains to HT-29 cell lines

Adhesion capabilities of two *B. subtilis* strains from *iru* and reference strain *B. clausii* UBB-07 were determined *in vitro* on cultured undifferentiated and differentiated human intestinal epithelial cells, mimicking an *in vivo* environment. Bacterial cells were used to infect HT-29 cell line monolayers at 100:1 MOI, incubated for 2 h, to create interaction with human enterocytes. Bacterial adhesion assessment based on microscopic random counting is likely to be prone to observer error. Also, adherence determination by extrapolation of bacteria fluorescence was not valid enough. As a result, data from total viable counts of adherent bacterial cell suspension obtained after HT-29 cell lysis only were analyzed.

Percentage adhesion expressed as the number of bacteria cells adhering to the epithelial cells relative to the number added is presented in Figure 4.27. For both undifferentiated and differentiated cells, *B. clausii* UBB-07 was the most adhesive strain; 0.45% and 0.38% respectively. The next was *B. subtilis* U170B, which had 0.15% and 0.12%, and then *B. subtilis* U146A with 0.12% and 0.06% respectively. Only *B. clausii* UBB-07 showed significant difference ($p<0.05$) in binding percentage for both undifferentiated and differentiated cells compared to other strains (Figure 4.27). However, no statistical difference ($p<0.05$) was found in percentage adhesion between undifferentiated and differentiated cells for each *Bacillus* strain (Figure 27).

4.8.7 Inhibitory effects of *Bacillus* strains on adhesion of *S. enterica* serovar Typhimurium MBU 1047 to HT-29 cell lines

The ability of *Bacillus* strains to prevent or reduce the adhesion of *S. enterica* serovar Typhimurium MBU 1047 to HT-29 cells was evaluated based on pre-incubation of bacilli with HT-29; co-infection of bacilli and *S. enterica* serovar Typhimurium MBU 1047, and pre-incubation of *S. enterica* serovar Typhimurium MBU 1047 with HT-29. *B. subtilis* U170B, *B. subtilis* U146A and *B. clausii* UBB-07 were able to hinder adhesion of *S. enterica*

serovar Typhimurium MBU 1047 to HT-29 cells by 22.05%, 28.72% and 34.15% respectively, when previously added (Figure 4.28).

In the co-incubation experiment, where both bacilli and *S. enterica* serovar Typhimurium MBU 1047 were provided equal opportunity for adherence to HT-29 cells, the strains prevented 20.51%, 24.10% and 32.31% of *S. enterica* serovar Typhimurium MBU 1047 from adhering to HT-29 cells (Figure 4.28). In the displacement assay, *B. subtilis* U170B, *B. subtilis* U146A and *B. clausii* UBB-07 displaced already adhered *S. enterica* serovar Typhimurium MBU by 18.46%, 17.14% and 22.05% respectively, though the level of inhibition of adhesion was weaker compared to exclusion and competition assays (Figure 4.28). In all the experimental assays conducted, *B. clausii* UBB-07 demonstrated the greatest inhibition of adhesion to HT-29 cells by *S. enterica* serovar Typhimurium MBU 1047 (Figure 4.28). However, test of statistical significance at $p<0.05$; Duncan within and between groups showed no difference in percentage of *S. enterica* serovar Typhimurium MBU 1047 inhibited from adhering to HT-29 cells.

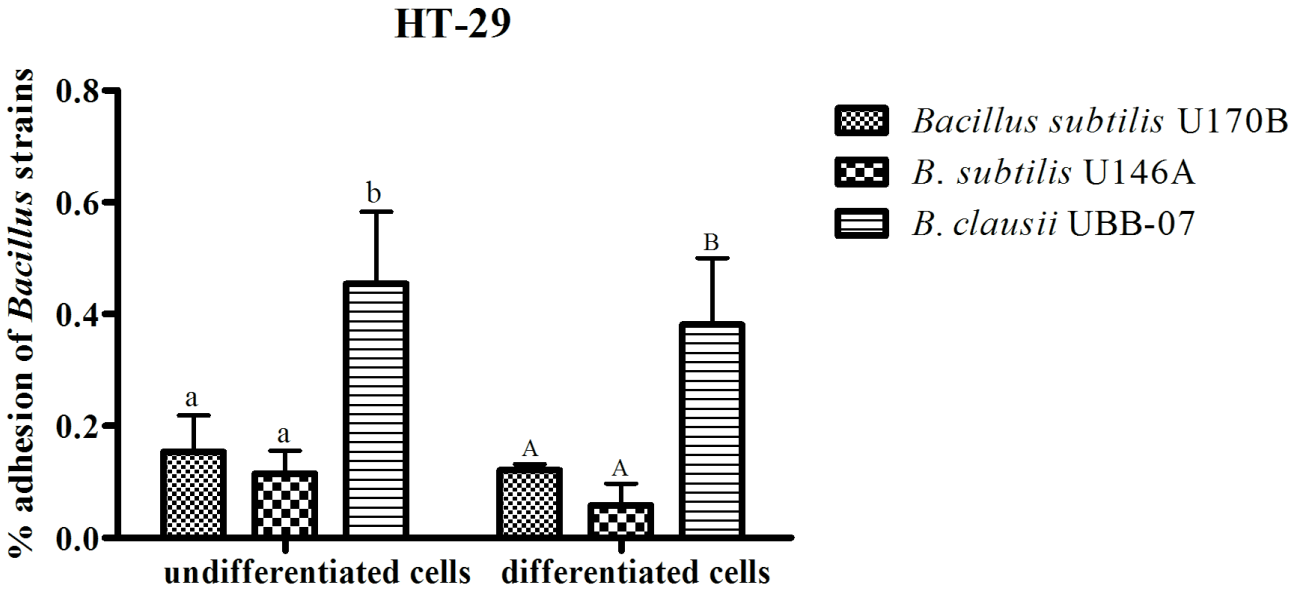


Figure 4.27. Percentage adhesion of *B. subtilis* strains U170B, U146A and *B. clausii* UBB-07 that bound to undifferentiated and differentiated cultured HT-29 cells. Bars with different alphabets within the same group differ significantly at $p<0.05$; Duncan test. Error bars represent standard error of mean (SEM) of two replicate values.

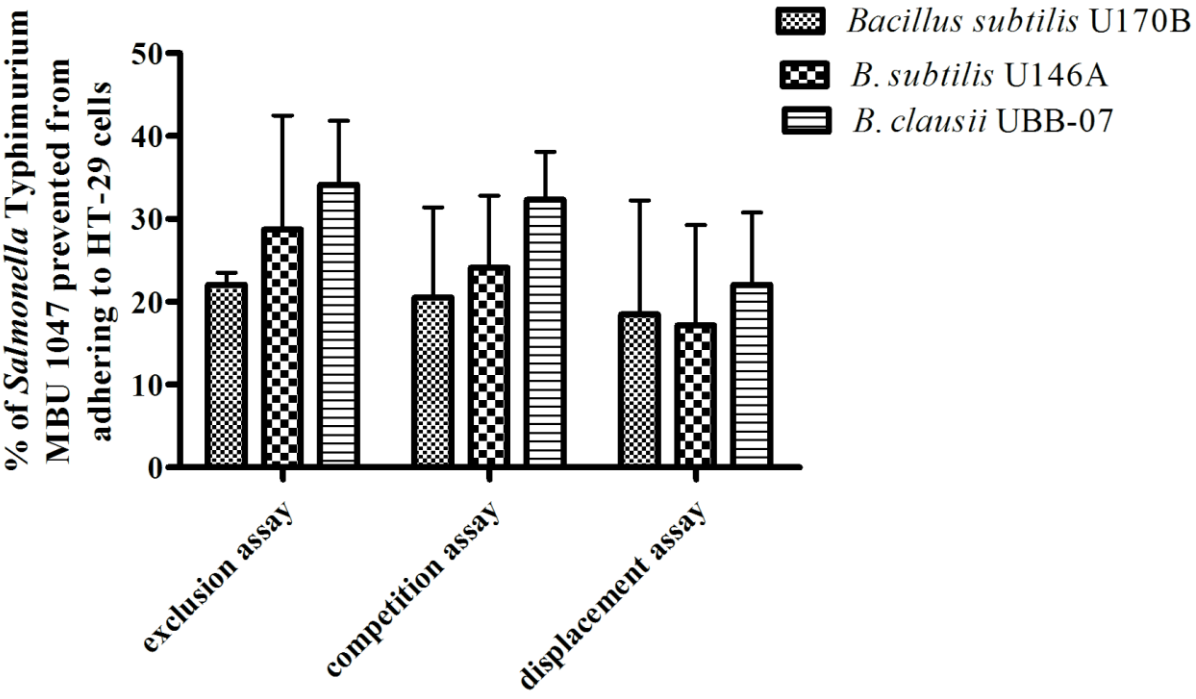


Figure 4.28. Inhibition of adhesion of *S. enterica* serovar Typhimurium MBU 1047 during exclusion, competition and displacement assays in the presence of *B. subtilis* strains, U170B, U146A and *B. clausii* UBB-07. Error bars represent standard error of mean (SEM) of two replicate values.

4.9 *In vitro* safety evaluation of *Bacillus* strains

4.9.1 Haemolytic activity

B. subtilis strains U170B and U146A isolated from *iru* did not lyse red blood cells when streaked on BHI agar containing sheep blood. This can be considered γ-haemolytic. *B. clausii* UBB-07 had α-haemolysis with partial clearance zone and greenish colouration around the region streaked. *B. cereus* U175 also from *iru* and haemolytic *B. cereus* MBU 1011 used as positive controls produced complete clear zone, indicating β-haemolysis.

4.9.2 Antibiotic susceptibility pattern

The antibiotic susceptibility pattern of the *Bacillus* strains were demonstrated combining standard disk and agar overlay diffusion methods. Seventeen different antibiotics were examined, including Gram-positive, Gram-negative, broad spectrum, β-lactams and aminoglycosides. *B. subtilis* U170B and *B. subtilis* U146A were sensitive to most of the antibiotics; however, the latter was resistant to methicillin (Table 4.6). On the contrary *B. clausii* UBB-07 was resistant to majority of the antibiotics used, but only sensitive to chloramphenicol.

4.9.3 Degradation of porcine mucin by plate assay

The potential of *Bacillus* strains to degrade mucin was qualitatively determined *in vitro*. Mucinolysis activity with zone of discolouration was detected around the colony of the faecal flora, as well as the bacilli, in varying zone of inhibition (Figure 4.29). *B. clausii* UBB-07 however exhibited higher mucin degradation activity when compared to other strains.

Table 4.6: Antibiotic sensitivity profiles of *B. subtilis* strains isolated from *iru* and reference probiotic *B. clausii* UBBC-07.

	Zone of inhibition (mm)‡		
Antibiotics (symbol, µg)	*B. subtilis* U170B	*B. subtilis* U146A	*B. clausii* UBB-07
Kanamycin (K, 30)	25.10±0.28$^{\Phi}$ (S)	27.40±0.71 (S)	16.35±0.07 (I)
Amoxyclav (AMC, 30)	37.15±0.92 (S)	40.00±0.85 (S)	3.20±0.99 (R)
Ampicillin (AMP, 10)	24.10±0.14 (S)	26.20±0.14 (S)	1.40±0.57 (R)
Chloramphenicol (C, 30)	25.15±0.07 (S)	29.30±0.28 (S)	25.00±0.99 (S)
Clindamycin (CD, 2)	19.10±0.14 (S)	34.25±0.35 (S)	11.25±0.78 (R)
Ciprofloxacin (CIP, 5)	33.15±0.35 (S)	38.15±0.78 (S)	14.25±0.50 (R)
Erythromycin (E, 15)	23.10±0.42 (S)	42.25±0.07 (S)	10.05±0.21 (R)
Gentamicin (GEN, 10)	27.20±0.28 (S)	24.15±0.35 (S)	13.15±0.07 (I)
Methicillin (MET, 5)	10.00±0.28 (I)	4.20±0.99 (R)	- (R)
Nalidixic Acid (NA, 30)	15.05±0.35 (I)	28.35±0.21 (S)	5.25±0.35 (R)
Penicillin G (P, 10 units)	39.20±0.57 (S)	34.35±0.35 (S)	- (R)
Rifampicin (RIF, 5)	24.20±0.28 (S)	25.45±0.35 (S)	5.25±0.50 (R)
Streptomycin (S, 10)	23.10±0.57 (S)	29.05±1.20 (S)	12.20±0.14 (I)
Tetracycline (TE, 30)	38.30±0.71 (S)	34.30±0.28 (S)	5.05±1.06 (R)
Trimethoprim (TR, 5)	24.20±0.14 (S)	24.15±0.64 (S)	- (R)
Vancomycin (VA, 30)	22.00±1.13 (S)	28.60±0.14 (S)	12.10±1.13 (R)
Norfloxacin (NX, 10)	36.05±0.50 (S)	32.35±0.21 (S)	11.60±0.42 (R)

‡Values have antibiotic disk (6mm) subtracted from them. $^{\Phi}$Mean with standard deviation (SD) of two replicate data. S, sensitive; I, intermediate; R, resistant as per CLSI.

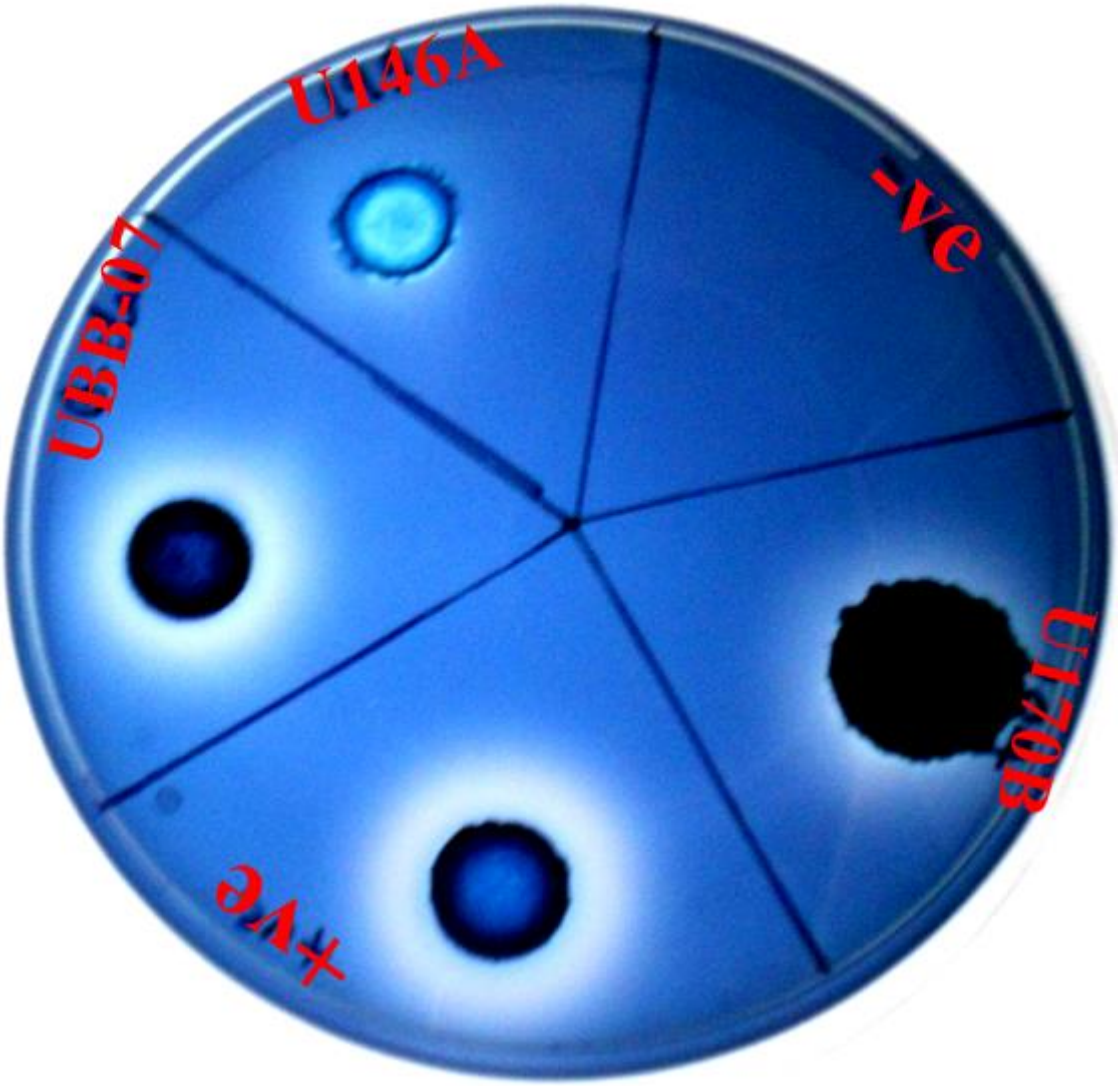


Plate 4.2. Mucin degradation assay in agarose Petri dish without glucose. Faecal flora (+ve) showed mucin degradation activity with discolouration zone (halo) around region of spot. Heat killed faecal flora (-ve) exhibited no activity. *B. subtilis* strains (U170B and U146A) displayed weak mucinolytic activity, while *B. clausii* UBB-07 showed higher discoloured zone.

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

5.0 Discussion

Fermented vegetable protein cotyledons processed into condiments are widely consumed in Africa and Asia. In many W. African countries they are produced from different legume substrates, consumed by over ca. 100 million people and significantly contribute to their nutrient intake, as well as enhancement of organoleptic properties of variety of dishes. Different challenges associated with traditional fermented food products have been previously identified (Oguntoyinbo *et al*., 2011; Oguntoyinbo, 2014). Significantly, production and preservation of traditional fermented foods around the world generally face nutritional limitations, safety and low shelf-life issues, which have been attributed to the process conditions during manufacturing.

Condiments are produced by natural, uncontrolled fermentation conditions, without conscious inoculation of substrates with defined starter cultures. This often leads to batch variations in microbial composition, resulting into products inconsistency, low shelf-life and incidences of safety issues in developing countries (Oguntoyinbo, 2014). Fermentation stage has been identified as a critical control point during condiments production that requires diligent selection of microbial starter cultures, to develop predictable fermentation process, improve safety and bring about value added products (Oguntoyinbo, 2012). Earlier studies poorly characterized the microbial profile and structure of traditional fermented protein seeds; these data were limited in methodology and reproducibility. The data were also generated from traditional microbiological cultivation methods and phenotypic tests, which provided limited information on taxonomic, genetic diversity and uncultivable microbes (Hong *et al*., 2012).

Dominance of aerobic and facultative anaerobic spore-forming *Bacillus* species during traditional legume fermentation processes for condiments production in W. Africa has been

consistently reported (Sanni *et al*., 1998; Ouoba *et al*., 2004). However, there is dearth of knowledge on the genomic diversity, sub-type, clonal relationship and phylogenetic of autochthonous *Bacillus* species in *iru*. Consequently, this study was conducted in order to provide adequate information on the bacterial community profile and population structure of *iru*, by applying a polyphasic strategy that combined culture-independent molecular techniques, traditional methods of microbial isolation, characterization and identification, and culture-based genomic DNA fingerprinting techniques, necessary for strains sub-typing of dominant *Bacillus* species in *iru*. This is aimed at screening and selection of suitable starter cultures with specific desirable fermentation features and functional probiotic properties that can be harnessed in pilot, cottage and other small-scale production of condiments in Nigeria.

In the culture-independent molecular analysis, the two DNA extraction protocols yielded suitable nucleic acid templates for PCR amplification; this first crucial step has been shown to be essential to profiling microbial community in earlier studies (Di Pinto *et al*., 2007). Results of the amplified PCR-DGGE products of V3 variable region of 16S rRNA gene separated in DGGE gel, showed no significant difference in DNA fragments and DGGE band patterns with the two methods of genomic DNA extraction used in this study. Mafra *et al*. (2008) earlier reported similar observations in a study involving comparison of four DNA extraction methods for downstream applications.

Analysis of similarities and differences in bacterial composition of the various *iru* samples obtained from different geographical regions revealed intra- and inter-specific variations, as established by Dice similarity coefficient and nMDS, an indication that the bacterial community profile of these condiments is diverse than earlier reported; having grouped into distinct clusters with varying diversity indexes. The different *iru* samples were produced by fermentation of the same substrate *P*. *biglobosa* seeds but under varying conditions, such as time, temperature, processing equipment etc. Therefore, diversity observed in the microbial

communities could be due to difference in fermentation conditions, which is natural and uncontrolled, without the use of any known specific bacterial isolate as starter culture. Other workers have also reported similar variation in the microbial composition of naturally fermented food materials (Ampe and Miambi, 2000; Oguntoyinbo *et al*., 2011).

DGGE gel patterns analysed for the various *iru* samples was completed by estimation of bacterial species abundance and biodiversity indexes based on Shannon-Weaver models. These ecological parameters were relatively high for all the condiments; however, *iru* sampled from Ilorin, North-Central Nigeria had the highest values, which implied that a great number of bacterial species were involved in the fermentation process. Again, this is not surprising since these condiments are produced under natural conditions with no specific microbial strain used to initiate fermentation. One of the advantages of DGGE fingerprinting technique is that it provides significant information on the ecology of microorganisms of any known environment, allowing for comparison of total microbial communities through biodiversity indexes determination (ben Omar and Ampe, 2000). This is not the case for conventional techniques, such as cultivation of bacteria on solid media, where biodiversity study is practically impossible, since majority of the microorganisms are yet to be cultured (Amann *et al*., 1995).

Another benefit of DGGE is that bands can be excised and sequenced for identification of microbial species present in a particular community. DNA sequencing of the major PCR-DGGE bands identified *B. subtilis* as the main bacterial species associated with *iru*, being consistently present and most frequently found in virtually all the samples analysed, an indication that this condiment is a preferential niche for *Bacillus* colonization. It also confirmed the persistence and relevance of *B. subtilis* in the fermentation process of *P. biglobosa* for *iru* production. This bacterium is a common soil microorganism; it must have been probably carried along with the raw legume seeds (*P. biglobosa*), establish survival by

producing endospores to withstand the adverse heating condition during *iru* production. It is widely recognized that one of the main sources of microorganisms in food fermentation, if starters are not employed, is the raw material used (Rantsiou *et al*., 2005). An underlining factor that also contributed to the predominance of *B. subtilis* was the visible intense band common to the various *iru* samples. The intensity of an individual band is thought to be a semi-quantitative measure of its abundance sequence in mixed complex microbial populations, and qualitative functional roles in a fermentation process (Muyzer *et al*., 1993; Nielsen *et al*., 2007).

The other DGGE bands identified were potential food-borne pathogens and contaminants such as *S.vitulinus, S. saprophyticus, B. thuringiensis, M. morganii, T. halophilus, S. jeotgali, U. thermosphaericus, B. parabrevis, Brevibacterium* sp. and uncultured bacteria clones. *Staphylococcus* species was found in 10 of the 16 *iru* samples, representing 63%. They are thought to be the second dominant bacterial flora after *Bacillus* species or co-dominance of bacilli, frequently isolated in viable counts in various indigenous African and Asian fermented condiments, using traditional culture-dependent techniques, involving phenotypic, biochemical and molecular characterization (Odunfa, 1981a; Obeta, 1983; Antai and Ibrahim, 1986; Odunfa and Komolafe, 1989; Achi, 1992; N'dir *et al*., 1997; Ogbonna *et al*., 2001; Ogunshe *et al*., 2006; Jeyaram *et al*., 2008; Parkouda *et al*., 2010; Chadare *et al*., 2011 ; Okorie and Olasupo, 2013). However, very few reports are available on the detection of these organisms in fermented vegetal protein seeds using molecular culture-independent methods. Kim *et al*. (2009) and Nam *et al*. (2012) applying PCR-DGGE and barcoded next-generation pyrosequencing respectively detected *S. gallinarum* and *S. saprophyticus* in soybean fermented *doenjang* samples analysed in South Korea. Meanwhile, the only study so far in W. Africa according to literature search, found no species of *Staphylococcus* in *tayohounta*, a

fermented food condiment in Benin Republic analysed using PCR-DGGE (Chadare *et al*., 2011).

Thus, information on the detection of *S.vitulinus* and *S. saprophyticus* in traditional African fermented flavouring condiment using PCR-DGGE is reported for the first time. The role of these microorganisms during legume seeds fermentation is poorly understood. However, they have been constantly isolated from fermented sausages, particularly, *S. xylosus*, justifying its wide use as starter culture in this product (Rantsiou and Cocolin, 2006). In this regard their contributions and functions in alkaline fermented condiments are worth investigating.

The incidence of *Staphylococcus* species in *iru* clearly raises concern regarding the microbiological safety of this condiment, and hazards inherent with consumption of naturally fermented food products where production processes are not controlled (Oguntoyinbo *et al*., 2011). Staphylococci are known to be normal flora of the human skin, this could be a source of these organisms in fermented condiments, contributed by the processors who handle the production processes, and regularly have manual contact with the final product. The amino acids, peptides and vitamins synthesized by proteolytic *Bacillus* species during fermentation of *P. biglobosa* have also been presumed to promote the growth of *Staphylococcus* species in *iru* (Odunfa and Komolafe, 1989).

Many *Staphylococcus* strains are capable of producing heat stable toxins, generally known as staphylococcal enterotoxins (SEs), and these may not be totally inactivated during normal process of cooking *P. biglobosa* for *iru* production. The enterotoxins have been implicated in various food poisonings, leading to a number of food-borne diseases and outbreaks (Balaban and Rasooly, 2000). To circumvent this and ensuring production of safe and consistent fermented condiments for the teeming consumers, quality control measures combining application of starter cultures and training and education of processors on improved

fermentation conditions based on GMPs, good hygienic practices (GHPs), and hazard analysis critical control points (HACCP) guidelines are suggested.

B. thuringiensis was occasionally found in the *iru* samples. Sequencing of clones of PCR-DGGE products of bacterial DNA also revealed the infrequent presence of *B. thuringiensis* in *tayohounta* (Chadare *et al*., 2011). *B. thuringiensis* strains belong to the *B. cereus* group and their virulence characteristics is well documented (Hsieh *et al*., 1999). The frequency of *M. morganii* band in the DGGE gel representing the bacterial composition of the various *iru* samples was as high as that of *S. vitulinus*. *M. morganii* is a Gram-negative, facultative anaerobe, straight rods bacterium, belonging to the *Enterobacteriaceae* family (Seija *et al*., 2015). It is ubiquitous in the environment, and also constitutes the normal flora of humans, mammals and reptiles' intestinal tract (O'Hara *et al*., 2000). This organism is an opportunistic pathogen, causing skin and soft tissue diseases, and other nosocomial infections such as urinary tract infections (McDermott and Mylotte, 1984; Chen *et al*., 2012). It has been earlier reported that members of the *Enterobacteriaceae* family namely *E. coli*, *Enterobacter cloacae*, *E. aerogenes*, *Klebsiella pneumoniae*, *Providencia rettgeri* and *Proteus mirabilis* contribute to the microbial ecology of naturally fermented protein seeds, especially at the early stage of the fermentation process (Nout *et al*., 1998; Achi, 1992; Han *et al*., 2001; Jeyaram *et al*., 2008; Parkouda *et al*., 2010).

In this study, *M. morganii* not previously identified in traditional fermented food condiments by culturing methods was detected in *iru* for the first time using PCR-DGGE. This bacterial species typically grows on blood agar, requiring selective enrichment and other growth conditions. They may be present in *iru* in viable counts, sub-lethal or injured form, and eventually enters a non-cultivable state (Cocolin and Ercolini, 2008). These perhaps may be the reason why it was not isolated hitherto with traditional nutrient culturing. The classical microbiological methods, based on isolation and plate counts has been repeatedly criticized

because only easily culturable microorganisms are isolated while others whose growth requirements are not readily accessible are missed out (Rantsiou and Cocolin, 2006). Hence, PCR-DGGE as applied in the present study has eliminated the prejudices inherent in cultural techniques (such as underestimation of microbial contents) and improved understanding of the bacterial population profile of *iru*. Some strains of *M. morganii* carry antibiotic-resistant plasmids and are naturally resistant to a number of antibiotics namely tetracycline, ampicillin, oxacillin, tigecycline, polymyxin B, nitrofuratoin, penicillin, aminopenicillin, amoxicillin-clavulanate, including first and second generation cephalosporins (Leclercq *et al*., 2013). Since there is possibility of acquired resistance of antibiotic genes by horizontal transfer that may occur among food-borne microbial strains, there is need to check the incidence of this organism in traditional fermented food products.

Of particular interest was the identification of *S. jeotgali* and *T. halophilus* in some *iru* samples. These moderately halophilic bacteria of the family *Staphylococcaceae* and LAB group respectively are one of the important microbial species in the production of oriental salted fermented foods such as soy sauce, fermented soybean paste and fermented seafood (Aslam *et al*., 2007; Kim *et al*., 2009; Kuda *et al*., 2014). The source of these bacteria in *iru* can be attributed to the salt added as preservative at the end of the production process; hence their active participation during fermentation of *P. biglobosa* is ruled out.

A number of studies have identified genera of LAB such as *Enterococcus* spp., *E. faecium, E. hirae, E. durans, E. casseliflavus, L. fermentum, L. agilis, L. mesenteroides, L. dextranicus, S. equinus, P. acidilactici, P. pentosaceus* and *W. confusa* in W. African fermented condiments, whose roles are yet to be ascertained (Antai and Ibrahim, 1986; Oguntoyinbo *et al*., 2007; Kpikpi *et al*., 2010; Ouoba *et al*., 2010; Parkouda *et al*., 2010; Chadare *et al*., 2011). Sometimes, these LAB may be from the additives used during condiments production or post-heating contaminants, for example, *kantong*, a Ghanaian condiment, made from

fermented *Ceiba pentandra* seeds with cassava flour, and rice straw used in covering steamed soybeans meant for fermentation of Korean *chungkookjang* (Hong *et al*., 2012; Kpikpi *et al*., 2014). These additives tend to provide fermentable sugar substrates for LAB to thrive.

In the culture-dependent analysis, an average population density of 10^6 of bacterial spore-formers was enumerated in the *iru* samples by selective isolation. This was within the limits obtained in other fermented condiments investigated (Oguntoyinbo *et al*., 2010; Chettri and Tamang, 2015). Mean pH of 7.2 was recorded for the various *iru* samples; confirming their alkalinity nature. Omafuvbe *et al*. (2004) reported pH value of 7.9 for *ogiri-ijebu* produced from melon seeds. Chettri and Tamang (2015) also noted similar pH values of 7.4 and 7.1 in *tungrymbai* and *bekang* respectively, naturally fermented soybean condiments commonly consumed in Meghalaya and Mizoram states of India. The proteolytic activities due to enzymatic hydrolysis of dominant *Bacillus* species, resulting in the production of amino acids and ammonia from proteins has been attributed to cause rise in pH during alkaline fermentation of vegetal protein seeds (Aderibigbe and Odunfa, 1990; Ouoba *et al*., 2003b). Common disparity was observed in the population counts and pH of the diverse *iru* sampled within and between the different locations, which may be largely due to factors such as spontaneous nature of the fermentation process, cultural practices of the producers, environmental influences among others.

The representative predominant bacterial colonies phenotyped based on cultural features, microscopic and biochemical tests presumptively identified the strains as *Bacillus* species and its closest relative members. Most of the leguminous protein seeds are long heated before fermentation; this may constitute a selective step for heat resistance bacteria, especially spore-formers, such as bacilli (Ouoba *et al*., 2008). High protein content of the raw material also favours the dominance of *Bacillus*, where they produce large amounts of protease enzyme, including the exothermic nature of the fermentation process. Previous studies in W.

Africa employed conventional phenotypic parameters for the identification of *Bacillus* species in fermented condiments (Odunfa, 1985a; Ikenebomeh, 1989; Okorie and Olasupo, 2013). Phenotypic characters have also been frequently used for bacterial characterization, where they form basis for numerical taxonomy (Vandamme *et al*., 1996).

Further characterization of two selected *Bacillus* strains with API 50 CHB sugar fermentation tests confirmed their identities as *B. subtilis*/*B. amyloliquefaciens*. This system of bacterial identification is however not reliable, insufficient, often misleading, and does not justify strains diversity, sub-type and clonal relationships. Interestingly, the two bacilli strains were able to ferment and hydrolyse lactose and raffinose; suggesting their potential to produce α-galactosidase require for hydrolyzing complex carbohydrates present in *P. biglobosa*. This can help to improve the nutritional contents of *iru* significantly, overcome problem of flatulence, diarrhoea and indigestion among consumers. *B. subtilis*/*B. amyloliquefaciens* U170B in addition utilized inulin, a prebiotic oligosaccharide compound that help to stimulate the growth and activities of intestinal beneficial microorganisms. This bacterium can be an appropriate probiotic candidate that can be considered during development of synbiotic. The two *B. subtilis*/*B. amyloliquefaciens* strains both produced amylase and proteinase enzymes, which indicate their potentials as fermenting starters for proteolysis needed for flavour development and release of amino acids during *iru* production.

Different comparative genomic typing techniques were employed in this study to differentiate species, strains diversity and sub-type, identification, as well as determination of phylogenetic relationship among autochthonous bacilli cultured from Africa and Asian traditional fermented condiments, including reference *Bacillus* strains. This was aimed at providing sufficient information that will guarantee maximization of technological advantages and desired functional properties of divergent strains when used as starter cultures to produce safe, consistent and acceptable condiments in W. Africa.

The ARDRA fingerprints differentiated *B. subtilis* phylogeny from *B. cereus* group, including other related genera. Oguntoyinbo *et al*. (2010) earlier used this technique to distinguish *B. cereus* group from other *Bacillus* species isolated from *okpehe*, fermented *Prosopis africana* seeds. However, their ARDRA digestion with *Hha*I, *Hinf*I and *Sau*3AI could not bring about species differentiation of the *B. subtilis* phylotype. Vaerewijck *et al*. (2001) also reported difficulty in separating *B. licheniformis* from *B. subtilis*. Identification based on Raman spectroscopy of closely related species of *B. subtilis* group failed to differentiate *B. subtilis* from *B. amyloliquefaciens* (Hutsebaut *et al*., 2006). In this present study a simple, rapid, reproducible and reliable ARDRA protocol simply based on *Rsa*I and *Cfo*I clearly differentiated closely related species of the *B. subtilis* group, in particular, *B. licheniformis* from *B. subtilis*, which hitherto was a difficult task.

ITS-PCR alone also allowed typing of *B. subtilis sensu lato* mainly at species level, with differentiation of *B. licheniformis* at subspecies level. This technique has been reportedly suitable for interspecies and occasional intraspecies differentiation of *B. subtilis* phylotype and LAB obtained from different sources, including fermented condiments (Johnson *et al*., 2000; Marten *et al*., 2000; Lei and Jacobsen, 2004; Ouoba *et al*., 2010; Ahaotu *et al*., 2013). The combined results of ITS-PCR and ITS-PCR-RFLP showed more species differentiation and higher intraspecies differences among *B. subtilis*, *B. amyloliquefaciens* and *B. pumilus* strains as previously observed (Jeyaram *et al*., 2008; 2010). Dendrogram obtained from combined gel fingerprints of ARDRA, ITS-PCR and ITS-PCR-RFLP indicated the predominance of *B. subtilis* strains in *iru,* which was earlier observed in the culture-independent molecular study. This finding is in agreement with previous reports on the prevalence of *B. subtilis* in similar traditional alkaline fermented condiments in Southeast Asia and W. Africa (Meerak *et al*., 2007, 2008; Ouoba *et al*., 2008; Oguntoyinbo *et al*., 2010).

RAPD-PCR analyses of OPA 18 and M13 primers revealed high strain level diversity among the dominant *Bacillus* species in *iru*, and these were completely diverged and phylogenetically different from *hawaijar, kinema* and other reference bacilli strains. Different studies have confirmed the effectiveness of RAPD-PCR for precise strains typing and heterogenicity delineation of *B. subtilis* and other bacterial strains belonging to the same species (Sarkar *et al*., 2002; Matarante *et al*., 2004; Inatsu *et al*., 2006; Jeyaram *et al*., 2008). Kwon *et al*. (2009) also developed a species-specific RAPD-PCR protocol that rapidly identified *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* isolated from *cheonggukjang*. The phylogenetic analysis based on 16S rRNA gene sequences showed genetic relationship among the bacilli strains and established that most of the species must have originated from common ancestor, adapted and domesticated into vegetable protein environments both in Africa and Asia. The species and strains divergent especially between *B. subtilis* and *B. cereus* groups may be due to horizontal gene transfer and/or homologous recombination, a phenomenon that is common among bacterial species (Van der Auwera *et al*., 2007; Manzano *et al*., 2009).

Phenotypic characteristics of these strains showed that *B. subtilis* are highly amylolytic and proteolytic, while *B. pumilus* are non-amylolytic but proteolytic; this is in agreement with previous bacilli phenotypic studies (Holt *et al*., 1994). Whereas, *B. cereus* are weakly proteolytic and amylolytic but must have acquired genes for production of toxins such as emetic and diarrhoeal over time via recombination and transposable elements (De Palmenaer *et al*., 2004; Oguntoyinbo and Sanni, 2007; Didelot *et al*., 2009). Apart from the dominant *Bacillus* species isolated from *iru*, another closely related genus, *Brevibacillus* was also detected; this confirmed the earlier reports of the implication of *B. bortelensis* in *soumbala* and *bikalga* (Ouoba *et al*., 2004, 2007a). Similarly, this bacterium was identified in the culture-independent molecular analysis of this study.

The results of the phylogenetic tree indicated five different clades, which clustered separately on the basis of species; it also established divergence, evolutionary and clonal relationships of bacilli from Africa, placing them on a global perspective with other strains. Limitation in the 16S rRNA gene sequences discrimination of *B. subtilis* and *B. amyloliquefaciens* was observed, which is in agreement with previous reports confirming the inability of this gene to differentiate closely related species of *Bacillus* because of their clonal and genetic relatedness (Stackebrandt *et al*., 2002; Santos and Ochman, 2004).

The *B. cereus* phylogeny showed no species differentiation even with the combined efforts of the various techniques employed (i.e. colony morphology and genomic characterization). This is perhaps because all the species in this group have high degree of sequence similarity, which makes them genetically related and indistinguishable from each other (Manzano *et al*., 2003). Several attempts were made previously using 16S rRNA gene sequence-based analysis; single-strand conformation polymorphisms of amplified 16S rRNA gene; virulence parameters; pulsed-field gel electrophoresis; ITS-PCR; restriction fragment length polymorphisms (RFLP), including DNA:DNA hybridization for possible discrimination of the *B. cereus* group (Harrell *et al*., 1995; Ramisse *et al*., 1996; Keim *et al*., 1997; Borin *et al*., 1997; Yamada *et al*., 1999; Wu *et al*., 2006; Ahaotu *et al*., 2013), but none of these was able to differentiate *B. cereus* from *B. thuringiensis*.

MLSA of chromosomally encoded housekeeping genes based on RAPD-PCR *Bacillus cereus sensu lato* groupings used in this study prove to be a better and promising technique for differentiation of closely related *B. cereus* phylogeny, as it clearly differentiated *B. cereus* from *B. thuringiensis*. This can have potential application during industrial processing of fermented foods. Other investigators have also used this technique to study the phylogenetic relationships in the *B. cereus* group obtained from different sources (Hoffmaster *et al*., 2004; Klee *et al*., 2006; Tourasse *et al*., 2011; Thorsen *et al*., 2015). Dendrogram constructed based

on *gyrB*, *glpF* and *gmk* genes revealed phylogenetic and clonal relationship of common ancestral origin between *B. cereus* and *B. thuringiensis*, confirming *B. cereus sensu stricto* as the major member of the *B. cereus sensu lato* associated with *iru*. Also, *B. cereus* and *B. thuringiensis* strains from the present study are totally diverged from other strains, information that shows that they are geographically domesticated.

Microbial cultures in traditional fermented foods have been found to enhance modification of substrates, preservation, improvements in nutrient contents, and development of food products with putative health promoting benefits needed for well-being (Saarela *et al*., 2002). In other words traditional fermented foods contain beneficial live microorganisms, in form of probiotics that can promote and maintain health; prevent and manage diseases. Bacilli, LAB and yeasts are the most commonly isolated microflora in fermented food products that have gained acceptable popularity because of their convincing and established beneficial effects on human health (Sorokulova *et al*., 2008; Generoso *et al*., 2010). For example, *Bacillus* species long consumed since ancient times in alkaline fermented condiments in Asian subcontinent, where they form dominant microbial flora, demonstrated beneficial health properties such as immunostimulatory, antimutagenic, antioxidative, anticancer and fibrinolytic activity (Lim *et al*., 1999; Lim *et al*., 2004; Ra *et al*., 2004; Hong *et al*., 2006; Kim *et al*., 2011).

In this study divergent *B. subtilis* strains from *iru*, *B. subtilis* U170B and *B. subtilis* U146A, in combination with reference *B. clausii* UBB-07 strain were evaluated for *in vitro* probiotic properties for the purpose of developing sustainable starter cultures for medium-scale industrial production of fermented vegetable protein seeds consumed as condiments in W. Africa, to contribute to improving the health of the consumers. To exert their physiological functions optimally probiotic strains must first be able to reach alive, in active form, and successfully survive the extreme harsh and stressed conditions during passage through the GIT (Taranto *et al*., 2006). That is, they should be able to express resistance to low gastric pH

of the stomach, which is the major host physico-chemical defence mechanism against ingested microorganisms, as well as, establish themselves in the bile salts environment along the upper region of the small intestine, and subsequently advanced to the large intestine (Kolida *et al*., 2006).

The *Bacillus* strains tested for survival under simulated stomach conditions consisting of pepsin and pH adjusted to 2.0 maintained satisfactory cell viability and adaptation at human body temperature (37^{o}C) for 3 h, with maximum of 1 Log CFU reduction. This insignificant loss in viable cells indicates the potential resistance of these strains to the acidic condition normally encountered during transit in the stomach. Their successful passage through the stomach suggests that they may eventually reach the small bowel to perform various probiotic functions. It has earlier been reported that the gastric contents of a healthy human is very acidic, with pH range of 2-2.5 (Fernandez *et al*., 2003), a similar pH value used in this study. The resistant nature of vegetative cells of *B. subtilis* DSM 5750 and *B. licheniformis* DSM 5749 in BioPlus® 2B, a probiotic product, to simulated human gastric juice of pH 2.2 has also been reported (Prieto *et al*., 2014), with a survival rate of 9%; the least being recorded in the present study. However, *B. subtilis* strain SC2362 almost completely lost its viability when analysed in a medium containing 1 mg/ml pepsin at pH 2.0, with significant reduction in cell counts and very negligible survival rate of 0.001% after 1 h (Duc *et al*., 2003a).

These results altogether clearly support the hypothesis that the ability of putative probiotic strains to survive the low acidic pH condition of the stomach is variable and strain-dependent (Charteris *et al*., 1998). The intrinsic resistance of *S. thermophilus* and lactococci strains to low pH solutions of 2.0 showed high loss of cell recovery and survivalbility (Vinderola and Reinheimer, 2003). Strains of *L. casei* were also highly susceptible to simulated gastric fluid exposure; an average of 4.2 Log cycle viable cell loss recorded (Corsetti *et al*., 2008; Zhang

et al., 2011). Ayeni *et al*. (2011) observed as much as 6 Log units loss of *L*. *paracasei* and *W. confusa* strains under SGJ of pH 2.0. The acidic pH stress was thereafter reduced to a lesser extent by the protective effect of skimmed milk incorporated in the formulated juice. Similar technique was previously demonstrated, where fresh cheese conferred high protection on lactic probiotic bacteria and supports their survival in the acidic environment of the stomach (Vinderola *et al*., 2000). *Bacillus* strains on the other hand do not require any protective food matrix/covering or microencapsulation and immobilized cell technologies to withstand adverse and other stressed environmental conditions, an innate unique advantage over *Lactobacillus*. Hence, commercially available probiotic products such as Enterogermina®, Biosporin® and BioPlus® 2B are administered in spore forms, because of their resilient nature to environmental stresses, stability, and storage at room temperature without any deleterious effects on viability.

Bacilli spores consist of several protective layers surrounding the nucleoid, which make them extremely resistant to unfavourable GI tract conditions (Henriques and Moran, 2000). There are evidence-based results that justified the germination, growth and re-sporulation of ingested *Bacillus* spores in the small intestine to elicit positive probiotic effects on the hosts (Hoa *et al*., 2001; Casula and Cutting, 2002; Duc *et al*., 2003a). Studies have shown the immunomodulation that is, stimulation of the gut-associated lymphoid tissue (GALT) and dissemination of *Bacillus* spores to the Peyer's patches and mesenteric lymph nodes (MLN) (Duc *et al*., 2003a, b).

In the simulated small intestinal fluid and pancreaticobiliary secretions of 0.3% (w/v) bile salts concentration, the *Bacillus* strains demonstrated marginal decrease in the final Log counts of vegetative cells (< 1 Log unit reduction) and persisted after 3 h (the average time food spends in the small intestine), with greater percentage survival rate compare to the SGJ condition. Similarly, Zhang *et al*. (2011) reported higher cell viability and < 1 Log cycle loss

of lactobacilli strains when challenged for 4 h in pancreatin solution (pH 8.0) and 0.3% bile salts compare to SGJ. *L. delbrueckii* subsp. *bulgaricus* CECT 4005T and *S. thermophilus* CECT 801 used for yoghurt production also resisted simulated intestinal conditions more than that of the stomach; none of the strains showed significant loss in cell viability after 6 h pancreatin exposure (García-Hernández *et al*., 2012). Evidence of these data confirmed the ability of probiotic strains to tolerate and survive more in bile salts condition than acidic environment. This observed phenomenon is similar to what happens in the upper bowel of the human GIT where microbial numbers are restricted and low in the stomach, due to increased peristalsis and high gastric juice pH, which gradually increases from the duodenum to the jejunum and eventually becomes alkaline at the ileum, to accommodate more genera of microorganisms (Willey *et al*., 2009).

In this study 0.3% (w/v) bile salts was used for probiotic screening, which is believed to be the mean intestinal bile concentration of the human gut (Gilliland *et al*., 1984; Goldin *et al*., 1992). A number of studies have also used this concentration for selecting bile resistant probiotic strains (Marteau *et al*., 1997; Mainville *et al*., 2005). Spore suspensions of the two *B. subtilis* strains from *iru* unfortunately were acutely susceptible to the simulated gut conditions, whereas *B. clausii* UBB-07 spores remained viable. The plausible reason for this may be that as frequently analysed laboratory strains, these bacteria must have lost one or more of their natural traits, which would have been responsible for persistence of spores within the GIT. Previously, *Bacillus* probiotic products carrying spores exhibited very high sensitivity to SGJ and SIF (Duc *et al*., 2004a). However, some other studies reported the viability of *Bacillus* spores in simulated gastric and 0.3% bile intestinal fluids during 3 h passage (Guo *et al*., 2006; Patel *et al*., 2009; Jung *et al*., 2012; Prieto *et al*., 2014).

One of the functional requirements of probiotics is the ability to show antagonism against pathogenic organisms via competitive exclusion, competition for available nutrients, and

production of antimicrobial/bactericidal substances such as organic acids (lactic and acetic acids), hydrogen peroxide (H_2O_2), antibiotics and bacteriocins, which suppress the growth and activities of food-borne pathogens and other harmful bacteria (Fuller, 1991; Saarela *et al*., 2000; Pinchuk *et al*., 2001). Probiotic microorganisms produce different antimicrobial compounds, which may be one of the mechanisms for elimination of pathogens in the GIT, needed for the restoration of a healthy microflora composition, and further expression of probiotic effects on the hosts (Ouwehand and Salminen, 1998).

In the antagonistic activity assay against food-borne pathogens, *B. subtilis* U170B and *B. subtilis* U146A fairly inhibited *B. cereus* MTCC 430 and *B. cereus* MBU 1011, closely related bacterial species, known to cause diarrhoeal and emetic food poisoning, while *B. clausii* UBB-07 showed stronger inhibitory spectrum on solid agar medium. Probiotic *B. pumilus* and *B. licheniformis* strains from seaweed earlier produced weak antimicrobial activity against food-borne pathogens (Prieto *et al*., 2014). The antibacterial properties of some potential probiotic lactobacilli and bifidobacteria strains tested against pathogenic bacteria, including *E. coli*, *E. cloacae*, *S. aureus*, *C. difficle* and *K. oxytoca* also showed negative to weak inhibition on agar spot assay (Jacobsen *et al*., 1999; Arboleya *et al*., 2011). Meanwhile, *B. subtilis* strains from the GI tracts of native and broiler chickens evaluated for probiotic functions for use as feed additive in piglet diets, exhibited higher inhibition growth against *B. cereus*, *E. coli*, *S.* Typhimurium, *S.* Enteritidis, *S. aureus*, *L. monocytogenes* and *Vibrio cholerae* (Guo *et al*., 2006; Thirabunyanon and Thongwittaya, 2012). Consequently, these results indicate the species and strain-specific nature of antimicrobial activities of probiotic bacterial strains.

None of the two *B. subtilis* strains produced inhibitory zone in the cell-free culture supernatants (CFCS), whereas *B. clausii* UBB-07 demonstrated antagonism under same test conditions. The reason for this may be due to the fact that the supernatants were crude in

nature, not concentrated, as was done in previous studies (Schillinger and Lücke, 1989; Oguntoyinbo *et al*., 2007); hence the negative results obtained do not necessarily mean that an antimicrobial compound was not produced. Toure *et al*. (2003) earlier reported failure of unconcentrated supernatants of bifidobacteria to produce inhibition against target organisms, which was recovered when concentrated. Also, the diameters of inhibition zones of *B. subtilis* 3 CFCS against *H. pylori* increased significantly when concentrated (Pinchuk *et al*., 2001). The antagonistic property of the cell-free extract in this study was not organic acid related, having an alkaline pH value of 7.4, which otherwise suggests the presence of bacteriocin-like metabolites. Bacteriocins-producing *B. subtilis*, *B. pumilus* and *B. licheniformis* strains in W. African fermented condiments have been found to possess antagonistic activities against food-borne pathogens and food spoilage microorganisms (Ouoba *et al*., 2007a; Compaoré *et al*., 2013).

Microbial adhesion to the human gastrointestinal mucus membrane, epithelial cells and other gut tissues, both *in vitro* and *in vivo*, is known to be a requisite for survival, persistence and colonization of the GIT for subsequent probiotic health functions (Ouwehand *et al*., 1999). Both adhesion and colonization of the target sites are important properties of bacterial strains to be considered as probiotics (Collado *et al*., 2007a). Bacterial adhesion to the mucosal epithelium layers lining the gut involves a complex multistep process in which both non-specific mechanisms and a specific ligand receptor play vital roles (Del Re *et al*., 2000). Non-specific binding is based on the cell surface characteristics, in particular, bacterial cell surface hydrophobicity, which has been implicated in the attachment of bacterial cells to host tissues (Cuperus *et al*., 1995; Schillinger *et al*., 2005).

In the present investigation, the affinity of *B. subtilis* strains in *iru* and *B. clausii* UBB-07 reference strain to liquid hydrocarbons was used to determine their hydrophobic adherence. Their adhesion to *n*-hexadecane, toluene and chloroform varied among strains tested,

depending on the adsorption strength at the aqueous-hydrocarbon interface. Strains-specific and species-specific characteristics of hydrophobic interactions among probiotic microorganisms had been earlier identified (Schar-Zammaretti and Ubbink, 2003). The hydrophobicity values obtained in this study are within the range reported for different probiotic bacteria (Rijnaarts *et al*., 1993; Schillinger *et al*., 2005).

Among the bacilli strains analysed, *B. subtilis* U146A possessed the highest cell surface hydrophobicity of 44.96%, 44.11% and 61.73% for *n*-hexadecane, toluene and chloroform respectively, which were far more than that of the reference probiotic *B. clausii* UBB-07. Thirabunyanon and Thongwittaya (2012) previously reported an average of 44% hydrophobicity with *n*-hexadecane for *Bacillus* strains isolated from GIT of chickens, which is consistent with the values stated above. Lee *et al*. (2007) however found higher percentage hydrophobicity of 64.04% for *B. polyfermenticus* SCD. In a related study involving LAB strains, hydrophobic values obtained in the presence of *n*-hexadecane were 37.7% and 46.8% for *L. plantarum* Lp9 and *L. johnsonii* LA1 respectively (Kaushik *et al*., 2009). Some lactobacilli, *L. acidophilus* and *L. amylovorous* strains however showed surface hydrophobicity as low as 2-5% (Schillinger *et al*., 2005; Ndahetuye *et al*., 2012), which is quite below the minimum value recorded in the present study. The moderately high percentage adherence of *B. subtilis* U146A to the three different hydrocarbons demonstrated its hydrophobic cell surface properties, which in turn indicated greater ability and tendency of this organism to likely adhere better to human epithelial cells compare to the other strains studied.

Microbial cell surface hydrophobicity based on adhesion to liquid hydrocarbon droplets has been recognized as a determinant and estimation of the ability of microorganisms to adhere to epithelial cells and other abiotic surfaces (Kiely and Olson, 2000). This may be reasonably accepted owing to the fact that the lipo-organic compounds of the bacterial cell wall can be

easily dissolved in the organic mucin layer of the gut, thereby facilitating their interactions and subsequent attachment to the mucosal cells. Hitherto, the chemical composition of the bacterial cell surfaces such as peptidoglycan layer, techoic acid, lipotechoic acid (LPA), lipopolysaccharides (LPS), glycoproteins, including adhesins, structures/appendages external to the cell wall e.g. capsules, surface layer (S-layer) proteins, slime layer, flagella, fibrils and fimbriae were shown to mediate attachment of bacterial cells to intestinal mammalian cells, and also contribute to their ability to partition between two immiscible liquids (i.e. *n*-hexadecane and aqueous phase) (Rojas *et al*., 2002; Ross and Jonsson, 2002; De-Vries *et al*., 2006).

Another bacterial cell surface property, autoaggregation, appeared to be necessary for adhesion and transient colonization of the GIT surfaces (Boris *et al*., 1997; Del Re *et al*., 2000). Bacterial cellular autoaggregation also enhances adequate mass formation and biofilms that tends to provide protective shield to the host gut, and barrier against colonization of mucosal surfaces by pathogens (Rickard *et al*., 2003; Ferreira *et al*., 2011). Again, *B. subtilis* U146A autoaggregated very rapidly, which increased with incubation time, in comparison with the other two *Bacillus* strains. This strain eventually exhibited maximum autoaggregating phenotype and percentage autoaggregation (68.14%), while the reference probiotic strain had the least (19.97%). Patel *et al*. (2009) also found *B. subtilis* DET6 from food wastes demonstrating peak autoaggregation value of 60%. However, Zhang *et al*. (2011) reported poor autoaggregation ranging from 8.63-12.93% in lactobacilli strains after 4 h incubation.

High bacterial autoaggregation property shown by *B. subtilis* U146A suggests the ability of this bacterial strain to compete for adhesion sites and nutrients, prevent pathogens colonization by competitive exclusion, and promote gut colonization of beneficial microorganisms (Cesena *et al*., 2001; Jankovic *et al*., 2003). Findings from different studies

indicate a direct relationship between autoaggregation and cell surface hydrophobicity, where probiotic strain with high autoaggregation ability also showed corresponding high hydrophobicity (Vandevoorde *et al*., 1992; Del Re *et al*., 2000; Collado *et al*., 2007c; Chen *et al*., 2010). Similar trend was observed here; *B. subtilis* U146A with the highest adhesion to hydrocarbon demonstrated the greatest autoaggregation capacity.

Coaggregation between different bacterial species and strains is of considerable significance in various ecological niches, especially the human gut, where probiotics are expected to be active in forming competitive barrier against pathogens (Jankovic *et al*., 2003; Schellenberg *et al*., 2006). A coaggregation assay developed was used to establish interbacterial adherence between *Bacillus* strains and *S. enterica* subsp. *enterica* serovar Typhimurium LT2, an important food-borne pathogen. The strains were able to entrap the pathogen in an *in vitro* suspension in a strain-dependent manner as previously observed (Collado *et al*., 2007c; Sadrani *et al*., 2014). However, *B. subtilis* U146A formed the strongest coaggregation phenotype with *S. enterica* subsp. *enterica* serovar Typhimurium LT2, with increased percentage coaggregation over time.

Coaggregation as a cell surface property enables probiotic strains to produce antimicrobial substances in close proximity, required for elimination of pathogens in the GIT (Reid *et al*., 1988). Collado *et al*. (2007c) and Xu *et al*. (2009) suggested a correlation between autoaggregation and coaggregation cell surface properties, where strains with higher autoaggregation showed greater coaggregation abilities among pathogens tested. In this study, *B. subtilis* U146A with the best autoaggregation potential also coaggregated well with *S. enterica* subsp. *enterica* serovar Typhimurium LT2 than the other strains. Thus, a positive correlation between bacterial surface characteristics (adhesion to hydrocarbons) and binding capabilities (autoaggregation and coaggregation) is hypothesized. The results of these three

parameters could be predictive in identifying potential probiotic *Bacillus* strain appropriate for human and animal use.

The adhesion of probiotic strains to intestinal micelles also prevents their immediate washout and elimination by peristalsis, providing a competitive advantage in this ecosystem, necessary to disrupt pathogen attachment, facilitate contact with GALT mediating local and systemic immune effects, including stable intestinal mucosal barrier (Salminen *et al*., 1996; Alander *et al*., 1997; Saarela *et al*., 2000). Challenges in studying bacterial adhesion and colonization *in vivo*, especially in humans, have led to the development of *in vitro* cellular model systems involving three human derived intestinal epithelial cell lines, namely HT-29, HT-29-MTX (a mucus secreting type) and Caco-2, which have been extensively and successfully used in the assessment of adhesion properties of a large number of candidate probiotic strains (Kimoto *et al*., 1999; Blum and Reniero, 2000). These cultured cell lines mimic the *in vivo* conditions, expressing the structural phenotypes, resemblance and functional differentiation typical of matured normal human enterocytes and goblet cells of the small intestine, including polarization, brush border microvilli at confluent (Wang *et al*., 2008; Moussavi *et al*., 2009).

In the present work, the adhesive characteristics of putative probiotic bacilli strains to both differentiated and undifferentiated HT-29 cells were evaluated. The percentage adhesion values between undifferentiated and differentiated cells for each *Bacillus* strain was not significantly different, which implies that any of these cell lines may be used for *in vitro* adhesion analysis. To obtain differentiated cells, undifferentiated HT-29 cell lines were treated with forskolin, leading to the production of microscopic villi structure typical of the mucus membrane.

Generally, the *Bacillus* strains poorly adhered to the epithelial cells, based on the adhesion percentage, as determined by the ratio of bacteria attached to the cells to that added. Their

adhesion to HT-29 though weak, they could still be preferred to non-adherent strains, in that they are more likely to get established and persist longer in the intestinal tract, and thus have better chances of showing immunomodulatory effects than non-adhering strains (Saarela *et al*., 2000). Probiotic microbial strains analysed elsewhere with poor adhesion values *in vitro* and/or *in vivo* were found to show positive health promoting effects in the hosts (Saarela *et al*., 2000). These results evidently indicate that the mechanisms by which probiotic microorganisms carry out their functions remain unclear, requiring some clarifications, especially clinical trials (Reid *et al*., 2003). Sorokulova *et al*. (2008) and Prieto *et al*. (2014) earlier reported poor adhesion properties as low as 0.01% and 0.001% for *B. subtilis* strains isolated from probiotic product and seaweed, tested using Caco-2 and HT-29 cells respectively. Binding of bifidobacteria isolates from human breast milk and *L. casei* strains to HT-29 were also reported to be relatively low, ranging from 0.08-0.10% (Bertazzoni-Minelli *et al*., 2004; Arboleya *et al*., 2011).

The adhesion pattern of *Bacillus* strains under investigation showed some variations depending on the origin of the strains. Adhesion of some commercial probiotic strains was similarly strain-specific, varying within the same species and genera (Collado *et al*., 2007b). *B. clausii* UBB-07 reference probiotic strain with the least overall cell surface characteristics significantly adhered better to HT-29 cell lines than the two *B. subtilis* strains from *iru*. On the other hand *B. subtilis* U146A with the maximum hydrophobic interactions, autoaggregation and coaggregation was the least adhered strain. Categorically, these findings established lack of direct relationship or correlation between bacterial cell surface properties and attachment to human epithelial surface receptors, where it may be expected that the strain possessing higher physicochemical properties would adhere more to HT-29 cell lines, and the one with lower properties would adhere less.

In agreement with these results, several other authors earlier found lack of correlation between cell surface hydrophobicity (as measured by microbial adhesion to *n*-hexadecane), autoaggregation or coaggregation, and adhesion to mucus and epithelial cells, claiming that these parameters do not correlate (Handley *et al*., 1987; Vinderola *et al*., 2004; Schillinger *et al*., 2005; Collado *et al*., 2007a; Alzate *et al*., 2008; Mathara *et al*., 2008; Todorov *et al*., 2008). One possible reason for this may be due to how adhesion abilities are determined; autoaggregation, coaggregation and adhesion to hydrocarbons are spectrophotometrically analysed, which may not be totally reliable, since the suspensions examined constitutes both dead and live cells, including other particles, captured by the light scattering effects, while adhesion to epithelial cell lines is usually by viable cell counts. Consequently, bacterial cell surface properties only may not be adequate in determining the adhesion indices of probiotic strains for *in vivo* applications. Conversely, a direct relationship between hydrophobicity, as well as autoaggregation and adhesion has been identified in a number of studies (Wadstrom *et al*., 1987; Del Re *et al*., 1998; Garriga *et al*., 1998; Pérez *et al*., 1998; Del Re *et al*., 2000; Ehrmann *et al*., 2002; Kos *et al*., 2003). *In vitro* adhesion properties of probiotic bacteria using human derived intestinal cell models have been demonstrated to correlate with *in vivo* colonization (Crociani *et al*., 1995; Jacobsen *et al*., 1999; Cesena *et al*., 2001). Thus, adherent *B. subtilis* strains in *iru*, as reported herein, may colonize the GIT, to prevent host-pathogen interactions, establishing probiotic-host influence.

The human intestinal bacteria community plays crucial roles in maintaining a healthy gut homeostatic equilibrium. Under certain conditions such as dysbiosis (i.e. bacterial imbalance), depending on the gut physiology of the host and diets intake, individual protective microflora may become depleted, resulting in a higher susceptibility of the GIT mucosal surfaces to enteropathogenic bacterial infections and diseases (Fooks and Gibson, 2002). In the current study the inhibitory effects and protective roles of *Bacillus* probiotic

strains against adhesion of enteric pathogen (*S. enterica* serovar Typhimurium MBU 1047), to cultured HT-29 intestinal cell monolayers was investigated.

Bacilli were administered simultaneously with, before or after *S. enterica* serovar Typhimurium MBU 1047 to evaluate their effectiveness in exclusion, competition and displacement assays, respectively on HT-29 cell lines. The results obtained indicate the potential capability of the three *Bacillus* strains to effectively interfere and hamper the adhesion of *S. enterica* serovar Typhimurium MBU 1047 to human intestinal epithelial cells. Prevention of attachment of food-borne pathogens may represent an operative probiotic therapy for combating food-borne diseases and other various infections. *Salmonella* spp. cause acute GIT illnesses, including diarrhoea that results in high rate of mortality and morbidity worldwide, with Typhimurium being one of the most frequently isolated serovars (Gorman and Adley, 2004). Previous reports confirmed the ability of bifidobacteria and lactobacilli to inhibit mucosal adhesion of enteropathogens (*E. coli* and *S. enterica* serotype Typhimurium) to epithelial cells *in vitro* (Bernet *et al*., 1993; Collado *et al*., 2005). However, other investigators reported increased adhesion of *E. coli* strains, *L. monocytogenes*, *S.* Typhimurium and *Shigella sonnei* to intestinal HT-29 cell lines in either pre-treatment, competition or displacement assay with probiotic lactobacilli and bifidobacteria strains (Tuomola *et al*., 1999; He *et al*., 2001; Collado *et al*., 2005; Gueimonde *et al*., 2006; Collado *et al*., 2007b; Zhang *et al*., 2010; Ayeni *et al*., 2011).

Hence, the ability to inhibit, compete and displace already adhered pathogens depends on the specific probiotic strains and pathogens analysed. It is interesting to note that the *B. subtilis* strains from *iru* were able to adhere to the HT-29 enterocyte layers to displace previously colonized *S. enterica* serovar Typhimurium, though at lower percentage adhesion inhibition compared to exclusion and competition assays, whose values were not significantly different. This information would assist in the efforts geared towards identifying *Bacillus* probiotic

candidates for future *in vivo* administrations in consumer foods for the management of infections caused by *Salmonella*, especially salmonellosis.

Microbial strains as probiotics for human use are preferably of human origin, since it is believed that probiotic microorganisms isolated from healthy humans are safer for consumption, and may adhere and colonize strongly the GIT than strains not from similar environment (Saarela *et al*., 2000; Dunne *et al*., 2001; Quigley, 2010). However, the European Food Safety Authority (EFSA) regulations and guidelines make no recommendation on strain origin, except that the source must be stated, including accurate taxonomic species characterization, identification and strain typing, as requisites for safety assurance (EFSA, 2012). In this study *B. subtilis* strains analysed for probiotic attributes were extensively characterized, and strains identities determined by applying phenotypic and polyphasic molecular sub-typing techniques according to the guidelines established by the FAO/WHO working group for evaluating the safety of potential probiotic strains (FAO/WHO, 2002; 2006). Safety assessment parameters such as haemolysis on blood agar, antibiotic resistance pattern and mucin degradation were also conducted on the *B. subtilis* strains in combination with reference *B. clausii* UBB-07.

Bacilli in *iru* were non-haemolytic whereas *B. clausii* UBB-07 displayed partial haemolysis. They were also sensitive to most of the common clinically important antibiotics, suggesting that these organisms can be safely used as starter cultures for human consumption. *B. subtilis* consumed in large counts in alkaline fermented condiments in the W. African subregion and Asian subcontinent has long history of safe use. To this end it was ascribed GRAS status by FDA and included in the inventory of Microbial Food Culture (MFC) by the International Dairy Federation, and also given a Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) (EFSA, 2005). *B. clausii* UBB-07 was however resistant to virtually all the antibiotics analysed. Enterogermina® *B. clausii* was earlier found to be

resistant to a number of antibiotics (Green et al., 1999; Hoa et al., 2000). Hence this organism is marketed as a commercial probiotic product, a more detailed safety examinations is pertinent. It would be important to check for the presence of transferable plasmids and mobile genetic elements linked with antibiotic resistance, since strains harbouring transmissible antibiotic resistance genes are not suitable for use as probiotics (Vankerckhoven *et al*., 2008).

5.1 Summary of Findings

	Specific Objectives	Findings
1.	Characterize the bacterial community profile of *iru* using culture-independent molecular methods.	(a) The bacterial composition in terms of DNA fragments of *iru* samples obtained from different geographical regions revealed intra- and inter-specific diversity. (b) Ecological parameters based on bacterial species richness (*R*) and diversity (*H*) indexes were relatively high for all the *iru* samples analysed. (c) DNA Sequences of the major PCR-DGGE bands identified *B. subtilis* as the predominant and persistent bacterial species associated with fermentation of *P. biglobosa* for *iru* production.
2.	Analyze the bacterial population density of *iru* obtained from different producers and retail markets.	An average population count of 10^6 of bacterial colonies was obtained in the various *iru* samples. Mean pH of 7.2 was also recorded.
3.	Evaluate the phenotypic features and identification of bacterial spore-formers isolated from *iru* using traditional culture-dependent techniques.	The representative bacterial colonies ca. 280 isolated from 26 *iru* samples were identified as *Bacillus* species and its closest relative members based on phenotypic characteristics such as cultural, microscopic and biochemical parameters.
4.	Sub-type the bacterial species associated with *iru* for strains differentiation.	(a) ARDRA, ITS-PCR, ITS-PCR-RFLP and RAPD-PCR combined with nucleotide sequences of almost complete 16S rRNA gene revealed high strain divergence and sub-types among the dominant *B. subtilis* strains. (b) MLSA of chromosomally encoded *gyrB*, *glpF* and *gmk* housekeeping genes separated *B. cereus* strains from *B. thuringiensis*.
5.	Investigate some *in vitro* probiotic properties of *Bacillus* strains isolated from *iru* that can be applied as probiotic-starter cultures for process optimization during small and intermediate industrial condiments production.	(a) *B. subtilis* strains from *iru* maintained satisfactory cell viability and adaptation during transit in the simulated stomach and small intestine conditions. (b) They demonstrated probiotic properties through adhesion and colonization of human derived intestinal epithelial cells for competitive exclusion of potential food-borne pathogens.

5.2 Conclusion

In conclusion, this study elucidated the understanding of the bacterial distribution, occurrence and diversity of naturally fermented *P. biglobosa* cotyledons for *iru* production in Nigeria. Divergent *B. subtilis* strains in *iru* demonstrated probiotic functions; they are traditionally consumed in large counts in fermented vegetable protein condiments in W. Africa. These strains survived and established themselves in the physiological conditions prevalent in the GIT, a prerequisite for promising probiotic candidate selection. In addition they adhered to human derived epithelial cells for colonization, competitively excluded food-borne pathogens, and considered safe with regard to their non-haemolytic activity and antibiotic susceptibility pattern. However, their technological properties need to be further investigated, to justify their development as cheap starter cultures for use during small- and large-scale industrial production of condiments with enhanced health benefits in W. Africa.

5.3 Contributions to Knowledge

1. The present study has improved understanding of the bacterial composition and diversity of *iru*, with the detection of bacterial flora such as *M. morganii*, *T. halophilus*, *S. jeotgali* and *U. thermosphaericus*, not previously reported.
2. This study has also revealed the distinct differentiation of closely related species of the *B. subtilis* phylogeny using ARDRA, based on *Rsa*I and *Cfo*I restriction enzymes, which hitherto had been a difficult task.
3. *B. cereus sensu stricto* was clearly distinguished from *B. thuringiensis* for the first time in fermented condiments study, using MLSA of chromosomally housekeeping genes.

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APPENDIX I

Preparation of reagents

A. Stock 0.5M EDTA, pH 8.0

186.1g disodium EDTA.2H_2O dissolves in a known quantity of Milli Q water. Adjust pH to 8.0 with NaOH and make up to 1000 ml with Milli Q water. EDTA will not dissolve until pH 8.0 is attained.

B. 40% acrylamide/bis-acrylamide (37.5:1)

Acrylamide	38.93g
Bis-acrylamide	1.07g

Make up to 100 ml with Milli Q water. Filter through 0.45 µm or 0.2 µm membrane filter and store at 4^oC.

C. 8% Denaturing Polyacrylamide gel

	25% (LD)	55% (HD)
Urea	1.575g (in 2 ml dH_2O)	3.465g (in 2 ml dH_2O)
Formamide	1.5 ml	3.3 ml
40% acrylamide	3 ml	3 ml
50X TAE (final conc. 1X)	0.3 ml	0.3 ml
	Make up vol. to 15 ml at this juncture	
10% APS	75 µl	75 µl
TEMED	15 µl	15 µl

APPENDIX II

Preparation of buffers

A. 0.1M Potassium Phosphate Buffer, pH 6.6

Stock solution of 1M Dibasic K_2HPO_4

47.64g dissolve in 350 ml Milli Q water

Stock solution of 1M Monobasic KH_2PO_4

45.64g dissolve in 200 ml Milli Q water

38 ml of 1M stock of K_2HPO_4 combine with 62 ml of 1M KH_2PO_4 give 0.1M Potassium Phosphate Buffer, 6.6

B. 5X TBE Buffer

Tris Base	54g
Boric acid	27.5g
0.5M EDTA (pH 8.0)	20 ml

Make up vol. to 1000 ml with Milli Q water

C. 5X TAE Buffer

Tris Base	242.0g
Acetic acid glacial	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

Make up vol. to 1000 ml with Milli Q water